

# Rsubread package: high-performance read alignment, quantification and mutation discovery

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14 September 2015

## 1 Introduction

This vignette provides a brief description to the Rsubread package. For more details, please refer to the Users Guide which can be brought up in your R session via the following commands:

```
> library(Rsubread)
> RsubreadUsersGuide()
```

The Rsubread package provides facilities for processing the read data generated by next-gen sequencing technologies. These facilities include quality assessment, read alignment, read summarization, exon-exon junction detection, absolute expression calling and SNP discovery. They can be used to analyze data generated from all major sequencing platforms including Illumina GA/HiSeq, Roche 454, ABI SOLiD and Ion Torrent.

The Subread aligner (**align** function) is a highly efficient and accurate aligner for mapping genomic DNA and RNA sequencing reads. It adopts a novel mapping paradigm called “seed-and-vote”. Under this paradigm, a number of 16mers (called seeds or sub-reads) are extracted from each read and they were mapped to the reference genome to vote for the mapping location of the read. Read mapping performed under this paradigm has been found to be more efficient and accurate than that carried out under the conventional “seed-and-extend” paradigm (Liao et al. 2013). This package also includes a program for detecting exon-exon junctions, **subjunc**, that makes use of the “seed-and-vote” paradigm as well.

An important step in processing next-gen sequencing data is to assign mapped reads to genomic features such as genes, exons, and promoters. This package includes a general-purpose read summarization function **featureCounts** that takes mapped reads as input and assigns them to genomic features. In-built annotations are provided for users convenience.

Different from microarray technologies, the next-gen sequencing technologies do not provide Present/Absent calls for genomic features such as genes. We have developed

an algorithm to use the background noise measured from the RNA-seq data to call absolutely expressed genes. The function `detectionCall` returns a detection p value for each gene from the read mapping results.

We have also developed a new SNP calling algorithm which is being implemented in function `exactSNPs`. Our results showed that it compared favorably to competing methods, but was an order of magnitude faster.

This package also includes some other useful functions such as quality assessment (`qualityScores`, `atgcContent`), duplicate read removal (`removeDupReads`) and mapping percentage calculation (`propmapped`).

## 2 Read alignment

An index needs to be built first and then alignments can be carried out. Building the index is an one-off operation. The generated index can be re-used in subsequent read alignments.

### Step 1: Index building

The Rsubread package includes a dummy reference sequence that was generated by concatenating 900 100bp reads that were taken from a pilot dataset generated from the SEquencing Quality Control (SEQC) project. We further extracted 100 reads from the same dataset and combine them with the 900 reads to make a read dataset for mapping. Below is the command for building an index for the reference sequence:

```
> library(Rsubread)
> ref <- system.file("extdata", "reference.fa", package="Rsubread")
> buildindex(basename="reference_index", reference=ref)

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Rsubread 1.32.4

//===== indexBuilder setting =====\\
||
||           Index name : reference_index           ||
||           Index space : base-space               ||
||           One block index : yes                  ||
||           Repeat threshold : 100 repeats          ||
||           Distance to next subread : 1            ||
||
||           Input files : 1 file in total           ||
||                   o reference.fa                 ||
||
||===== http://subread.sourceforge.net/ =====//

//===== Running =====\\
||
```

```

|| Check the integrity of provided reference sequences ... ||
|| No format issues were found ||
|| Scan uninformative subreads in reference sequences ... ||
|| 8%, 0 mins elapsed, rate=6.7k bps/s, total=0m ||
|| 16%, 0 mins elapsed, rate=13.4k bps/s, total=0m ||
|| 24%, 0 mins elapsed, rate=20.0k bps/s, total=0m ||
|| 33%, 0 mins elapsed, rate=26.7k bps/s, total=0m ||
|| 41%, 0 mins elapsed, rate=33.3k bps/s, total=0m ||
|| 49%, 0 mins elapsed, rate=39.8k bps/s, total=0m ||
|| 58%, 0 mins elapsed, rate=46.4k bps/s, total=0m ||
|| 66%, 0 mins elapsed, rate=52.8k bps/s, total=0m ||
|| 74%, 0 mins elapsed, rate=59.3k bps/s, total=0m ||
|| 83%, 0 mins elapsed, rate=65.8k bps/s, total=0m ||
|| 91%, 0 mins elapsed, rate=72.3k bps/s, total=0m ||
|| 99%, 0 mins elapsed, rate=78.6k bps/s, total=0m ||
|| 1 uninformative subreads were found. ||
|| These subreads were excluded from index building. ||
|| Build the index... ||
|| 8%, 0 mins elapsed, rate=828.4k bps/s, total=0m ||
|| 16%, 0 mins elapsed, rate=745.6k bps/s, total=0m ||
|| 24%, 0 mins elapsed, rate=721.5k bps/s, total=0m ||
|| 33%, 0 mins elapsed, rate=693.6k bps/s, total=0m ||
|| 41%, 0 mins elapsed, rate=703.4k bps/s, total=0m ||
|| 49%, 0 mins elapsed, rate=699.0k bps/s, total=0m ||
|| 58%, 0 mins elapsed, rate=695.9k bps/s, total=0m ||
|| 66%, 0 mins elapsed, rate=701.7k bps/s, total=0m ||
|| 74%, 0 mins elapsed, rate=699.0k bps/s, total=0m ||
|| 83%, 0 mins elapsed, rate=696.8k bps/s, total=0m ||
|| 91%, 0 mins elapsed, rate=701.0k bps/s, total=0m ||
|| 99%, 0 mins elapsed, rate=699.0k bps/s, total=0m ||
|| Save current index block... ||
|| [ 0.0% finished ] ||
|| [ 10.0% finished ] ||
|| [ 20.0% finished ] ||
|| [ 30.0% finished ] ||
|| [ 40.0% finished ] ||
|| [ 50.0% finished ] ||
|| [ 60.0% finished ] ||
|| [ 70.0% finished ] ||
|| [ 80.0% finished ] ||
|| [ 90.0% finished ] ||
|| [ 100.0% finished ] ||
|| ||
|| Total running time: 0.2 minutes. ||
|| Index reference_index was successfully built! ||
|| ||
\\===== http://subread.sourceforge.net/ =====//

```

The generated index files were saved to the current working directory. Rsubread creates a hash table for indexing the reference genome. Keys in the hash table are the 16bp sequences and hash values are their corresponding chromosomal locations. Color space index can be built by setting the `colorsapce` argument to `TRUE`.

A unique feature of Rsubread is that it allows users to control the computer memory usage in read mapping process. Users can do this by tuning the amount of memory (in MB) to be used in read mapping.

## Step 2: read mapping

After the index was successfully built, we map the read dataset (including 1,000 reads) to the reference sequence:

```
> reads <- system.file("extdata","reads.txt.gz",package="Rsubread")
> align(index="reference_index",readfile1=reads,output_file="alignResults.BAM",phredOffset=64)
```

```
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Rsubread 1.32.4
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```
//===== subread-align setting =====\\
||
|| Function      : Read alignment (RNA-Seq)
|| Input file    : reads.txt.gz
|| Output file   : alignResults.BAM (BAM)
|| Index name    : reference_index
||
|| -----
||
||                      Threads : 1
||                      Phred offset : 64
||                      Min votes : 3 / 10
||                      Max mismatches : 3
||                      Max indel length : 5
||                      Report multi-mapping reads : yes
|| Max alignments per multi-mapping read : 1
||
\\===== http://subread.sourceforge.net/ =====//

//===== Running (06-Mar-2019 19:23:54, pid=61164) =====\\
||
|| The input file contains base space reads.
|| The range of Phred scores observed in the data is [2,34]
|| Load the 1-th index block...
||
||                      Completed successfully.
||
\\=====//

//===== Summary =====\\
||
||      Total reads : 1,000
||      Mapped : 904 (90.4%)
||      Uniquely mapped : 904
||      Multi-mapping : 0
||
||      Unmapped : 96
||
||      Indels : 0
||
||      Running time : 0.0 minutes
||
\\===== http://subread.sourceforge.net/ =====//

Category alignResults.BAM
1      Total_reads      1000
2      Mapped_reads     904
3      Uniquely_mapped_reads 904
4      Multi_mapping_reads 0
```

```

5      Unmapped_reads      96
6      Indels              0

```

Map paired-end reads:

```

> reads1 <- system.file("extdata","reads1.txt.gz",package="Rsubread")
> reads2 <- system.file("extdata","reads2.txt.gz",package="Rsubread")
> align(index="reference_index",readfile1=reads1,readfile2=reads2,
+ output_file="alignResultsPE.BAM",phredOffset=64)

```

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Rsubread 1.32.4

```

```

//===== subread-align setting =====\\
||
|| Function      : Read alignment (RNA-Seq)
|| Input file 1  : reads1.txt.gz
|| Input file 2  : reads2.txt.gz
|| Output file   : alignResultsPE.BAM (BAM)
|| Index name    : reference_index
||
|| -----
||
|| Threads      : 1
|| Phred offset  : 64
|| # of extracted subreads : 10
||   Min read1 vote : 3
||   Min read2 vote : 1
||   Max fragment size : 600
||   Min fragment size : 50
||   Max mismatches : 3
||   Max indel length : 5
|| Report multi-mapping reads : yes
|| Max alignments per multi-mapping read : 1
||
|| \\===== http://subread.sourceforge.net/ =====//
//===== Running (06-Mar-2019 19:23:56, pid=61164) =====\\
||
|| The input file contains base space reads.
|| The range of Phred scores observed in the data is [2,34]
|| Load the 1-th index block...
||
|| Completed successfully.
||
|| \\=====//
//===== Summary =====\\
||
|| Total fragments : 1,000
||   Mapped : 909 (90.9%)
|| Uniquely mapped : 909
||   Multi-mapping : 0
||
|| Unmapped : 91
||
|| Correctly paired : 897
|| Not mapped in pairs : 12

```

```

|| Only one end mapped : 10 ||
|| Multi-chromosomes : 0 ||
|| Different strands : 0 ||
|| Not in PE distance : 2 ||
|| Abnormal order : 0 ||
|| ||
|| Indels : 0 ||
|| ||
|| Running time : 0.0 minutes ||
|| ||
\\===== http://subread.sourceforge.net/ =====//

```

	Category	alignResultsPE.BAM
1	Total_fragments	1000
2	Mapped_fragments	909
3	Uniquely_mapped_fragments	909
4	Multi_mapping_fragments	0
5	Unmapped_fragments	91
6	Properly_paired_fragments	897
7	Singleton_fragments	10
8	More_than_one_chr_fragments	0
9	Unexpected_strandness_fragments	0
10	Unexpected_template_length	2
11	Inversed_mapping	0
12	Indels	0

### 3 Counting mapped reads for genomic features

The `featureCounts` function is a general-purpose read summarization function that assigns mapped reads (RNA-seq or gDNA-seq reads) to genomic features such as genes, exons, promoters, gene bodies and genomic bins.

This function takes as input a set of files that contain read mapping results and an annotation file that includes genomic features. It automatically detects the format of input read files (supported formats include SAM and BAM). For paired end reads, it automatically re-orders the reads if reads from the same pair were found not to be next to each other in the input.

In-built NCBI RefSeq gene annotations for genomes mm9, mm10 and hg19 are provided for convenience. These annotations include chromosomal coordinates of exons of each gene. When these annotations are used for summarization, only reads overlapping with exons will be counted by `featureCounts`. Users can use `getInBuiltAnnotation` function to retrieve these annotations.

Below gives the example code of assigning reads and fragments, generated in the last section, to two artificial genes. Assign single end reads to genes:

```

> ann <- data.frame(
+ GeneID=c("gene1", "gene1", "gene2", "gene2"),
+ Chr="chr_dummy",
+ Start=c(100, 1000, 3000, 5000),
+ End=c(500, 1800, 4000, 5500),
+ Strand=c("+", "+", "-", "-"),
+ stringsAsFactors=FALSE)
> ann

```

```

GeneID      Chr Start  End Strand
1 gene1 chr_dummy 100 500 +
2 gene1 chr_dummy 1000 1800 +
3 gene2 chr_dummy 3000 4000 -
4 gene2 chr_dummy 5000 5500 -

> fc_SE <- featureCounts("alignResults.BAM",annot.ext=ann)

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Rsubread 1.32.4

//===== featureCounts setting =====\\
||
||          Input files : 1 BAM file
||                      S alignResults.BAM
||
||          Annotation : R data.frame
||
||      Dir for temp files : .
||          Threads : 1
||          Level : meta-feature level
||          Paired-end : no
||          Multimapping reads : counted
||      Multi-overlapping reads : not counted
||          Min overlapping bases : 1
||
||\\===== http://subread.sourceforge.net/ =====//

//===== Running =====\\
||
|| Load annotation file .Rsubread_UserProvidedAnnotation_pid61164 ...
||      Features : 4
||      Meta-features : 2
||      Chromosomes/contigs : 1
||
|| Process BAM file alignResults.BAM...
||      Single-end reads are included.
||      Assign alignments to features...
||      Total alignments : 1000
||      Successfully assigned alignments : 31 (3.1%)
||      Running time : 0.00 minutes
||
||\\===== http://subread.sourceforge.net/ =====//

> fc_SE

$counts
      X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResults.BAM
gene1                                     14
gene2                                     17

$annotation
      GeneID      Chr      Start      End Strand Length
1 gene1 chr_dummy;chr_dummy 100;1000 500;1800 ++ 1202
2 gene2 chr_dummy;chr_dummy 3000;5000 4000;5500 -;- 1502

$targets
[1] "X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResults.BAM"

```

```

$stat
      Status
1      Assigned
2      Unassigned_Unmapped
3      Unassigned_MappingQuality
4      Unassigned_Chimera
5      Unassigned_FragmentLength
6      Unassigned_Duplicate
7      Unassigned_MultiMapping
8      Unassigned_Secondary
9      Unassigned_NonSplit
10     Unassigned_NoFeatures
11     Unassigned_Overlapping_Length
12     Unassigned_Ambiguity
X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResults.BAM
1                                           31
2                                           96
3                                           0
4                                           0
5                                           0
6                                           0
7                                           0
8                                           0
9                                           0
10                                          873
11                                          0
12                                          0

```

Assign fragments (read pairs) to the two genes:

```
> fc_PE <- featureCounts("alignResultsPE.BAM",annot.ext=ann,isPairedEnd=TRUE)
```

```

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Rsubread 1.32.4

```

```

//===== featureCounts setting =====\\
||
||      Input files : 1 BAM file                ||
||                  P alignResultsPE.BAM        ||
||
||      Annotation : R data.frame                ||
||      Dir for temp files : .                   ||
||      Threads : 1                             ||
||      Level : meta-feature level               ||
||      Paired-end : yes                         ||
||      Multimapping reads : counted             ||
||      Multi-overlapping reads : not counted    ||
||      Min overlapping bases : 1                ||
||
||      Chimeric reads : counted                 ||
||      Both ends mapped : not required          ||
||
||===== http://subread.sourceforge.net/ =====\\

//===== Running =====\\
||
|| Load annotation file .Rsubread_UserProvidedAnnotation_pid61164 ... ||

```



```

|| Features : 4 ||
|| Meta-features : 2 ||
|| Chromosomes/contigs : 1 ||
||
|| Process BAM file alignResultsPE.BAM... ||
|| Paired-end reads are included. ||
|| Assign alignments (paired-end) to features... ||
|| Total alignments : 1000 ||
|| Successfully assigned alignments : 35 (3.5%) ||
|| Running time : 0.00 minutes ||
||
||
\\===== http://subread.sourceforge.net/ =====//

> fc_PE

$counts
  X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResultsPE.BAM
gene1 16
gene2 19

$annotation
  GeneID      Chr      Start      End Strand Length
1 gene1 chr_dummy;chr_dummy 100;1000 500;1800 ++ 1202
2 gene2 chr_dummy;chr_dummy 3000;5000 4000;5500 -;- 1502

$targets
[1] "X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResultsPE.BAM"

$stat
      Status
1      Assigned
2  Unassigned_Unmapped
3  Unassigned_MappingQuality
4  Unassigned_Chimera
5  Unassigned_FragmentLength
6  Unassigned_Duplicate
7  Unassigned_MultiMapping
8  Unassigned_Secondary
9  Unassigned_NonSplit
10 Unassigned_NoFeatures
11 Unassigned_Overlapping_Length
12 Unassigned_Ambiguity
  X.private.tmp.RtmptJk6iP.Rbuilddc945cfec1f1.Rsubread.vignettes.alignResultsPE.BAM
1 35
2 91
3 0
4 0
5 0
6 0
7 0
8 0
9 0
10 874
11 0
12 0

```

## 4 Finding exon junctions

The RNA-seq technology provides a unique opportunity to identify the alternative splicing events that occur during the gene transcription process. The `subjunc` function can

be used to detect exon-exon junctions. It first extracts a number of subreads (16mers) from each read, maps them to the reference genome and identifies the two best mapping locations for each read (representing potential locations of exons spanned by the read). Then, it builds a junction table including all putative junctions. Finally, it carries out a verification step to remove false positives in junction detection by realigning all the reads. The donor ('GT') and receptor sites('AG'), are required to be present when calling exon-exon junctions. Output of this function includes the discovered exon-exon junctions and also read mapping results.

## 5 Base quality scores

Quality scores give the probabilities of read bases being incorrectly called, which is useful for examining the quality of sequencing data. The `qualityScores` function can be used to quickly retrieve and display the quality score data extracted from a read file.

```
> x <- qualityScores(filename=reads,offset=64,nreads=1000)

qualityScores Rsubread 1.32.4

Scan the input file...
Totally 1000 reads were scanned; the sampling interval is 1.
Now extract read quality information...

Completed successfully. Quality scores for 999 reads (equally spaced in the file) are returned.

> x[1:10,1:10]

      1  2  3  4  5  6  7  8  9 10
[1,] 33 33 33 20 20 24 31 15 21 16
[2,] 33 33 30 33 33 30 34 30 32 28
[3,] 32 33 33 32 33 33 33 20 32 24
[4,] 33 33 33 33 33 30 29 34 31 25
[5,] 33 33 33 33 33 34 34 34 33 30
[6,] 33 30 31 24 24 28 33 33 30 32
[7,] 33 33 33 33 30 28 17 25 31 33
[8,] 33 32  2  2  2  2  2  2  2  2
[9,] 33 33 33 34 33 33 31 33 33 33
[10,] 33 33 33 33 28 24 33 33 33 28
```

## 6 GC content

The `atgcContent` function returns fractions of A, T, G and C bases at each base location of reads or in the entire dataset.

## 7 Mapping percentage

Function `propmapped` returns the proportion of mapped reads included in a SAM/BAM file. For paired end reads, it can return the proportion of mapped fragments (ie. read pairs).

```
> propmapped("alignResults.BAM")
```

```

                                     Samples
1 /private/tmp/RtmptJk6iP/Rbuild945cfec1f1/Rsubread/vignettes/alignResults.BAM
  NumTotal NumMapped PropMapped
1      1000       904      0.904
```

## 8 Citation

Yang Liao, Gordon K Smyth and Wei Shi (2013). The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. *Nucleic Acids Research*, 41(10):e108.

Yang Liao, Gordon K Smyth and Wei Shi (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics*, 30(7):923-30

## 9 Authors

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## 10 Contact

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