

Introduction to *customProDB*

Xiaojing Wang, Bing Zhang

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1 Introduction

Mass spectrometry (MS)-based proteomics technology is widely used in biological researches. For peptide and protein identification, sequence database search is the most popular method. We recently showed that a sample-specific protein database derived from RNA-Seq data could better

approximate the real protein pool and thus improve protein identification. With continuously decreasing cost, more and more groups have started multilayer experiment designs that profile both proteome and transcriptome of the same cohort of samples in order to gain a comprehensive understanding of cellular systems. To facilitate such efforts, we have developed this R package *customProDB*, which is dedicated to the generation of customized databases from RNA-Seq data for proteomics searches.

We designed this package based on a few assumptions (1) undetected or lowly expressed transcripts are less likely to produce detectable proteins, thus excluding them would improve sensitivity and specificity; (2) each sample has a unique set of SNPs, mutations, gene fusions, alternative splicing etc, including them in them in the protein database would allow the identification of sample specific proteins. This is particularly useful in cancer studies, in which tumors typically carry oncogenic genomic alterations.

To filter out undetected or lowly expressed transcripts, the package provides functions to either calculate the RPKM (Reads Per Kilobase per Million mapped reads) values, or accept user-provided measurements from other sources such as the FPKM (Fragments per kilobase of exon per million fragments mapped) from cufflinks. Users may specify a expression threshold, subsequently a FASTA file is generated for proteins that pass the threshold.

customProDB allows users to incorporate variations identified from RNA-seq data into the FASTA database. It annotates all SNVs with their proper locations and functional consequences in transcripts. Non-synonymous coding variations are introduced to protein sequences to create variant protein entries. Aberrant proteins resulted from short INDELs are also predicted and added to the variation database.

One important application of RNA-Seq is to identify previously unannotated structures, such as novel exons, alternative splice variants and gene fusions. The package provides a function to classify splice junctions identified from RNA-Seq data, and then uses three-frame translation to generate peptides that cross the novel junctions. Similarly, fusion genes can also be incorporated into the FASTA database.

This document provides a step by step tutorial of customized database generation.

2 Preparing annotation files

To map RNA-Seq information to the protein level, numerous pieces of genome annotation information are needed, such as genome elements region boundary, protein coding sequence, protein sequence and known SNPs et al. It is possible to manually download these data from different public resources (e.g. NCBI, UCSC and ENSEMBL) and then parse them to an appropriate format. But to make the process more efficient and autonomous, we provide two functions to prepare the gene/transcript annotation files. Users should use the same version of annotations through the entire dataset(s) analysis. All the annotations are saved to a specified directory for latter use.

The dbSNP data is huge and is getting larger and larger. These two functions only download the data in coding region for performance reasons. Table 1 shows which dbSNP version to choose for a specified genome.

Genome	Ensembl dataset	Ensembl version	dbSNP version
hg19	hsapiens_gene_ensembl	v55-now	snp131/snp132/snp135/snp137
hg19	hsapiens_gene_ensembl	v54	snp130
mm10	mmusculus_gene_ensembl	v69-now	snp137
mm9	mmusculus_gene_ensembl	v54-v68	snp128

Table 1: Choose dbSNP version

2.1 Refseq annotation from UCSC table browser

The `PrepareAnnotationRefseq` function downloads annotations from the UCSC table browser through *rtracklayer*, extracts and derives the relevant information and then saves them as the required R data structure. However, this function is not totally the automatic, it requires users to download coding sequence and protein sequence FASTA files from UCSC table browser. Since Refseq updates from time to time, we suggest generating the FASTA file the same day as running this function.

The bullet list below summarizes the steps to download coding sequence FASTA files.

- Go to UCSC Table Browser
- Choose genome
- Choose assembly
- Group — Genes and Gene Prediction Tracks
- Track — RefSeq Genes
- Table — refGene
- Region — genome (If you only need some genes, choose paste list or upload list)
- Output format — sequence
- Then choose genomic — CDS exons — one FASTA record per gene
- Press 'get sequence' button

Downloading protein sequence FASTA file is the same as above, just choose 'protein' instead of 'genomic' after clicking the 'get output' button.

```
> library(customProDB)

> transcript_ids <- c("NM_001126112", "NM_033360", "NR_073499", "NM_004448",
+                   "NM_000179", "NR_029605", "NM_004333", "NM_001127511")
> pepfasta <- system.file("extdata", "refseq_pro_seq.fasta",
+                          package="customProDB")
> CDSfasta <- system.file("extdata", "refseq_coding_seq.fasta",
+                          package="customProDB")
> annotation_path <- tempdir()
```

```
> PrepareAnnotationRefseq(genome='hg19', CDSfasta, pepfasta, annotation_path,
+                          dbsnp = NULL, transcript_ids=transcript_ids,
+                          splice_matrix=FALSE, COSMIC=FALSE)
```

2.2 ENSEMBL annotation from BIOMART

An alternative resource for annotation is ENSEMBL. The `PrepareAnnotationEnsembl` function downloads the annotation from ENSEMBL through *biomaRt*. This process may take several hours if users choose to download the whole dataset. The ENSEMBL version number can be specified in the `host` in `useMart` function. It took about 1.5 hour to prepare all annotations for ENSEMBL v66 in our tests.

```
> ensembl <- useMart("ENSEMBL_MART_ENSEMBL", dataset="hsapiens_gene_ensembl",
+                   host="feb2012.archive.ensembl.org", path="/biomart/martservice",
+                   archive=FALSE)
> annotation_path <- tempdir()
> transcript_ids <- c("ENST00000234420", "ENST00000269305", "ENST00000445888",
+                   "ENST00000257430", "ENST00000457016", "ENST00000288602",
+                   "ENST00000269571", "ENST00000256078", "ENST00000384871")
> PrepareAnnotationEnsembl(mart=ensembl, annotation_path=annotation_path,
+                          splice_matrix=FALSE, dbsnp=NULL,
+                          transcript_ids=transcript_ids, COSMIC=FALSE)
```

3 Building database from a single sample

After preparing all the annotation files, there are usually three steps to build a customized database. Users could choose one or multiple steps according to the research interest.

3.1 Filtering based on transcript expression

For a given BAM file, the `calculateRPKM` function computes the RPKM for each transcript based on reads mapped to the exon region. The output is a numeric vector. Users should make sure that the chromosome name in annotation and the BAM file are consistent, otherwise errors will be raised.

After getting RPKMs, users may check the distribution and choose a cutoff to retain relatively highly expressed transcripts that are more likely to produce proteins that are detectable in shotgun proteomics.

```
> load(system.file("extdata/refseq", "exon_anno.RData", package="customProDB"))
> bamFile <- system.file("extdata/bams", "test1_sort.bam", package="customProDB")
> load(system.file("extdata/refseq", "ids.RData", package="customProDB"))
> RPKM <- calculateRPKM(bamFile, exon, proteincodingonly=TRUE, ids)
```

Alternatively, users could input the calculated RPKM/FPKM from other software output rather than to calculate from BAM file, such as the cufflinks output. The cutoff can be defined based on a specific RPKM/FPKM value or a specific percentile. The default cutoff is '30%', which means

that only the top 70% transcripts with the largest RPKM values are retained. Then the `Outputproseq` function could output a FASTA format file containing protein sequences with corresponding transcript RPKM/FPKM values above the cutoff.

```
> load(system.file("extdata/refseq", "proseq.RData", package="customProDB"))
> outf1 <- paste(tempdir(), '/test_rpkm.fasta', sep='')
> Outputproseq(RPKM, 1, proteinseq, outf1, ids)
```

3.2 Variation annotation

First, users can input variations from a single VCF file using `InputVcf`. The package generates a list of `GRanges` object as output. It works for VCF file containing either one or multiple samples.

```
> # single sample
> vcffile <- system.file("extdata/vcfs", "test1.vcf", package="customProDB")
> vcf <- InputVcf(vcffile)
> length(vcf)
```

```
[1] 1
```

```
> vcf[[1]][1:3]
```

GRanges with 3 ranges and 40 metadata columns:

	seqnames	ranges	strand	REF	ALT						
	<Rle>	<IRanges>	<Rle>	<character>	<character>						
T/C	chr1	[32386425, 32386425]	*	T	C						
G/T	chr1	[32507666, 32507666]	*	G	T						
A/C	chr1	[32524459, 32524459]	*	A	C						
	QUAL	FILTER	DP	DP4.DP4	DP4.DP4.1	DP4.DP4.2					
	<numeric>	<character>	<integer>	<integer>	<integer>	<integer>					
T/C	24.00	.	3	0	0	0					
G/T	6.20	.	5	3	0	2					
A/C	3.54	.	5	1	2	0					
	DP4.DP4.3	MQ	FQ	AF1	AC1	G3.G3					
	<integer>	<integer>	<numeric>	<numeric>	<numeric>	<numeric>					
T/C	3	50	-36.00	1.0000	2	<NA>					
G/T	0	50	8.65	0.4999	1	<NA>					
A/C	2	50	5.47	0.4998	1	<NA>					
	G3.G3.1	G3.G3.2	HWE	CLR	UGT	CGT					
	<numeric>	<numeric>	<numeric>	<integer>	<character>	<character>					
T/C	<NA>	<NA>	<NA>	<NA>	<NA>	<NA>					
G/T	<NA>	<NA>	<NA>	<NA>	<NA>	<NA>					
A/C	<NA>	<NA>	<NA>	<NA>	<NA>	<NA>					
	PV4.PV4	PV4.PV4.1	PV4.PV4.2	PV4.PV4.3	INDEL	PC2.PC2					
	<numeric>	<numeric>	<numeric>	<numeric>	<logical>	<integer>					
T/C	<NA>	<NA>	<NA>	<NA>	FALSE	<NA>					

```

G/T      1      0.0620      1      0.36      FALSE      <NA>
A/C      1      0.0021      1      1.00      FALSE      <NA>
  PC2.PC2.1  PCHI2  QCHI2  PR  GT  GQ
  <integer> <numeric> <integer> <integer> <character> <character>
T/C      <NA>      <NA>      <NA>      <NA>      1/1      15
G/T      <NA>      <NA>      <NA>      <NA>      0/1      36
A/C      <NA>      <NA>      <NA>      <NA>      0/1      30
  DP.1  SP  PL  PL.1  PL.2
  <character> <character> <character> <character> <character>
T/C      <NA>      <NA>      56      9      0
G/T      <NA>      <NA>      35      0      78
A/C      <NA>      <NA>      31      0      98
  PL.3  PL.4  PL.5
  <character> <character> <character>
T/C      56      9      0
G/T      35      0      78
A/C      31      0      98
---
seqlengths:
  chr1 chr12 chr17  chr2  chr5  chr7  chr9
  NA   NA   NA   NA   NA   NA   NA

```

```

> # multiple samples in one VCF file
> vcffile <- system.file("extdata", "test_mul.vcf", package="customProDB")
> vcfs <- InputVcf(vcffile)

```

After reading the VCF file, users should choose the functions corresponding to different variation types, SNVs or INDELS. Although the package focuses on protein coding transcripts, we intentionally implemented several functions to examine where the SNVs are located, how many of them are located in the protein coding transcript regions, etc. The `Varlocation` functions classifies variations into eight categories, see Table 2.

Label	Description
Intergenic	Out of transcripts boundary
Intron_nonprocoding	Located in introns of non-coding transcripts
Exon_nonprocoding	Located in exons of non-coding transcripts
Intron	Located in introns of protein coding transcripts
5'UTR	Located in 5utr region of protein coding transcripts
3'UTR	Located in 3utr region of protein coding transcripts
Coding	Located in coding region of protein coding transcripts
Unknown	No annotation for this chromosome

Table 2: Definition of genomic locations of variations

```

> table(values(vcf[[1]])[['INDEL']])

```

```
FALSE TRUE
54      7
```

```
> index <- which(values(vcf[[1]])[['INDEL']]==TRUE)
> indelvcf <- vcf[[1]][index]
> index <- which(values(vcf[[1]])[['INDEL']]==FALSE)
> SNVvcf <- vcf[[1]][index]
> load(system.file("extdata/refseq", "ids.RData", package="customProDB"))
> txdb <- loadDb(system.file("extdata/refseq", "txdb.sqlite", package="customProDB"))
> SNVloc <- Varlocation(SNVvcf,txdb,ids)
> indelloc <- Varlocation(indelvcf,txdb,ids)
> table(SNVloc[, 'location'])
```

3'UTR	Coding	Intergenic
11	11	25
Intron	Intron_nonprocoding	
5	2	

For those variations labeled with 'Coding', the `Positionincoding` function computes the position of variation in the coding sequence of each transcript. The `dbSNP rsid` and `COSMIC_id` can also be retrieved if they are available.

```
> load(system.file("extdata/refseq", "exon_anno.RData", package="customProDB"))
> load(system.file("extdata/refseq", "dbsnpinCoding.RData", package="customProDB"))
> load(system.file("extdata/refseq", "cosmic.RData", package="customProDB"))
> postable_snv <- Positionincoding(SNVvcf, exon, dbsnpinCoding, COSMIC=cosmic)
> postable_snv
```

	genename	txname	txid	proname	chr	strand	pos
1	KRAS	NM_033360	6	NP_203524	chr12	-	25368462
2	ERBB2	NM_004448	7	NP_004439	chr17	+	37866082
3	MSH6	NM_000179	2	NP_000170	chr2	+	48010558
4	MSH6	NM_000179	2	NP_000170	chr2	+	48018081
5	MSH6	NM_000179	2	NP_000170	chr2	+	48018221
6	MSH6	NM_000179	2	NP_000170	chr2	+	48027990
7	APC	NM_001127511	3	NP_001120983	chr5	+	112162854
8	APC	NM_001127511	3	NP_001120983	chr5	+	112164561
9	APC	NM_001127511	3	NP_001120983	chr5	+	112175639
10	APC	NM_001127511	3	NP_001120983	chr5	+	112176559
11	APC	NM_001127511	3	NP_001120983	chr5	+	112176756

	refbase	varbase	pincoding	rsid	COSMIC_id
1	C	T	483	rs4362222	<NA>
2	G	A	591	<NA>	COSM260714
3	C	A	186	rs1042820	<NA>
4	A	G	276	rs1800932	<NA>
5	C	T	416	<NA>	<NA>

```

6      G      T      2868      <NA> COSM172960
7      T      C      1404      rs2229992      <NA>
8      G      A      1581      rs351771      <NA>
9      C      T      4294      rs121913332   COSM19149
10     T      G      5214      rs866006      <NA>
11     T      A      5411      rs459552      <NA>

```

```

> postable_indel <- PositioninCoding(indelvcf, exon)
> postable_indel

```

```

  gene_name      tx_name txid      pr_name chr strand      pos
1      APC NM_001127511    3 NP_001120983 chr5      + 112154737
2      APC NM_001127511    3 NP_001120983 chr5      + 112175897
  rebase varbase pin_coding
1      CT      C      954
2      GAA     GA     4552

```

3.2.1 SNVs

Variations can be divided into SNVs and INDELS. There are different consequences for SNVs. By taking outputs of function `PositioninCoding`, function `aaVariation` is used to predict the consequences of the SNVs in a protein sequence, i.e. synonymous or non-synonymous.

The non-synonymous variations are labeled as either AposB (A is the reference codon and B is the variation codon, e.g., E13V) or nonsense.

```

> load(system.file("extdata/refseq", "pro_codingseq.RData", package="customProDB"))
> txlist <- unique(postable_snv[, 'txid'])
> codingseq <- pro_codingseq[pro_codingseq[, 'tx_id'] %in% txlist,]
> mtab <- aaVariation(postable_snv, codingseq)
> mtab

```

```

  txid gene_name      tx_name      pr_name chr strand      pos
1      2      MSH6      NM_000179      NP_000170 chr2      + 48010558
2      2      MSH6      NM_000179      NP_000170 chr2      + 48018081
3      2      MSH6      NM_000179      NP_000170 chr2      + 48018221
4      2      MSH6      NM_000179      NP_000170 chr2      + 48027990
5      3      APC NM_001127511 NP_001120983 chr5      + 112162854
6      3      APC NM_001127511 NP_001120983 chr5      + 112164561
7      3      APC NM_001127511 NP_001120983 chr5      + 112175639
8      3      APC NM_001127511 NP_001120983 chr5      + 112176559
9      3      APC NM_001127511 NP_001120983 chr5      + 112176756
10     6      KRAS      NM_033360      NP_203524 chr12     - 25368462
11     7      ERBB2      NM_004448      NP_004439 chr17     + 37866082
  rebase varbase pin_coding      rsid COSMIC_id refcode varcode
1      C      A      186      rs1042820      <NA>      CGC      CGA
2      A      G      276      rs1800932      <NA>      CCA      CCG

```


3	C	T	416	<NA>	<NA>	ACA	ATA
4	G	T	2868	<NA>	COSM172960	GAG	GAT
5	T	C	1404	rs2229992	<NA>	TAT	TAC
6	G	A	1581	rs351771	<NA>	GCG	GCA
7	C	T	4294	rs121913332	COSM19149	CGA	TGA
8	T	G	5214	rs866006	<NA>	TCT	TCG
9	T	A	5411	rs459552	<NA>	GTC	GAC
10	C	T	483	rs4362222	<NA>	AGG	AGA
11	G	A	591	<NA>	COSM260714	CCG	CCA

	vartype	aaref	aapos	aavar
1	synonymous	R	62	R
2	synonymous	P	92	P
3	non-synonymous	T	139	I
4	non-synonymous	E	956	D
5	synonymous	Y	468	Y
6	synonymous	A	527	A
7	non-synonymous	R	1432	*
8	synonymous	S	1738	S
9	non-synonymous	V	1804	D
10	synonymous	R	161	R
11	synonymous	P	197	P

Then `OutputVarproseq` function replace the reference amino acid with the variation, and output a FASTA file containing those variant proteins. There are several options for output, users could choose either put all the SNVs of a protein into the sequence or put one SNVs each time.

```
> outfile <- paste(tempdir(), '/test_snv.fasta', sep='')
> load(system.file("extdata/refseq", "proseq.RData", package="customProDB"))
> OutputVarproseq(mtab, proteinseq, outfile, ids)
```

3.2.2 INDELS

Short insertion/deletion may led to frame shift thus produce aberrant proteins. We provide a function `OutputabrrrentPro` to generate a FASTA file containing such proteins.

```
> txlist_indel <- unique(postable_indel[, 'txid'])
> codingseq_indel <- procodingseq[procodingseq[, 'tx_id'] %in% txlist_indel, ]
> outfile <- paste(tempdir(), '/test_indel.fasta', sep='')
> Outputaberrant(postable_indel, coding=codingseq_indel, proteinseq=proteinseq,
+               outfile=outfile, ids=ids)
```

3.3 Splice junction analysis

One important application of RNA-Seq is the identification of previously unannotated structures, such as novel exons, alternative splicing and gene fusions. `Bed2Range` is used to input a BED file. Based on a BED file that contains splice junctions from RNA-Seq data, the function `JunctionType`

classifies all the junctions into six categories, Table 3. The category 'connect two known exon' is further divided into known junction, novel alternative splicing and gene fusion. Users need to set the parameter `splice_matrix` to `TRUE` when preparing the annotation files if planning to do junction analysis in this section.

Label	sub-label
connect two known exon	known junction
connect two known exon	alternative splicing
connect two known exon	gene fusion
connect one known exon and one region overlap with known exon	
connect one known exon and one non-exon region	
connect two regions both overlaped with known exons	
connect one region overlap with known exon and one non-exon region	
connect two non-exon region	

Table 3: Junction Type

A complete BED file is required for this function. The output of function `JunctionType` provides more detailed information of the junction, such as transcript source et al.

```
> bedfile <- system.file("extdata/beds", "junctions1.bed", package="customProDB")
> jun <- Bed2Range(bedfile, skip=1, covfilter=5)
> jun
```

GRanges with 56 ranges and 8 metadata columns:

	seqnames	ranges	strand		id	
	<Rle>	<IRanges>	<Rle>		<character>	
[1]	chr1	[32479978, 32495899]	+		JUNC00002865	
[2]	chr1	[32496023, 32497125]	+		JUNC00002866	
[3]	chr1	[32497241, 32498789]	+		JUNC00002868	
[4]	chr1	[32498935, 32502511]	+		JUNC00002869	
[5]	chr1	[32502644, 32503436]	+		JUNC00002871	
...	
[52]	chr17	[7578554, 7579312]	-		JUNC00041584	
[53]	chr17	[7579590, 7579700]	-		JUNC00041585	
[54]	chr17	[7579721, 7579839]	-		JUNC00041586	
[55]	chr17	[7579940, 7590695]	-		JUNC00041587	
[56]	chr17	[7591879, 7591966]	+		JUNC00041588	
	cov	part1_len	part2_len	part1_sta	part1_end	part2_sta
	<integer>	<numeric>	<numeric>	<numeric>	<numeric>	<numeric>
[1]	8	69	44	32479910	32479978	32495899
[2]	13	73	72	32495951	32496023	32497125
[3]	20	66	66	32497176	32497241	32498789
[4]	29	68	74	32498868	32498935	32502511
[5]	48	73	72	32502572	32502644	32503436
...

```

[52]      19      74      58  7578481  7578554  7579312
[53]      35      75      25  7579516  7579590  7579700
[54]      25      22      56  7579700  7579721  7579839
[55]      29      66      67  7579875  7579940  7590695
[56]       6      54      62  7591826  7591879  7591966

```

```

      part2_end
      <numeric>
 [1] 32495942
 [2] 32497196
 [3] 32498854
 [4] 32502584
 [5] 32503507
 ...      ...
[52] 7579369
[53] 7579724
[54] 7579894
[55] 7590761
[56] 7592027
---
```

```

seqlengths:
  chr1 chr17 chr2 chr5 chr7 chr9
   NA   NA   NA   NA   NA   NA

```

```

> load(system.file("extdata/refseq", "spllicemax.RData", package="customProDB"))
> load(system.file("extdata/refseq", "ids.RData", package="customProDB"))
> junction_type <- JunctionType(jun, spllicemax, txdb, ids)
> junction_type[10:19,]

```

	seqnames	start	end	width	strand	id	cov
10	chr2	48032846	48033343	498	+	JUNC00057364	12
11	chr2	48033497	48033591	95	+	JUNC00057365	10
12	chr2	48035386	48035468	83	-	JUNC00057367	9
13	chr5	112200429	112203101	2673	+	JUNC00080007	23
14	chr7	140706335	140710219	3885	-	JUNC00096159	15
15	chr9	86584295	86585077	783	-	JUNC00101237	14
16	chr9	86585246	86585652	407	-	JUNC00101239	171
17	chr9	86585734	86585812	79	-	JUNC00101240	80
18	chr9	86585827	86586188	362	-	JUNC00101241	121
19	chr17	37856564	37863243	6680	+	JUNC00043382	57

	part1_len	part2_len	part1_sta	part1_end	part2_sta	part2_end
10	65	28	48032782	48032846	48033343	48033370
11	43	64	48033455	48033497	48033591	48033654
12	75	53	48035312	48035386	48035468	48035520
13	74	67	112200356	112200429	112203101	112203167
14	53	66	140706283	140706335	140710219	140710284
15	60	72	86584236	86584295	86585077	86585148

```

16      69      73 86585178 86585246 86585652 86585724
17      68      16 86585667 86585734 86585812 86585827
18      16      75 86585812 86585827 86586188 86586262
19      74      74 37856491 37856564 37863243 37863316
      part1_type      part2_type part1_exon
10 known exon (same end) known exon (same start)      18
11 known exon (same end) known exon (same start)      19
12      non-exon region      non-exon region      NA
13      non-exon region      non-exon region      NA
14      non-exon region      non-exon region      NA
15      non-exon region      non-exon region      NA
16      non-exon region      non-exon region      NA
17      non-exon region      non-exon region      NA
18      non-exon region      non-exon region      NA
19 known exon (same end) known exon (same start)      61
      part2_exon      jun_type tx_id_part1 tx_name_part1
10      19      known junction      2      NM_000179
11      20      known junction      2      NM_000179
12      NA connect two non-exon region      <NA>      <NA>
13      NA connect two non-exon region      <NA>      <NA>
14      NA connect two non-exon region      <NA>      <NA>
15      NA connect two non-exon region      <NA>      <NA>
16      NA connect two non-exon region      <NA>      <NA>
17      NA connect two non-exon region      <NA>      <NA>
18      NA connect two non-exon region      <NA>      <NA>
19      62      known junction      7      NM_004448
      ge_name_part1 tx_id_part2 tx_name_part2 ge_name_part2
10      MSH6      2      NM_000179      MSH6
11      MSH6      2      NM_000179      MSH6
12      <NA>      <NA>      <NA>      <NA>
13      <NA>      <NA>      <NA>      <NA>
14      <NA>      <NA>      <NA>      <NA>
15      <NA>      <NA>      <NA>      <NA>
16      <NA>      <NA>      <NA>      <NA>
17      <NA>      <NA>      <NA>      <NA>
18      <NA>      <NA>      <NA>      <NA>
19      ERBB2      7      NM_004448      ERBB2

```

```
> table(junction_type[, 'jun_type'])
```

```

connect a known exon and a region overlap with known exon      1
      connect two non-exon region      9
      known junction      46

```

Except for 'known junction', all others are treated as putative novel junctions. Then all putative novel junctions are three-frame translated into peptides using the function `OutputNovelJun`. The reference genome sequence is required when using this function.

```
> outf_junc <- paste(tempdir(), '/test_junc.fasta', sep='')
> library('BSgenome.Hsapiens.UCSC.hg19')
> OutputNovelJun <- OutputNovelJun(junction_type, Hsapiens, outf_junc,
+     proteinseq)
```

4 Building database from multiple samples

We provide two functions to help generate a consensus database from multiple samples, especially for a group of similar samples. Even though deep sequencing reveals large scales of heterogeneity, consensus protein database consisting of the commonly expressed proteins and SNVs from a group of samples with similar genetic background will help identify subtype specific proteins.

4.1 Filtering based on transcript expression in multiple samples

The function `OutputsharedPro` outputs proteins with expression level above the cutoff in multiple samples. Unlike `Outputproseq` that uses vector as input, the function `Outputsharedpro` uses expression matrix as input. Users need to specify both the value of sample number and the RPKM cutoff when calling this function. Users could generate RPKM matrix from multiple BAM files as follows, or use RPKM matrix generated by other programs.

```
> path <- system.file("extdata/bams", package="customProDB")
> bamFile<- paste(path, '/', list.files(path, pattern="*bam$"), sep='')
> rpkm <- sapply(bamFile, function(x)
+     calculateRPKM(x, exon, proteincodingonly=TRUE, ids))
> #colnames(rpkm) <- c('1', '2', '3')
> #rpkm
> outfile <- paste(tempdir(), '/test_rpkm_share.fasta', sep='')
> pro <- OutputsharedPro(rpkm, cutoff=1, share_sample=2, proteinseq,
+     outfile, ids)
```

4.2 Variations occurred in multiple samples

The function `Multiple_VCF` outputs variations occurred in more than k samples, with the k specified by a user input parameter. When recurrent variations are identified, the following analysis is the same as shown in the 'Variation annotation' section.

```
> path <- system.file("extdata/vcfs", package="customProDB")
> vcfFiles<- paste(path, '/', list.files(path, pattern="*vcf$"), sep='')
> vcfs <- lapply(vcfFiles, function(x) InputVcf(x))
> shared <- Multiple_VCF(vcfs, share_num=2)
> shared
```

GRanges with 62 ranges and 3 metadata columns:

	seqnames	ranges	strand	REF
	<Rle>	<IRanges>	<Rle>	<character>
test.T/C	chr1	[32386425, 32386425]	*	T
test.G/T	chr1	[32507666, 32507666]	*	G
test.A/C	chr1	[32524459, 32524459]	*	A
test.G/A	chr1	[32622505, 32622505]	*	G
test.CAA/C	chr12	[25357574, 25357576]	*	CAA
...
test.G/C	chr9	[86593314, 86593314]	*	G
test.C/T	chr9	[86595070, 86595070]	*	C
test.G/A	chr9	[86595498, 86595498]	*	G
test.T/A	chr5	[112154737, 112154737]	*	T
test.G/T	chr5	[112175897, 112175897]	*	G

	ALT	INDEL
	<character>	<logical>
test.T/C	C	FALSE
test.G/T	T	FALSE
test.A/C	C	FALSE
test.G/A	A	FALSE
test.CAA/C	C	TRUE
...
test.G/C	C	FALSE
test.C/T	T	FALSE
test.G/A	A	FALSE
test.T/A	A	FALSE
test.G/T	T	FALSE

seqlengths:

chr1	chr12	chr17	chr2	chr5	chr7	chr9
NA	NA	NA	NA	NA	NA	NA

4.3 Junctions occurred in multiple samples

The function SharedJunc outputs splice junctions occurred in more than k samples, with the k specified by a user input parameter. When recurrent junctions are ready, the following analysis is the same as shown in the 'Splice junction analysis' section.

```
> path <- system.file("extdata/beds", package="customProDB")
> bedFiles<- paste(path, '/', list.files(path, pattern="*bed$"), sep='')
> juncs <- lapply(bedFiles, function(x) Bed2Range(x, skip=1, covfilter=5))
> sharedjun <- SharedJunc(juncs, share_num=2, ext_up=100, ext_down=100)
> sharedjun
```

GRanges with 55 ranges and 8 metadata columns:

seqnames	ranges	strand	id	cov
----------	--------	--------	----	-----

	<Rle>	<IRanges>	<Rle>		<character>	<integer>
[1]	chr1	[32479978, 32495899]	+		JUNC1	3
[2]	chr1	[32496023, 32497125]	+		JUNC2	3
[3]	chr1	[32497241, 32498789]	+		JUNC3	2
[4]	chr1	[32498935, 32502511]	+		JUNC4	2
[5]	chr1	[32502644, 32503436]	+		JUNC5	3
...
[51]	chr17	[7578554, 7579312]	-		JUNC51	3
[52]	chr17	[7579590, 7579700]	-		JUNC52	2
[53]	chr17	[7579721, 7579839]	-		JUNC53	3
[54]	chr17	[7579940, 7590695]	-		JUNC54	3
[55]	chr17	[7591879, 7591966]	+		JUNC55	3

	part1_len	part2_len	part1_sta	part1_end	part2_sta	part2_end
	<numeric>	<numeric>	<numeric>	<integer>	<integer>	<numeric>
[1]	101	101	32479878	32479978	32495899	32495999
[2]	101	101	32495923	32496023	32497125	32497225
[3]	101	101	32497141	32497241	32498789	32498889
[4]	101	101	32498835	32498935	32502511	32502611
[5]	101	101	32502544	32502644	32503436	32503536
...
[51]	101	101	7578454	7578554	7579312	7579412
[52]	101	101	7579490	7579590	7579700	7579800
[53]	101	101	7579621	7579721	7579839	7579939
[54]	101	101	7579840	7579940	7590695	7590795
[55]	101	101	7591779	7591879	7591966	7592066

seqlengths:

chr1	chr17	chr2	chr5	chr7	chr9
NA	NA	NA	NA	NA	NA

5 Two integrated functions

We provide two integrated functions for the one-step generation of customized databases.

`easyrun` generates a customized database from single sample.

```
> bamFile <- system.file("extdata/bams", "test1_sort.bam",
+                         package="customProDB")
> vcffile <- system.file("extdata/vcfs", "test1.vcf", package="customProDB")
> bedfile <- system.file("extdata", "junctions.bed", package="customProDB")
> annotation_path <- system.file("extdata/refseq", package="customProDB")
> outfile_path <- tempdir()
> outfile_name='test'
> easyRun(bamFile, RPKM=NULL, vcffile, annotation_path, outfile_path,
+         outfile_name, rpkm_cutoff=1, INDEL=TRUE, lablersid=TRUE, COSMIC=TRUE,
+         nov_junction=FALSE)
```

`easyrun_mul` generates a consensus database from multiple samples.

```
> bampath <- system.file("extdata/bams", package="customProDB")
> vcfile_path <- system.file("extdata/vcfs", package="customProDB")
> annotation_path <- system.file("extdata/refseq", package="customProDB")
> outfile_path <- tempdir()
> outfile_name <- 'mult'
> easyRun_mul(bampath, RPKM_mtx=NULL, vcfile_path, annotation_path, rpkm_cutoff=1,
+             share_num=2, var_shar_num=2, outfile_path, outfile_name, INDEL=TRUE,
+             lablersid=TRUE, COSMIC=TRUE, nov_junction=FALSE)
```

6 FASTA file format

The primary outputs of this package are FASTA files. Related information, such as gene symbol, gene description, variation position, change status, and corresponding dbSNP ID (if required and available), are included in the sequence header for interpretation of the search result. There are four types of headers in the FASTA file.

6.1 Normal proteins passing the expression cutoff

The header starts with RefSeq protein id, followed by RPKM/FPKM value in each sample (separated by ';') and the average RPKM/FPKM, RefSeq transcript id, gene symbol and description.

```
> outfile_path <- system.file("extdata/tmp", package="customProDB")
> readLines(file(paste(outfile_path, '/test_rpkm.fasta', sep=''), 'rt'), 1)
```

```
[1] ">NP_004439 |148172.2567|NM_004448|ERBB2|receptor tyrosine-protein kinase erbB-2 isoform a p
```

6.2 Variant Proteins induced by SNVs

The variation information, including variation position, amino acid change status and corresponding dbSNP ID (if available), is added to the RefSeq protein id followed by '_'. Different variations are separated by ','.

```
> readLines(file(paste(outfile_path, '/test_snv.fasta', sep=''), 'rt'), 1)
```

```
[1] ">NP_000170_T139I,E956D |15810.2686|NM_000179|MSH6|DNA mismatch repair protein Msh6"
```

6.3 Aberrant proteins induced by INDELS

The INDEL information is added to protein id followed by '_'. Here the INDELS position represents the position where this INDELS occurs in a coding sequence, not the position in protein sequence, which is different from proteins with SNVs.

```
> readLines(file(paste(outfile_path, '/test_indel.fasta', sep=''), 'rt'), 1)
```

```
[1] ">NP_004439_3508:CCC>C |148172.2567|NM_004448|ERBB2|receptor tyrosine-protein kinase erbB-2
```


6.4 Novel junction peptides

The junction id, genomic position, coverage (For single sample, it's the reads coverage. For multiple samples, it's the sample coverage), ORF, the source of left/right part and the junction type are added to the ID line of the FASTA file.

```
> readLines(file(paste(outfile_path, '/test_junc.fasta', sep=''), 'rt'), 1)
[1] ">JUNC00041588|6|ORF1|Junpos:18-19|+|NA|NA|connect two non-exon region"
```

7 Session Information

R version 3.0.1 (2013-05-16)

Platform: x86_64-redhat-linux-gnu (64-bit)

locale:

```
[1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
[3] LC_TIME=en_US.UTF-8      LC_COLLATE=C
[5] LC_MONETARY=en_US.UTF-8  LC_MESSAGES=en_US.UTF-8
[7] LC_PAPER=C                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  stats      graphics  grDevices  utils      datasets
[7] methods   base
```

other attached packages:

```
[1] BSgenome.Hsapiens.UCSC.hg19_1.3.19
[2] BSgenome_1.29.0
[3] GenomicFeatures_1.13.15
[4] Rsamtools_1.13.17
[5] Biostrings_2.29.6
[6] GenomicRanges_1.13.18
[7] XVector_0.1.0
[8] customProDB_1.1.1
[9] biomaRt_2.17.1
[10] AnnotationDbi_1.23.15
[11] Biobase_2.21.4
[12] IRanges_1.19.11
[13] BiocGenerics_0.7.2
```

loaded via a namespace (and not attached):

```
[1] DBI_0.2-7          RCurl_1.95-4.1
[3] RSQLite_0.11.4    VariantAnnotation_1.7.28
[5] XML_3.96-1.1      bitops_1.0-5
```

```
[7] plyr_1.8           rtracklayer_1.21.5
[9] stats4_3.0.1       stringr_0.6.2
[11] tools_3.0.1        zlibbioc_1.7.0
```