

/VAS : Identification of genetic Variants affecting Alternative Splicing

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1 Introduction

Alternative splicing controls relative expression ratios of mature mRNA isoforms from a single gene. Mapping studies of Splicing Quantitative Trait Loci (SQTl), a genetic variant affecting the alternative splicing, are important steps to understand gene regulations and protein activity [1]. We present an effective and user-friendly computational tool to detect SQTls using transcript expression data from RNA-seq and genotype data, both measured on the same sample. As RNA sequencing (RNA-seq) provides insight into relatively precise measurements of expression level of transcript isoforms from a gene, it is a useful tool to analyze complicated biological phenomenon of RNA transcripts including the alternative splicing [2]. The mapping analysis uses two statistical models : Linear regression model [3] and/or Generalized linear mixed model [5].

2 The input data set

The next subsection introduces the input data. To run this tool, two experimental data sets (an expression data frame from RNA-seq and a genotype data frame) are required. Moreover, we also need a data frame for positions of SNP markers and GTF file for transcript models. As any other genome-wide analyses, it is recommended to use as many samples as possible, usually of population scale, in order to guarantee a statistically significant result.

2.1 The genotype data

The genotype data should be prepared as a simple matrix data. Each column represents an individual and its name should match that of the expression matrix described below (2.2)

	ind1	ind2	ind3	ind4
SNP1	AA	AA	AT	TT
SNP2	CG	CC	GG	CG
SNP3	TT	TT	AT	TT

2.2 The expression data

The expression matrix must comprise expression values of transcripts from RNA-seq. We may obtain them by using alignment tools such as cufflinks. Each column represents an individual and its name should match that of the genotype matrix described above (2.1)

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	ind1	ind2	ind3	ind4
transcript1	10.5	15.4	6.7	12.4
transcript2	6.4	7.2	4.5	9.2
transcript3	15.4	14.5	13.2	17.8

2.3 The SNP marker position data

To search SNPs affecting alternative splicing, a data frame comprising genomic location of each SNP is required. It consists of following columns: SNP (SNP marker name), CHR(chromosome number), and locus(SNP position).

SNP	CHR	locus
SNP1	1	4964005
SNP2	1	23513047

2.4 The transcripts model data

We need a reference GTF (General Feature Format) file including information about gene structures such as the positions of exons, introns, and transcripts of genes. The GTF file must be `TxDb` object from the *GenomicFeatures* package [4].

3 The example dataset : data from Geuvadis RNA sequencing project of 1000 Genome samples

This example uses filtered data from an origin data generated by Geuvadis RNA sequencing project, available at <http://www.geuvadis.org/web/geuvadis/RNAseq-project> [6]. The example expression data includes transcripts of 11 randomly selected genes. The genotype data comprises SNPs in those genes.

4 Loading data

For this analysis, you need to load the *IVAS* package, SNP data, expression data, SNP position data, and `TxDb` object from GTF.

Loading *IVAS* package :

```
> library(IVAS)
```

Loading expression data :

```
> data(sampleexp)
```

Loading SNP data :

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```
> data(samplesnp)
```

Loading SNP position data :

```
> data(samplesnplocus)
```

Loading TxDb object :

```
> sampleDB <- system.file("extdata","sampleDB", package="IVAS")
> sample.Txdb <- loadDb(sampleDB)
```

If you want to create the TxDb object from a GTF file, you need to use the `makeTxDbFromGFF` function in the `GenomicFeatures` package.

5 The ASdb object

The ASdb object is a `s4` type class object, and the object is used by the IVAS package to store the results from functions in this IVAS package. The functions of IVAS will save their results by adding a slot. Each slot contains a list object consisting of three elements named as "ES", "ASS", and "IR" for each alternatively splicing pattern type (i.e. ES, ASS, and IR means exon skipping, alternative splice site, and intron retention, respectively).

5.1 Searching alternatively spliced exons based on a reference transcript model.

The `Splicingfinder` function tabulates patterns of alternatively spliced exons. This needs the TxDb object from `makeTxDbFromGFF` by reading a reference GTF file for reference transcript models. The `Splicingfinder` function categorizes alternatively spliced exons into four types of AS patterns (i.e. exon skipping, alternative 3-prime splice site, alternative 5-prime splice site, and intron retention). The result will be saved in the "SplicingModel" slot of ASdb.

To use this function :

```
> ASdb <- Splicingfinder(GTFdb=sample.Txdb,calGene=NULL,Ncor=1,out.dir=NULL)

[1] "-----Processing : chr 2 -----"
[1] "-----Processing : chr 3 -----"
[1] "-----Processing : chr 6 -----"
[1] "-----Processing : chr 8 -----"
[1] "-----Processing : chr 9 -----"
[1] "-----Processing : chr 11 -----"
[1] "-----Processing : chr 17 -----"
[1] "-----Processing : chr 19 -----"

> ASdb
```

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```
Splicing Models : ES = 182 Rows & ASS = 11 Rows & IR = 2 Rows
#ASdb object with SplicingModel

> head(slot(ASdb,"SplicingModel")$"ASS")
```

	Index	EnsID	Nchr	Strand	ShortEX
1	"ASS1"	"ENSG00000186001"	"3"	"+"	"197562545-197562609"
2	"ASS2"	"ENSG00000183826"	"6"	"-"	"38565686-38565833"
3	"ASS3"	"ENSG00000183826"	"6"	"-"	"38565686-38565833"
4	"ASS4"	"ENSG00000172728"	"8"	"-"	"33319006-33319245"
5	"ASS5"	"ENSG00000172728"	"8"	"-"	"33318930-33319243"
6	"ASS6"	"ENSG00000166263"	"17"	"+"	"53076993-53077203"

	LongEX	ShortNeighborEX	LongNeighborEX
1	"197562545-197562693"	"197566192-197566268"	"197566192-197566268"
2	"38565686-38565897"	"38607576-38607924"	"38607576-38607700"
3	"38565686-38565897"	"38607576-38607924"	"38580610-38580809"
4	"33318890-33319243"	"33310734-33311028"	"33310734-33311028"
5	"33318890-33319243"	"33310734-33311028"	"33310734-33311028"
6	"53076987-53077203"	"53076706-53076812"	"53076706-53076812"

	Short_des	Long_des	ShortNeighbor_des
1	"197562545-197562609"	"197562545-197562693"	"197566192-197566268"
2	"38565686-38565833"	"38565686-38565897"	"38607576-38607924"
3	"38565686-38565833"	"38565686-38565897"	"38607576-38607924"
4	"33319006-33319245"	"33318890-33319243"	"33310734-33311028"
5	"33318930-33319243"	"33318890-33319243"	"33310734-33311028"
6	"53076993-53077203"	"53076987-53077203"	"53076706-53076812"

	LongNeighbor_des	Types
1	"197566192-197566268"	"A5SS"
2	"38580610-38580809,38607576-38607700"	"A5SS"
3	"38580610-38580809,38607576-38607700"	"A5SS"
4	"33310734-33311028"	"A3SS"
5	"33310734-33311028"	"A3SS"
6	"53076706-53076812"	"A3SS"

You are able to define only a single gene if the single gene is inputted. The first column, named by "Index", is a generally used as an identifier and commonly used in other functions of IVAS.

5.2 Estimating expression ratio of AS exons with a data set including FPKM values of transcripts

The `RatioFromFPKM` function calculates expression ratio between transcripts with and without alternatively spliced exons. First, `RatioFromFPKM` divides the isoforms from a single gene into two groups: transcripts with and without an alternatively spliced

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exon. Then, the ratio of the group totals of transcript FPKM values is calculated. The `RatioFromFPKM` requires expression data set of transcript FPKM values and `ASdb` with the "SplicingModel" slot. The result will be saved in the "Ratio" slot of `ASdb`

```
> ASdb <- RatioFromFPKM(GTFdb=sample.Txdb,ASdb=ASdb,Total.expdata=sampleexp,
+   CalIndex="ASS7",Ncor=1,out.dir=NULL)
> ASdb
```

Splicing Models : ES = 182 Rows & ASS = 11 Rows & IR = 2 Rows

Ratio : ES = 0 Rows by 0 samples & ASS = 1 Rows by 78 samples & IR = 0 Rows by 0 samples

#ASdb object with SplicingModel & Ratio

```
> head(slot(ASdb,"Ratio")$"ASS")
```

	Index	EnsID	Nchr	Strand	ShortEX	LongEX
1	"ASS7"	"ENSG00000170889"	"19"	"+"	"54704610-54704756"	"54704740-54704829"
		ShortNeighborEX		LongNeighborEX	Short_TX	
1	"54705028-54705149"	"54705028-54705149"			"ENST00000302907 ENST00000391751"	
		Long_TX		Types	NA06984	
1	"ENST00000391752 ENST00000402367"	"A5SS"			"0.0370610175563808"	
		NA06986		NA06989	NA06994	
1	"0.0754673755080699"	"0.431995041306961"			"0.352248098956179"	
		NA07037		NA07048	NA07051	NA07056
1	"0.615508066951179"	"0.2535297934717"			"0.396359920018477"	"0.229019337579839"
		NA07347		NA07357	NA10847	
1	"0.147021679772774"	"0.294091318766693"			"0.0835188716083212"	
		NA10851		NA11829	NA11830	
1	"0.030954840680335"	"0.0174246902189581"			"0.030532429762246"	
		NA11831		NA11843	NA11892	NA11893
1	"0.15728432880497"	"0.215269984759597"			"0.136324142619792"	"0.38436403201718"
		NA11894		NA11920	NA11930	
1	"0.212453507751045"	"0.0333217262293684"			"0.0681235360867984"	
		NA11931		NA11992	NA11993	
1	"0.0248643687301508"	"0.245183066925427"			"0.276368360584032"	
		NA11994		NA11995	NA12004	
1	"0.114340505887804"	"0.0688073456257966"			"0.0218694795539099"	
		NA12006		NA12043	NA12044	
1	"0.719903020532971"	"0.0253480818915533"			"0.0691011133998759"	
		NA12045		NA12058	NA12144	
1	"0.269579049697507"	"0.35466877311412"			"0.495392792194793"	
		NA12154		NA12155	NA12249	
1	"0.0353058516847381"	"0.0356549500182518"			"0.332527122764556"	
		NA12272		NA12273	NA12275	
1	"0.547392066663861"	"0.0461822327489977"			"0.134086715517285"	
		NA12282		NA12283	NA12286	
1	"0.584161781407799"	"0.0376982756006002"			"0.242375101101388"	
		NA12287		NA12340	NA12341	

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```
1 "0.298063167714008" "0.344188773640231" "0.0398630023057284"
  NA12342          NA12347          NA12348
1 "0.189423547621436" "0.304069583466665" "0.0175489426208136"
  NA12383          NA12399          NA12400
1 "0.0285158376928488" "0.0208852172856115" "0.162129766403219"
  NA12413          NA12489          NA12546
1 "0.272617060489197" "0.102893668902972" "0.0480414433339426"
  NA12716          NA12717          NA12718
1 "0.38349497995995" "0.180658216895047" "0.269490808164129"
  NA12749          NA12750          NA12751
1 "0.0967290881103072" "0.226219189907337" "0.024698616842959"
  NA12761          NA12763          NA12775
1 "0.167469444660537" "0.0288808382631836" "0.475715991162855"
  NA12777          NA12778          NA12812
1 "0.281683152810493" "0.00965356629010742" "0.0351200909069428"
  NA12814          NA12815          NA12827
1 "0.583521251701744" "0.500254757772165" "0.313010002548281"
  NA12829          NA12830          NA12842
1 "0.035631624411982" "0.0102342527143945" "0.684792301681123"
  NA12843          NA12872          NA12873
1 "0.251585485678078" "0.220474614626964" "0.353762604584194"
  NA12874          NA12889          NA12890
1 "0.0362672467194004" "0.222764959132477" "0.27775528737905"
```

In this example, we will estimate ratio in the "ASS7" index among splicing models in ASdb.

5.3 Finding SQTls

Using "SplicingModel" and "Ratio" slots in ASdb from [Splicingfinder](#) and [RatioFromFPKM](#), respectively, the [sQTlsFinder](#) function can identify significant SNPs associated with alternative splicing rate (ratio). The result will be saved in the "sQTls" slot of ASdb

```
> ASdb <- sQTlsFinder(ASdb=ASdb, Total.snpdata=samplesnp,
+   Total.snplocus=samplesnplocus, method="lm", Ncor=1)
> ASdb
```

Splicing Models : ES = 182 Rows & ASS = 11 Rows & IR = 2 Rows

Ratio : ES = 0 Rows by 0 samples & ASS = 1 Rows by 78 samples & IR = 0 Rows by 0 samples

sQTls : ES = 0 Rows & ASS = 1 Rows & IR = 0 Rows

#ASdb object with SplicingModel & Ratio & sQTls

```
> head(slot(ASdb, "sQTls")$"ASS")
```

	SNP	Index	EnsID	Strand	Nchr	Types
[1,]	"rs3810232"	"ASS7"	"ENSG00000170889"	"+"	"19"	"A5SS"

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```
      ShortEX      LongEX      ShortNeighborEX
[1,] "54704610-54704756" "54704740-54704829" "54705028-54705149"
      LongNeighborEX      pByGeno      FdrByGeno      diff
[1,] "54705028-54705149" "3.98508717225338e-13" "3.98508717225338e-13" "diff"
      met
[1,] "lm"
```

In this example, we will run the function with the linear regression model. `sQTLsFinder` shows chromosome numbers during mapping analysis.

6 Identification of SQTLs using multiple cores

`Splicingfinder`, `RatioFromFPKM`, and `sQTLsFinder` functions provide to use multi-thread through `foreach` function. The last argument "Ncor" of the functions denotes the number of threads.

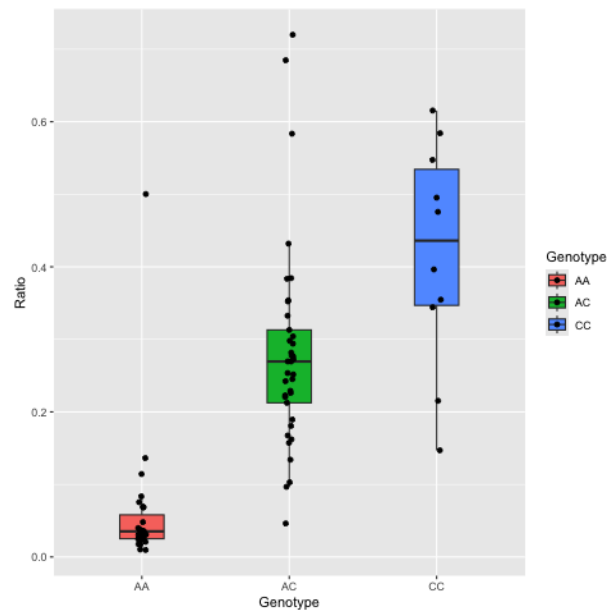
```
> ASdb <- Splicingfinder(GTFdb=sample.Txdb,calGene=NULL,Ncor=4)
> ASdb <- RatioFromFPKM(GTFdb=sample.Txdb,ASdb=ASdb,Total.expdata=sampleexp,Ncor=4)
> ASdb <- sQTLsFinder(ASdb=ASdb,Total.snpdata=samplesnp,
+   Total.snplocus=samplesnplocus,method="lm",Ncor = 4)
> ASdb
```

7 Visualizing the result

To visualize the results into boxplot, the `IVAS` package provides the `saveBplot` function. Using the data frame from the output of `sQTLsFinder` function, `saveBplot` can make the boxplot.

```
> saveBplot(ASdb=ASdb,Total.snpdata=samplesnp,Total.snplocus=samplesnplocus,
+   CalIndex="ASS7",out.dir="./result")
```


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The output png files are saved in "result" folder.

8 Session Information

R version 4.5.0 RC (2025-04-04 r88126)

Platform: aarch64-apple-darwin20

Running under: macOS Ventura 13.7.1

Matrix products: default

BLAS: /Library/Frameworks/R.framework/Versions/4.5-arm64/Resources/lib/libRblas.0.dylib

LAPACK: /Library/Frameworks/R.framework/Versions/4.5-arm64/Resources/lib/libRlapack.dylib; LAPACK

locale:

[1] C/en_US.UTF-8/en_US.UTF-8/C/en_US.UTF-8/en_US.UTF-8

time zone: America/New_York

tzcode source: internal

attached base packages:

[1] stats4 stats graphics grDevices utils datasets methods

[8] base

other attached packages:

[1] IVAS_2.28.0 ggplot2_3.5.2 GenomicFeatures_1.60.0

[4] AnnotationDbi_1.70.0 Biobase_2.68.0 GenomicRanges_1.60.0

[7] GenomeInfoDb_1.44.0 IRanges_2.42.0 S4Vectors_0.46.0

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```
[10] BiocGenerics_0.54.0    generics_0.1.3

loaded via a namespace (and not attached):
 [1] tidyselect_1.2.1      ggfortify_0.4.17
 [3] farver_2.1.2          dplyr_1.1.4
 [5] blob_1.2.4            Biostrings_2.76.0
 [7] bitops_1.0-9          fastmap_1.2.0
 [9] RCurl_1.98-1.17       GenomicAlignments_1.44.0
[11] XML_3.99-0.18         digest_0.6.37
[13] lifecycle_1.0.4       KEGGREST_1.48.0
[15] RSQLite_2.3.9         magrittr_2.0.3
[17] compiler_4.5.0        rlang_1.1.6
[19] tools_4.5.0           yaml_2.3.10
[21] rtracklayer_1.68.0    knitr_1.50
[23] labeling_0.4.3        S4Arrays_1.8.0
[25] bit_4.6.0             curl_6.2.2
[27] DelayedArray_0.34.0   abind_1.4-8
[29] BiocParallel_1.42.0   withr_3.0.2
[31] purrr_1.0.4           grid_4.5.0
[33] colorspace_2.1-1      MASS_7.3-65
[35] scales_1.3.0          iterators_1.0.14
[37] SummarizedExperiment_1.38.0 cli_3.6.4
[39] rmarkdown_2.29        crayon_1.5.3
[41] reformulas_0.4.0      httr_1.4.7
[43] rjson_0.2.23          minqa_1.2.8
[45] DBI_1.2.3             cachem_1.1.0
[47] stringr_1.5.1         splines_4.5.0
[49] parallel_4.5.0        BiocManager_1.30.25
[51] XVector_0.48.0        restfulr_0.0.15
[53] matrixStats_1.5.0     vctrs_0.6.5
[55] boot_1.3-31           Matrix_1.7-3
[57] jsonlite_2.0.0        bit64_4.6.0-1
[59] foreach_1.5.2         tidyr_1.3.1
[61] glue_1.8.0            nloptr_2.2.1
[63] codetools_0.2-20      stringi_1.8.7
[65] gtable_0.3.6          BiocIO_1.18.0
[67] UCSC.utils_1.4.0      lme4_1.1-37
[69] munsell_0.5.1         tibble_3.2.1
[71] pillar_1.10.2         htmltools_0.5.8.1
[73] GenomeInfoDbData_1.2.14 R6_2.6.1
[75] Rdpack_2.6.4          doParallel_1.0.17
[77] evaluate_1.0.3        lattice_0.22-7
[79] rbibutils_2.3         png_0.1-8
[81] Rsamtools_2.24.0      memoise_2.0.1
[83] BiocStyle_2.36.0      Rcpp_1.0.14
```

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[85] nlme_3.1-168	gridExtra_2.3
[87] SparseArray_1.8.0	xfun_0.52
[89] MatrixGenerics_1.20.0	pkgconfig_2.0.3

References

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- [2] Joseph K. Pickrell, John C. Marioni, Athma A. Pai, Jacob F. Degner, Barbara E. Engelhardt, Everlyne Nkadori, Jean-Baptiste Veyrieras, Matthew Stephens, Yoav Gilad, Jonathan K. Pritchard. 2010. Understanding mechanisms underlying human gene expression variation with RNA sequencing. *Nature* 464, 768-722.
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- [6] Tuuli Lappalainen, et al. 2013. Transcriptome and genome sequencing uncovers functional variation in humans. *Nature* 501, 506-511.