

Introduction to *customProDB*

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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. Use the code below to check the current dbSNP versions for a specified genome provided by the UCSC table browser.

```
> library('rtracklayer')
> session <- browserSession()
> genome(session) <- 'hg19'
> dbsnps <- trackNames(session)[grep('snp', trackNames(session), fixed=T)]
> dbsnps
```

```
SNPedia
"snpedia"
```

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, ClinVar=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. Go to website <http://useast.ensembl.org/info/website/archives/index.html> to check the currently available archives. It took about 1.5 hour to prepare all annotations for ENSEMBL v82 in our tests.

```
> ensembl <- useMart("ENSEMBL_MART_ENSEMBL", dataset="hsapiens_gene_ensembl",
+   host="may2015.archive.ensembl.org", path="/biomart/martservice",
+   archive=FALSE)
> annotation_path <- tempdir()
> transcript_ids <- c("ENST00000234420", "ENST00000269305", "ENST00000445888",
+   "ENST00000257430", "ENST00000508376", "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"))
> outfl <- paste(tempdir(), '/test_rpkm.fasta', sep='')
> Outputproseq(RPKM, 1, proteinseq, outfl, ids)
```



```

                <numeric> <logical> <integer> <integer> <numeric>
chr1:32386425_T/C      NA      FALSE      <NA>      <NA>      NA
chr1:32507666_G/T     0.36   FALSE      <NA>      <NA>      NA
chr1:32524459_A/C     1.00   FALSE      <NA>      <NA>      NA
                QCHI2      PR      GT      GQ
                <integer> <integer> <character> <character>
chr1:32386425_T/C     <NA>   <NA>      1/1      15
chr1:32507666_G/T     <NA>   <NA>      0/1      36
chr1:32524459_A/C     <NA>   <NA>      0/1      30
                DP.1      SP      PL      PL.1
                <character> <character> <character> <character>
chr1:32386425_T/C     <NA>   <NA>      56      9
chr1:32507666_G/T     <NA>   <NA>      35      0
chr1:32524459_A/C     <NA>   <NA>      31      0
                PL.2      PL.3      PL.4      PL.5
                <character> <character> <character> <character>
chr1:32386425_T/C     0      56      9      0
chr1:32507666_G/T     78     35      0      78
chr1:32524459_A/C     98     31      0      98

```

```

-----
seqinfo: 7 sequences from an unspecified genome; no seqlengths

```

```

> # 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 1.

| Label | Description |
|------------------|--|
| Intergenic | Out of transcripts boundary |
| Intron_noncoding | Located in introns of non-coding transcripts |
| Exon_noncoding | 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 1: 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
      refbase 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", "procodingseq.RData", package="customProDB"))
> txlist <- unique(postable_snv[, 'txid'])
> codingseq <- procodingseq[procodingseq[, '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
      refbase 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 2. 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 2: 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 object with 56 ranges and 8 metadata columns:

```
      seqnames      ranges strand |      id      cov
      <Rle>      <IRanges> <Rle> | <character> <integer>
[1]   chr1 32479978-32495899      + | JUNC00002865      8
[2]   chr1 32496023-32497125      + | JUNC00002866     13
[3]   chr1 32497241-32498789      + | JUNC00002868     20
[4]   chr1 32498935-32502511      + | JUNC00002869     29
[5]   chr1 32502644-32503436      + | JUNC00002871     48
...     ...
[52] chr17  7578554-7579312      - | JUNC00041584     19
[53] chr17  7579590-7579700      - | JUNC00041585     35
[54] chr17  7579721-7579839      - | JUNC00041586     25
[55] chr17  7579940-7590695      - | JUNC00041587     29
[56] chr17  7591879-7591966      + | JUNC00041588      6
      part1_len part2_len part1_sta part1_end part2_sta part2_end
      <numeric> <numeric> <numeric> <numeric> <numeric> <numeric>
[1]         69         44  32479910  32479978  32495899  32495942
[2]         73         72  32495951  32496023  32497125  32497196
[3]         66         66  32497176  32497241  32498789  32498854
[4]         68         74  32498868  32498935  32502511  32502584
[5]         73         72  32502572  32502644  32503436  32503507
...     ...
[52]         74         58   7578481   7578554   7579312   7579369
```

```
[53]      75      25  7579516  7579590  7579700  7579724
[54]      22      56  7579700  7579721  7579839  7579894
[55]      66      67  7579875  7579940  7590695  7590761
[56]      54      62  7591826  7591879  7591966  7592027
```

seqinfo: 6 sequences from an unspecified genome; no seqlengths

```
> load(system.file("extdata/refseq", "splicemax.RData", package="customProDB"))
> load(system.file("extdata/refseq", "ids.RData", package="customProDB"))
> junction_type <- JunctionType(jun, splicemax, 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='')
> rpkms <- sapply(bamFile, function(x)
+               calculateRPKM(x, exon, proteincodingonly=TRUE, ids))
> #colnames(rpkms) <- c('1', '2', '3')
> #rpkms
> outfile <- paste(tempdir(), '/test_rpkm_share.fasta', sep='')
> pro <- OutputsharedPro(rpkms, 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")
> vcfsFiles<- paste(path, '/', list.files(path, pattern="*vcf$"), sep='')
> vcfs <- lapply(vcfsFiles, function(x) InputVcf(x))
> shared <- Multiple_VCF(vcfs, share_num=2)
> shared
```

GRanges object with 62 ranges and 3 metadata columns:

| | seqnames | ranges | strand | |
|---------------------------|-------------|-------------------|-----------|----|
| | <Rle> | <IRanges> | <Rle> | |
| test.chr1:32386425_T/C | chr1 | 32386425 | * | |
| test.chr1:32507666_G/T | chr1 | 32507666 | * | |
| test.chr1:32524459_A/C | chr1 | 32524459 | * | |
| test.chr1:32622505_G/A | chr1 | 32622505 | * | |
| test.chr12:25357574_CAA/C | chr12 | 25357574-25357576 | * | |
| ... | ... | ... | ... | .. |
| test.chr9:86593314_G/C | chr9 | 86593314 | * | |
| test.chr9:86595070_C/T | chr9 | 86595070 | * | |
| test.chr9:86595498_G/A | chr9 | 86595498 | * | |
| test.chr5:112154737_T/A | chr5 | 112154737 | * | |
| test.chr5:112175897_G/T | chr5 | 112175897 | * | |
| | REF | ALT | INDEL | |
| | <character> | <character> | <logical> | |

```

test.chr1:32386425_T/C      T      C      FALSE
test.chr1:32507666_G/T      G      T      FALSE
test.chr1:32524459_A/C      A      C      FALSE
test.chr1:32622505_G/A      G      A      FALSE
test.chr12:25357574_CAA/C   CAA    C      TRUE
...
test.chr9:86593314_G/C      G      C      FALSE
test.chr9:86595070_C/T      C      T      FALSE
test.chr9:86595498_G/A      G      A      FALSE
test.chr5:112154737_T/A     T      A      FALSE
test.chr5:112175897_G/T     G      T      FALSE

```

seqinfo: 7 sequences from an unspecified genome; no seqlengths

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 object with 55 ranges and 8 metadata columns:

```

      seqnames      ranges strand |      id      cov
      <Rle>        <IRanges> <Rle> | <character> <numeric>
[1]   chr1 32479978-32495899      + |   JUNC1      8
[2]   chr1 32496023-32497125      + |   JUNC2     13
[3]   chr1 32497241-32498789      + |   JUNC3     20
[4]   chr1 32498935-32502511      + |   JUNC4     29
[5]   chr1 32502644-32503436      + |   JUNC5     48
...
[51]  chr17  7578554-7579312      - |  JUNC51     19
[52]  chr17  7579590-7579700      - |  JUNC52     35
[53]  chr17  7579721-7579839      - |  JUNC53     25
[54]  chr17  7579940-7590695      - |  JUNC54     29
[55]  chr17  7591879-7591966      + |  JUNC55      6
      part1_len part2_len part1_sta part1_end part2_sta part2_end
      <numeric> <numeric> <numeric> <integer> <integer> <numeric>
[1]      69      44  32479910  32479978  32495899  32495944
[2]      73      72  32495951  32496023  32497125  32497198
[3]      66      66  32497176  32497241  32498789  32498856
[4]      68      74  32498868  32498935  32502511  32502586

```

```

[5]      73      72 32502572 32502644 32503436 32503509
...      ...      ...      ...      ...      ...
[51]     74     58 7578481 7578554 7579312 7579371
[52]     75     25 7579516 7579590 7579700 7579726
[53]     22     56 7579700 7579721 7579839 7579896
[54]     66     67 7579875 7579940 7590695 7590763
[55]     54     62 7591826 7591879 7591966 7592029

```

seqinfo: 6 sequences from an unspecified genome; no seqlengths

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")
> vcffile_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, vcffile_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"
```

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 Under development (unstable) (2022-10-25 r83175)
Platform: x86_64-pc-linux-gnu (64-bit)
Running under: Ubuntu 22.04.1 LTS
```

```
Matrix products: default
BLAS: /home/biocbuild/bbs-3.17-bioc/R/lib/libRblas.so
```


LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.10.0

locale:

```
[1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C
[3] LC_TIME=en_GB            LC_COLLATE=C
[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] stats4      stats      graphics  grDevices  utils      datasets
[7] methods    base
```

other attached packages:

```
[1] BSgenome.Hsapiens.UCSC.hg19_1.4.3
[2] BSgenome_1.67.0
[3] Biostrings_2.67.0
[4] XVector_0.39.0
[5] GenomicFeatures_1.51.0
[6] customProDB_1.39.0
[7] biomaRt_2.55.0
[8] AnnotationDbi_1.61.0
[9] Biobase_2.59.0
[10] rtracklayer_1.59.0
[11] GenomicRanges_1.51.0
[12] GenomeInfoDb_1.35.0
[13] IRanges_2.33.0
[14] S4Vectors_0.37.0
[15] BiocGenerics_0.45.0
```

loaded via a namespace (and not attached):

```
[1] KEGGREST_1.39.0          SummarizedExperiment_1.29.0
[3] AhoCorasickTrie_0.1.2    rjson_0.2.21
[5] lattice_0.20-45         RMariaDB_1.2.2
[7] vctrs_0.5.0             tools_4.3.0
[9] bitops_1.0-7            generics_0.1.3
[11] curl_4.3.3              parallel_4.3.0
[13] tibble_3.1.8            fansi_1.0.3
[15] RSQLite_2.2.18          blob_1.2.3
[17] pkgconfig_2.0.3         Matrix_1.5-1
[19] dbplyr_2.2.1            assertthat_0.2.1
[21] lifecycle_1.0.3         GenomeInfoDbData_1.2.9
[23] compiler_4.3.0          stringr_1.4.1
[25] Rsamtools_2.15.0        progress_1.2.2
```

| | | |
|------|--------------------------|-----------------------|
| [27] | codetools_0.2-18 | RCurl_1.98-1.9 |
| [29] | yaml_2.3.6 | pillar_1.8.1 |
| [31] | crayon_1.5.2 | ellipsis_0.3.2 |
| [33] | BiocParallel_1.33.0 | DelayedArray_0.25.0 |
| [35] | cachem_1.0.6 | tidyselect_1.2.0 |
| [37] | digest_0.6.30 | stringi_1.7.8 |
| [39] | VariantAnnotation_1.45.0 | dplyr_1.0.10 |
| [41] | restfulr_0.0.15 | fastmap_1.1.0 |
| [43] | grid_4.3.0 | cli_3.4.1 |
| [45] | magrittr_2.0.3 | utf8_1.2.2 |
| [47] | XML_3.99-0.12 | rappdirs_0.3.3 |
| [49] | filelock_1.0.2 | prettyunits_1.1.1 |
| [51] | bit64_4.0.5 | lubridate_1.8.0 |
| [53] | httr_1.4.4 | matrixStats_0.62.0 |
| [55] | bit_4.0.4 | png_0.1-7 |
| [57] | hms_1.1.2 | memoise_2.0.1 |
| [59] | BiocIO_1.9.0 | BiocFileCache_2.7.0 |
| [61] | rlang_1.0.6 | Rcpp_1.0.9 |
| [63] | glue_1.6.2 | DBI_1.1.3 |
| [65] | xml2_1.3.3 | plyr_1.8.7 |
| [67] | R6_2.5.1 | MatrixGenerics_1.11.0 |
| [69] | GenomicAlignments_1.35.0 | zlibbioc_1.45.0 |