

# Subread/Rsubread Users Guide

Subread v1.4.3/Rsubread v1.12.6

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# Contents

<b>1</b>	<b>Introduction</b>	<b>3</b>
<b>2</b>	<b>Preliminaries</b>	<b>5</b>
2.1	Citation . . . . .	5
2.2	Download and installation . . . . .	5
2.2.1	SourceForge Subread package . . . . .	5
2.2.2	Bioconductor Rsubread package . . . . .	6
2.3	How to get help . . . . .	7
<b>3</b>	<b>The seed-and-vote mapping paradigm</b>	<b>8</b>
3.1	Seed-and-vote . . . . .	8
3.2	Indel detection . . . . .	9
3.2.1	Detection of short indels . . . . .	9
3.2.2	Detection of long indels . . . . .	10
3.3	Detection of canonical exon-exon junctions . . . . .	10
3.4	Fusion detection . . . . .	11
3.5	Read re-alignments . . . . .	12
3.6	Recommended alignment setting . . . . .	12
<b>4</b>	<b>Mapping reads generated by genomic DNA sequencing technologies</b>	<b>13</b>
4.1	A quick start for using SourceForge Subread package . . . . .	13
4.2	A quick start for using Bioconductor Rsubread package . . . . .	14
4.3	Index building . . . . .	14
4.4	Read mapping . . . . .	15
4.5	Mapping quality scores . . . . .	19
<b>5</b>	<b>Mapping reads generated by RNA sequencing technologies</b>	<b>20</b>
5.1	A quick start for using SourceForge Subread package . . . . .	20
5.2	A quick start for using Bioconductor Rsubread package . . . . .	21
5.3	Local read alignment . . . . .	22
5.4	Global read alignment . . . . .	22

<b>6</b>	<b>Read summarization</b>	<b>23</b>
6.1	Introduction . . . . .	23
6.2	featureCounts . . . . .	24
6.2.1	Input data . . . . .	24
6.2.2	Annotation format . . . . .	24
6.2.3	Single and paired-end reads . . . . .	25
6.2.4	Features and meta-features . . . . .	25
6.2.5	Overlap of reads with features . . . . .	26
6.2.6	Multiple overlaps . . . . .	26
6.2.7	Program usage . . . . .	26
6.3	A quick start for featureCounts in SourceForge Subread . . . . .	30
6.4	A quick start for featureCounts in Bioconductor Rsubread . . . . .	31
<b>7</b>	<b>SNP calling</b>	<b>32</b>
7.1	Algorithm . . . . .	32
7.2	exactSNP . . . . .	32
<b>8</b>	<b>Case studies</b>	<b>35</b>
8.1	A Bioconductor R pipeline for analyzing RNA-seq data . . . . .	35

# Chapter 1

## Introduction

The Subread/Rsubread packages comprise a suite of high-performance software programs for processing next-generation sequencing data. Main programs included in the packages are Subread aligner, Subjunc aligner, **featureCounts** read quantification program and **exactSNP** program for discovering SNPs and indels. This document provides a detailed description to the programs included in the packages.

**Subread** and **Subjunc** aligners adopt a mapping paradigm called “seed-and-vote” [1]. This is an elegantly simple multi-seed strategy for mapping reads to a reference genome. This strategy chooses the mapped genomic location for the read directly from the seeds. It uses a relatively large number of short seeds (called subreads) extracted from each read and allows all the seeds to vote on the optimal location. When the read length is <160 bp, overlapping subreads are used. More conventional alignment algorithms are then used to fill in detailed mismatch and indel information between the subreads that make up the winning voting block. The strategy is fast because the overall genomic location has already been chosen before the detailed alignment is done. It is sensitive because no individual subread is required to map exactly, nor are individual subreads constrained to map close by other subreads. It is accurate because the final location must be supported by several different subreads. The strategy extends easily to find exon junctions, by locating reads that contain sets of subreads mapping to different exons of the same gene. It scales up efficiently for longer reads.

**Subread** is a general-purpose read aligner. It can be used to align reads generated from both genomic DNA sequencing and RNA sequencing technologies. It been successfully used in a number of high-profile studies [2, 3, 4, 5, 6]. **Subjunc** is specifically designed to detect exon-exon junctions and to perform full alignments for RNA-seq reads. Note that **Subread** performs local alignments for RNA-seq reads. Both **Subread** and **Subjunc** detect insertions, deletions, fusions and they perform read alignments after detecting these genomic mutation events. **Subjunc** also perform read re-alignments after detecting the exon-exon junctions.

The **featureCounts** program is designed to assign mapped reads or fragments (paired-end data) to genomic features such as genes, exons and promoters. It is a light-weight read counting program suitable for count both gDNA-seq and RNA-seq reads for genomic features[7]. Also included in this software suite is a very efficient SNP caller – **exactSNP**. **exactSNP** measures local background noise for each candidate SNP and then uses that information to accurately

call SNPs.

These software programs support a variety of sequencing platforms including Illumina GA/HiSeq, ABI SOLiD, Life Science 454, Helicos Heliscope and Ion Torrent. They are released in two packages – SourceForge Subread package and Bioconductor Rsubread package.

# Chapter 2

## Preliminaries

### 2.1 Citation

If you use Subread or Subjunc aligners, please cite:

Liao Y, Smyth GK and Shi W. The Subread aligner: fast, accurate and scalable read mapping by seed-and-vote. Nucleic Acids Research, 41(10):e108, 2013  
<http://nar.oxfordjournals.org/content/early/2013/04/03/nar.gkt214.abstract>

If you use featureCounts, please cite:

Liao Y, Smyth GK and Shi W. featureCounts: an efficient general-purpose program for assigning sequence reads to genomic features. Bioinformatics, accepted on Nov 7, 2013, doi: 10.1093/bioinformatics/btt656  
<http://bioinformatics.oxfordjournals.org/cgi/reprint/btt656?ijkey=ZzPz96t2lqzAH6F&keyref>

### 2.2 Download and installation

#### 2.2.1 SourceForge Subread package

##### Installation from a binary distribution

This is the easiest way to install the Subread package onto your computer. Download a Subread binary distribution that suits your operating system, from the SourceForge website <http://subread.sourceforge.net>. The operating systems currently being supported include multiple variants of Linux (Debian, Ubuntu, Fedora and Cent OS) and Mac OS X. Both 64-bit and 32-bit machines are supported. The executables can be found in the 'bin' directory of the binary package.

To install **Subread** package for other operating systems such as FreeBSD and Solaris, you will have to install them for the source.

### Installation from the source package

Download **Subread** source package from the SourceForge website <http://subread.sourceforge.net> to your local directory. Type the following command to uncompress it:

```
tar zxvf subread-1.x.x.tar.gz
```

Enter the **src** subdirectory under the home directory of this package and then issue the following command to install it on a Linux operating system:

```
make -f Makefile.Linux
```

To install it on a Mac OS X operating system, issue the following command:

```
make -f Makefile.MacOS
```

To install it on a FreeBSD operating system, issue the following command:

```
make -f Makefile.FreeBSD
```

To install it on Oracle Solaris or OpenSolaris computer operating systems, issue the following command:

```
make -f Makefile.SunOS
```

A new subdirectory called **bin** will be created under the home directory of the software package, and the executables generated from the compilation will be saved to that subdirectory. To enable easy access to these executables, you may copy them to a system directory such as **/usr/bin** or add the path to them to your search path (your search path is usually specified in the environment variable **'PATH'**).

### 2.2.2 Bioconductor Rsubread package

You have to get R installed on my computer to install this package. Launch an R session and issue the following command to install it:

```
source("http://bioconductor.org/biocLite.R")
biocLite("Rsubread")
```

Alternatively, you may download the **Rsubread** source package directly from <http://bioconductor.org/packages/release/bioc/html/Rsubread.html> and install it to your R from the source.

## 2.3 How to get help

Bioconductor mailing list (<http://bioconductor.org/>) and SeqAnswer forum (<http://www.seqanswers.com>) are the best places to get help and to report bugs. Alternatively, you may contact Wei Shi (shi at wehi dot edu dot au) directly.

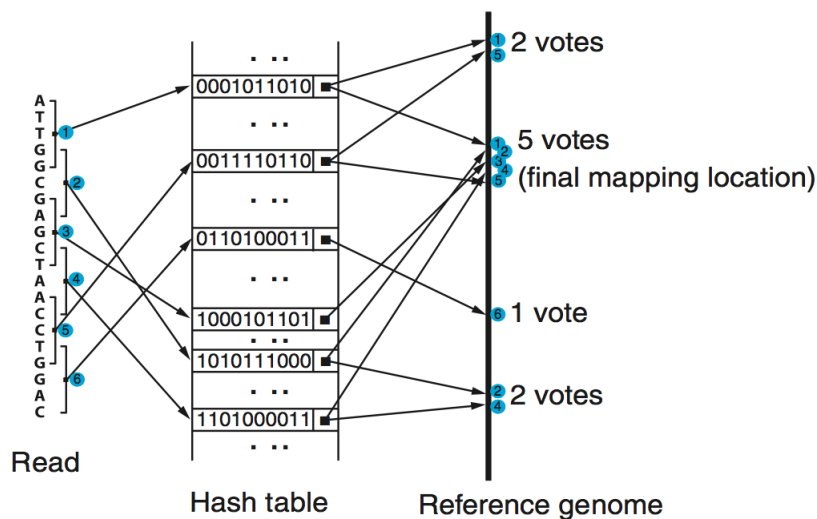


# Chapter 3

## The seed-and-vote mapping paradigm

### 3.1 Seed-and-vote

We have developed a new read mapping paradigm called “seed-and-vote” for efficient, accurate and scalable read mapping [1]. The seed-and-vote strategy uses a number of overlapping seeds from each read, called *subreads*. Instead of trying to pick the best seed, the strategy allows all the seeds to vote on the optimal location for the read. The algorithm then uses more conventional alignment algorithms to fill in detailed mismatch and indel information between the subreads that make up the winning voting block. The following figure illustrates the proposed seed-and-vote mapping approach with an toy example.



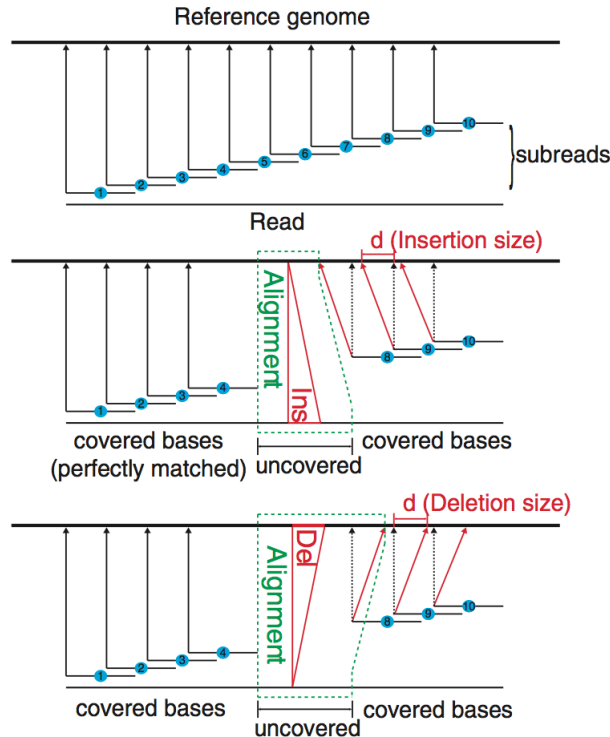
Two aligners have been developed under the seed-and-vote paradigm, including **Subread** and **Subjunc**. **Subread** is a general-purpose read aligner, which can be used to map both genomic DNA-seq and RNA-seq read data. Its running time is determined by the number of *subreads* extracted from each read, not by the read length. Thus it has an excellent mapping scalability, ie. its running time has only very modest increase with the increase of read length.

**Subread** uses the largest mappable region in the read to determine its mapping location, therefore it automatically determines whether a global alignment or a local alignment should be found for the read. For the exon-spanning reads in a RNA-seq dataset, **Subread** performs local alignments for them to find the target regions in the reference genome that have the largest overlap with them. Note that **Subread** does not perform global alignments for the exon-spanning reads and it soft clips those read bases which could not be mapped. However, the **Subread** mapping result is sufficient for carrying out the gene-level expression analysis using RNA-seq data, because the mapped read bases can be reliably used to assign reads, including both exonic reads and exon-spanning reads, to genes.

To get the full alignments for exon-spanning RNA-seq reads, the **Subjunc** aligner can be used. **Subjunc** is designed to discover exon-exon junctions from using RNA-seq data, but it performs full alignments for all the reads at the same time. The **Subjunc** mapping results should be used for detecting genomic variations in RNA-seq data, allele-specific expression analysis and exon-level gene expression analysis. The Section 3.3 describes how exon-exon junctions are discovered and how exon-spanning reads are aligned using the seed-and-vote paradigm.

## 3.2 Indel detection

### 3.2.1 Detection of short indels



The seed-and-vote paradigm is very powerful in detecting indels (insertions and deletions). The figure below shows how we use the *subreads* to confidently detect short indels. When

there is an indel existing in a read, mapping locations of subreads extracted after the indel will be shifted to the left (insertion) or to the right (deletion), relative to the mapping locations of subreads at the left side of the indel. Therefore, indels in the reads can be readily detected by examining the difference in mapping locations of the extracted subreads. Moreover, the number of bases by which the mapping location of subreads are shifted gives the precise length of the indel. Since no mismatches are allowed in the mapping of the subreads, the indels can be detected with a very high accuracy.

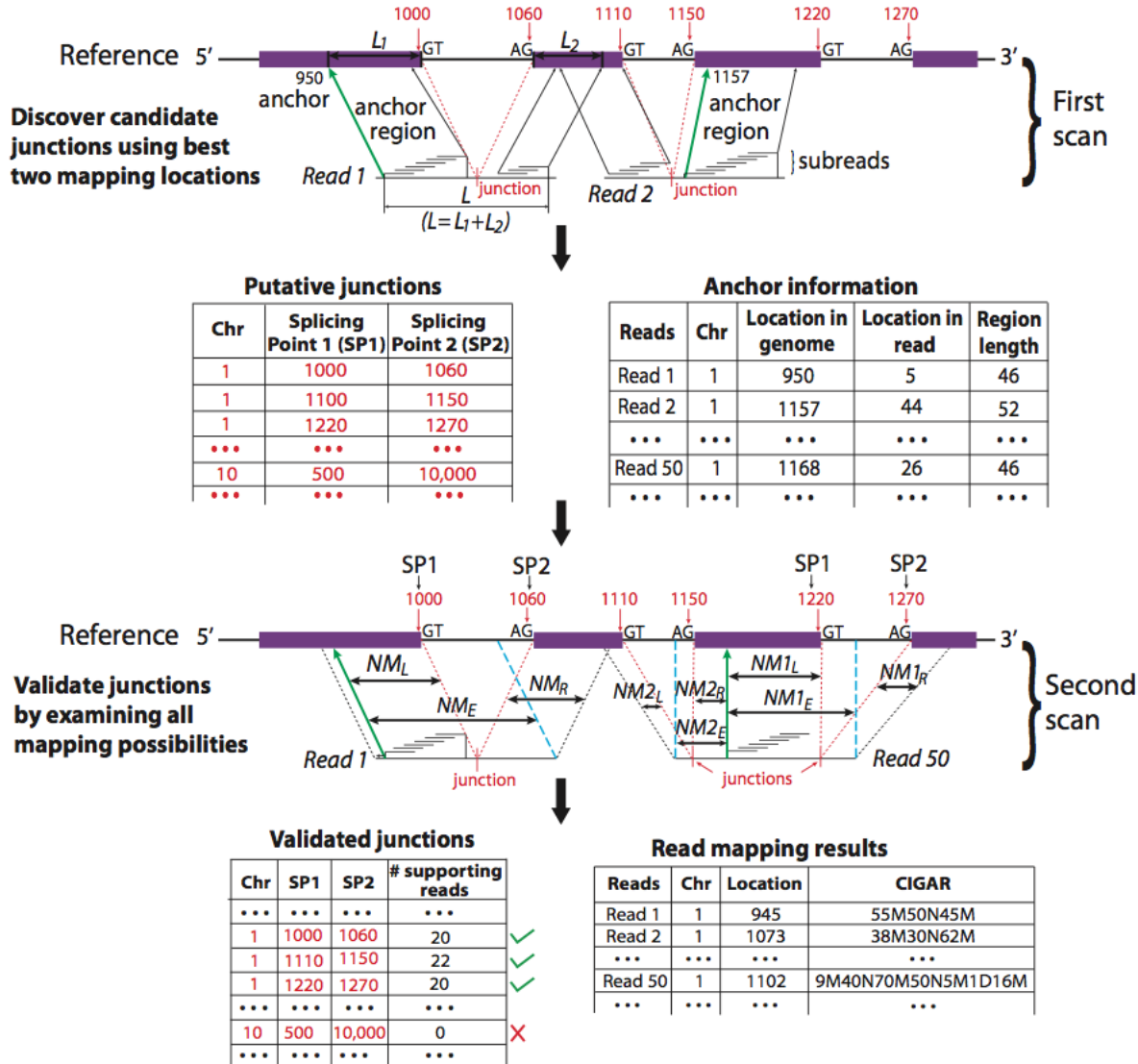
### 3.2.2 Detection of long indels

Detection of long indels is performed by using read assembly. When the specified indel length ('-I' option in SourceForge C or 'indels' paradigm in Rsubread) is greater than 16, the Subread and Subjunc will automatically start the read assembly procedure to identify indels of up to 200bp long.

## 3.3 Detection of canonical exon-exon junctions

The seed-and-vote paradigm is also very useful in detecting exon-exon junctions, because the short subreads extracted across the entire read can be used to detect short exons in a sensitive and accurate way. The figure below shows the schematic of detecting exon-exon junctions and mapping RNA-seq reads by Subjunc, which uses this paradigm.

The first scan detects all possible exon-exon junctions using the mapping locations of the subreads extracted from each read. Matched donor ('GT') and receptor ('AG') sites are required for calling junctions. Exons as short as 16bp can be detected in this step. The second scan verifies the putative exon-exon junctions discovered from the first scan by performing re-alignments for the junction reads. The output from Subjunc includes the list of verified junctions and also the mapping results for all the reads.



### 3.4 Fusion detection

Subjunc can detect genomic fusion events such as chimera in both RNA sequencing and genomic DNA sequencing data. It performs fusion detection in a manner similar to what it does for exon-exon junction detection, but it allows the same read to be splitted across different chromosomes. It also allows a read to be splitted across different strands on the same chromosome. It does not require donor/receptor sites when calling fusions. Non-canonical exon-exon junctions, which have donor/receptor sites other than GT/AG, may also be reported when subjunc tries to detect fusions.

For any read that maps to two or more chromosomes, maps to different strands of the same chromosome or spans a regions wider than  $2^{27}$  bases, Subjunc uses optional fields in the SAM/BAM output file to report the secondary alignments of the read. The primary alignment

of the read is saved in the main fields of the same record. The following tags are used for secondary alignments in the optional fields: CC(chromosome name), CP(mapping position), CG(CIGAR string) and CT(strand). Note that a fusion or junction read is always saved in a single record in SAM/BAM output.

### 3.5 Read re-alignments

Both **Subread** and **Subjunc** aligners re-align the reads after identifying indels, fusions and exon-exon junctions (**subjunc** only) from the data. They make use of the flanking window approach to identify indels, fusions and exon junctions. This is a highly accurate approach since it requires the identified indels, fusions or exon junctions to be flanked by perfectly matched subreads (16mers) at both of their sides. These discovered indels, fusions and exon junctions are then used for the re-alignments of all the reads. This approach is very effective in mapping the reads that contain indels, fusions or exon junctions at locations close to the ends of reads.

### 3.6 Recommended alignment setting

It is recommended to turn on `-u` option (reporting uniquely mapped reads only) and also `-H` option (breaking ties using Hamming distance when there is more than one best mapping location), when running **Subread** and **Subjunc** aligners. This should give the most accurate mapping results with little or no cost to the mapping percentage. This is the default setting used in **align** and **subjunc** functions in **Rsubread** package (`unique=TRUE` and `tieBreakHamming=TRUE`).

# Chapter 4

## Mapping reads generated by genomic DNA sequencing technologies

### 4.1 A quick start for using SourceForge Subread package

An index must be built for the reference first and then the read mapping can be performed.

#### Step 1: Building an index

Build a base-space index (default). You can provide a list of FASTA files or a single FASTA file including all the reference sequences.

```
subread-buildindex -o my_index chr1.fa chr2.fa ...
```

#### Step 2: Aligning the reads

Map single-end reads using 5 threads:

```
subread-align -T 5 -i my_index -r reads.txt -o subread_results.sam
```

Detect indels of up to 16bp:

```
subread-align -I 16 -i my_index -r reads.txt -o subread_results.sam
```

Report up to three best mapping locations:

```
subread-align -B 3 -i my_index -r reads.txt -o subread_results.sam
```

Report uniquely mapped reads only:

```
subread-align -u -i my_index -r reads.txt -o subread_results.sam
```

Map paired-end reads:

```
subread-align -d 50 -D 600 -i my_index -r reads1.txt -R reads2.txt  
-o subread_results.sam
```

## 4.2 A quick start for using Bioconductor Rsubread package

An index must be built for the reference first and then the read mapping can be performed.

### Step 1: Building an index

To build the index, you must provide a single FASTA file (eg. “genome.fa”) which includes all the reference sequences.

```
library(Rsubread)
buildindex(basename="my_index",reference="genome.fa")
```

### Step 2: Aligning the reads

Map single-end reads using 5 threads:

```
align(index="my_index",readfile1="reads.txt",output_file="rsubread.sam",nthreads=5)
```

Detect indels of up to 16bp:

```
align(index="my_index",readfile1="reads.txt",output_file="rsubread.sam",indels=16)
```

Report up to three best mapping locations:

```
align(index="my_index",readfile1="reads.txt",output_file="rsubread.sam",nBestLocations=3)
```

Report uniquely mapped reads only:

```
align(index="my_index",readfile1="reads.txt",output_file="rsubread.sam",unique=TRUE)
```

Map paired-end reads:

```
align(index="my_index",readfile1="reads1.txt",readfile2="reads2.txt",output_file="rsubread.sam",
minFragLength=50,maxFragLength=600)
```

## 4.3 Index building

The `subread-buildindex` (`buildindex` function in `Rsubread`) program builds an base-space or color-space index using the reference sequences. The reference sequences should be in FASTA format (the header line for each chromosomal sequence starts with “>”).

This program extracts all the 16 mer sequences from the reference genome at a 2bp interval and then uses them to build a hash table. Keys in the hash table are unique 16 mers and values are their chromosomal locations. Table 1 describes the arguments used by the `subread-buildindex` program.

Table 1: Arguments used by the **subread-buildindex** program (**buildindex** function in **Rsubread**). Arguments in parenthesis in the first column are used by **buildindex**.

Arguments	Description
<b>-o</b> < <i>basename</i> > ( <b>basename</b> )	Specify the base name of the index to be created.
<b>-f</b> < <i>int</i> > ( <b>TH_subread</b> )	Specify the threshold for removing uninformative subreads (highly repetitive 16mers). Subreads will be excluded from the index if they occur more than threshold number of times in the reference genome. Default value is 24.
<b>-M</b> < <i>int</i> > ( <b>memory</b> )	Specify the Size of requested memory(RAM) in megabytes, 8000MB by default. With the default value, the index built for a mammalian genome (eg. human or mouse genome) will be saved into one block, enabling the fastest mapping speed to be achieved. The amount of memory used is $\sim 7600$ MB for mouse or human genome (other species have a much smaller memory footprint), when performing read mapping. Using less memory will increase read mapping time.
<b>-c</b> ( <b>colorspace</b> )	Build a color-space index.
chr1.fa, chr2.fa, ... ( <b>reference</b> )	Give names of chromosome files. Note that in <b>Rsubread</b> , only a single FASTA file including all reference sequences should be provided.

## 4.4 Read mapping

The **subread-align** program (**align** in **Rsubread**) extracts a number of subreads from each read and then uses these subreads to vote for the mapping location of the read. It uses the “seed-and-vote” paradigm for read mapping. **subread-align** program automatically determines if a read should be globally aligned or locally aligned, making it particularly powerful for mapping RNA-seq reads. Table 2 describes the arguments used by the **subread-align** program (and also the **subjunc** program). These arguments are used by the read mapping programs included in both SourceForge **Subread** package and Bioconductor **Rsubread** package, although argument names are different in these two packages (arguments names used by Bioconductor **Rsubread** are included in parenthesis).



Table 2: arguments used by the `subread-align/subjunc` programs included in the SourceForge **Subread** package. Arguments in parenthesis in the first column are the equivalent arguments used in Bioconductor **Rsubread** package.

Arguments	Description
<code>-i &lt; index &gt;</code> ( <code>index</code> )	Specify the base name of the index.
<code>-r &lt; input &gt;</code> ( <code>readfile1</code> )	Give the name of input file(s) (multiple files are allowed to be provided to <code>align</code> and <code>subjunc</code> functions in <b>Rsubread</b> ). For paired-end read data, this gives the first read file and the other read file should be provided via the <code>-R</code> option. By default, format of the input file(s) is FASTQ (including FASTA). Other allowed formats include gzipped FASTQ (FASTA), SAM and BAM. See options below for how to specify these formats.
<code>-R &lt; input &gt;</code> ( <code>readfile2</code> )	Provide the name of the second reads file from paired-end data. The program will then be switched to paired-end read mapping mode.
<code>-o &lt; output &gt;</code> ( <code>output_file</code> )	Give the name of the output file (SAM format).
<code>--gzFASTQinput</code> ( <code>input_format="gzFASTQ"</code> )	specify that the input read data are in gzipped FASTQ or gzipped FASTA format.
<code>--SAMinput</code> ( <code>input_format="SAM"</code> )	specify that the input read data are in SAM format.
<code>--BAMinput</code> ( <code>input_format="BAM"</code> )	specify that the input read data are in BAM format.
<code>--BAMoutput</code> ( <code>output_format</code> )	specify that mapping results are saved into a BAM format file.
<code>-n &lt; int &gt;</code> ( <code>nsubreads</code> )	Specify the number of subreads extracted from each read, 10 by default.
<code>-m &lt; int &gt;</code> ( <code>TH1</code> )	Specify the consensus threshold, which is the minimal number of consensus subreads required for reporting a hit. The consensus subreads are those subreads which vote for the same location in the reference genome for the read. If pair-end read data are provided, at least one of the two reads from the same pair must satisfy this criteria. 3 by default.
<code>-p &lt; int &gt;</code> ( <code>TH2</code> )	Specify the minimum number of consensus subreads both reads from the same pair must have. This argument is only applicable for paired-end read data. The value of this argument should not be greater than that of ‘-m’ option, so as to rescue those read pairs in which one read has a high mapping quality but the other does not. 1 by default.

-d < <i>int</i> > (minFragLength)	Specify the minimum fragment/template length, 50 by default. Note that if the two reads from the same pair do not satisfy the fragment length criteria, they will be mapped individually as if they were single-end reads.
-D < <i>int</i> > (maxFragLength)	Specify the maximum fragment/template length, 600 by default.
-S < <i>ff:fr:rf</i> > (PE_orientation)	Specify the orientation of the two reads from the same pair. It has three possible values including 'fr', 'ff' and 'rf'. Letter 'f' denotes the forward strand and letter 'r' the reverse strand. 'fr' by default (ie. the first read in the pair is on the forward strand and the second read on the reverse strand).
-I < <i>int</i> > (indels)	Specify the number of INDEL bases allowed in the mapping. 5 by default. Indels of up to 200bp long can be detected.
-u (unique)	Output the uniquely mapped reads only.
-Q (codetieBreakQS)	Use mapping quality scores to break ties when more than one best mapping location is found.
-H (tieBreakHamming)	Use Hamming distance to break ties when more than one best mapping location is found.
* -B < <i>int</i> > (nBestLocations)	Specify the maximal number of equally-best mapping locations allowed to be reported for a read. Its value has to be within the range of 1 to 16. The default value is 1. The number of equally-best locations reported for a read will be less than or equal to the specified value. For example, if a read has two equally-best mapping locations, but the 'B' was set to 5, then only two locations will be reported for this read.
-P < 3 : 6 > (phredOffset)	Specify the format of Phred scores used in the input data, '3' for phred+33 and '6' for phred+64. '3' by default. For <code>align</code> function in <code>Rsubread</code> , the possible values are '33' (for phred+33) and '64' (for phred+64). '33' by default.
-T < <i>int</i> > (nthreads)	Specify the number of threads/CPU's used for mapping. 1 by default.
-b (color2base)	Output base-space reads instead of color-space reads in the mapping output. Note that the mapping itself will still be performed at color-space. This option is only applicable for color-space read mapping.
* -G < <i>int</i> > (DP_GapOpenPenalty)	Specify the penalty for opening a gap when applying the Smith-Waterman dynamic programming to detecting indels. -2 by default.
* -E < <i>int</i> > (DP_GapExtPenalty)	Specify the penalty for extending the gap when performing the Smith-Waterman dynamic programming. 0 by default.
* -X < <i>int</i> > (DP_MismatchPenalty)	Specify the penalty for mismatches when performing the Smith-Waterman dynamic programming. 0 by default.

* <b>-Y</b> < <i>int</i> > ( <b>DP_MatchScore</b> )	Specify the score for the matched base when performing the Smith-Waterman dynamic programming. 2 by default.
<b>--trim5</b> < <i>int</i> > ( <b>nTrim5</b> )	trim off < <i>int</i> > number of bases from 5' end of each read. 0 by default.
<b>--trim3</b> < <i>int</i> > ( <b>nTrim3</b> )	trim off < <i>int</i> > number of bases from 3' end of each read. 0 by default.
<b>--rg-id</b> < <i>string</i> > ( <b>readGroupID</b> )	specify the read group ID. If specified, the read group ID will be added to the read group header field and also to each read in the mapping output.
<b>--rg</b> < <i>string</i> > ( <b>readGroup</b> )	add a < <i>tag : value</i> > to the read group (RG) header in the mapping output.
** <b>--dnaseq</b> ( <b>DNaseq</b> )	Specify that the input read data are genomic DNA sequencing data. This option should only be used with <b>subjunc</b> . When specified, <b>subjunc</b> will perform read alignments and also detect fusion events such as chimeras. When a read is mapped to more than one chromosome, its secondary alignments will be saved to the following optional fields along with the main fields of the same record in the SAM/BAM output: CC(Chr), CP(Position), CG(CIGAR) and CT(strand).
** <b>--allJunctions</b> ( <b>reportAllJunctions</b> )	This option should only be used with <b>subjunc</b> for the mapping of RNA-seq data. If specified, the <b>subjunc</b> will output non-canonical exon-exon junctions and fusions (eg. chimeras), in addition to the canonical exon-exon junctions. Chimeric reads are reported in the same format as that in ' <b>--dnaseq</b> ' option.
* <b>--reportFusions</b> ( <b>reportFusions</b> )	This option should only be used with <b>subread-align</b> for the mapping of genomic DNA-seq data. If specified, <b>subread-align</b> will report discovered fusion events such as chimeras. Fusions are reported in the same format as that used in ' <b>--dnaseq</b> ' option.
<b>-v</b>	Output version of the program.

\* *Arguments used by subread-align only.*

\*\* *Arguments used by subjunc only.*

## 4.5 Mapping quality scores

Both Subread and Subjunc aligners output a mapping quality score for each mapped read, defined by

$$\text{MQS} = 100 + \frac{100}{l} \left\{ \sum_{i \in b_m} (1 - p_i) - \sum_{i \in b_{mm}} (1 - p_i) \right\}$$

where  $l$  is the read length,  $p_i$  is the base-calling  $p$ -value for the  $i$ th base in the read,  $b_m$  is the set of locations of matched bases, and  $b_{mm}$  is the set of locations of mismatched bases.

Base-calling  $p$  values can be readily computed from the base quality scores. High quality bases have low base-calling  $p$  values. Read bases which were found to be insertions are treated as matched bases in the MQS calculation. The MQS is a read-length normalized value, which is in the range of 0 to 200. If a read can be best mapped to more than one location, its MQS will be divided by the number of such locations.

# Chapter 5

## Mapping reads generated by RNA sequencing technologies

### 5.1 A quick start for using SourceForge **Subread** package

An index must be built for the reference first and then the read mapping and/or junction detection can be carried out.

#### Step 1: Building an index

The following command can be used to build a base-space index. You can provide a list of FASTA files or a single FASTA file including all the reference sequences.

```
subread-buildindex -o my_index chr1.fa chr2.fa ...
```

For more details about index building, see Section 4.3.

#### Step 2: Aligning the reads

##### Subread

For the purpose of differential expression analysis (ie. discovering differentially expressed genes), we recommend you to use the **Subread** aligner. **Subread** carries out local alignments for RNA-seq reads. The commands used by **Subread** to align RNA-seq reads are the same as those used to align gDNA-seq reads. Below is an example of using **Subread** to map single-end RNA-seq reads.

```
subread-align -i my_index -r rnaseq-reads.txt -o subread_results.sam
```

Another RNA-seq aligner included in this package is the **Subjunc** aligner. **Subjunc** not only performs read alignments but also detects exon-exon junctions. The main difference between

**Subread** and **Subjunc** is that **Subread** does not attempt to detect exon-exon junctions in the RNA-seq reads. For the alignments of the exon-spanning reads, **Subread** just uses the largest mappable regions in the reads to find their mapping locations. This makes **Subread** more computationally efficient. The largest mappable regions can then be used to reliably assign the reads to their target genes by using a read summarization program (eg. **featureCounts**, see Section 6.2), and differential expression analysis can be readily performed based on the read counts yielded from read summarization. Therefore, **Subread** is sufficient for read mapping if the purpose of the RNA-seq analysis is to perform a differential expression analysis. Also, **Subread** could report more mapped reads than **Subjunc**. For example, the exon-spanning reads that are not aligned by **Subjunc** due to the lack of GT/AG splicing signals (this is the only donor/receptor site accepted by **Subjunc**) could be aligned by **Subread**, as long as they have a good match with the target region.

## Subjunc

For other purposes of the RNA-seq data analyses such as exon-exon junction detection and genomic mutation detection, in which reads need to be fully aligned (especially the exon-spanning reads), **Subjunc** aligner should be used. Below is an example command of using **Subjunc** to perform global alignments for paired-end RNA-seq reads. Note that there are two files included in the output: one containing the discovered exon-exon junctions (BED format) and the other containing the mapping results for reads (SAM or BAM format).

```
subjunc -i my_index -r rnaseq-reads1.txt -R rnaseq-reads2.txt -o subjunc_result
```

## 5.2 A quick start for using Bioconductor Rsubread package

An index must be built for the reference first and then the read mapping can be performed.

### Step 1: Building an index

To build the index, you must provide a single FASTA file (eg. “genome.fa”) which includes all the reference sequences.

```
library(Rsubread)
buildindex(basename="my_index",reference="genome.fa")
```

### Step 2: Aligning the reads

Please refer to Section 5.1 for difference between **Subread** and **Subjunc** in mapping RNA-seq data. Below is an example for mapping a single-end RNA-seq dataset using **Subread**. Useful information about **align** function can be found in its help page (type **?align** in your R prompt).

```
align(index="my_index",readfile1="rnaseq-reads.txt",output_file="subread_results.sam")
```

Below is an example for mapping a single-end RNA-seq dataset using **Subjunc**. Useful information about **subjunc** function can be found in its help page (type `?subjunc` in your R prompt).

```
subjunc(index="my_index",readfile1="rnaseq-reads.txt",output_file="subjunc_results.sam")
```

## 5.3 Local read alignment

The **Subread** and **Subjunc** can both be used to map RNA-seq reads to the reference genome. If the goal of the RNA-seq data is to perform expression analysis, eg. finding genes expressing differentially between different conditions, then **Subread** is recommended. **Subread** performs fast local alignments for reads and reports the mapping locations that have the largest overlap with the reads. These reads can then be assigned to genes for expression analysis. For this type of analysis, global alignments for the exon-spanning reads are not required because local alignments are sufficient to get reads to be accurately assigned to genes.

However, for other types of RNA-seq data analyses such as exon-exon junction discovery, genomic mutation detection and allele-specific gene expression analysis, global alignments are required. The next section describes the **Subjunc** aligner, which performs global alignments for RNA-seq reads.

## 5.4 Global read alignment

**Subjunc** aligns each exon-spanning read by firstly using a large number of subreads extracted from the read to identify multiple target regions matching the selected subreads, and then using the splicing signals (donor and receptor sites) to precisely determine the mapping locations of the read bases. It also includes a verification step to compare the quality of mapping reads as exon-spanning reads with the quality of mapping reads as exonic reads to finally decide how to best map the reads. Reads may be re-aligned if required.

Output of **Subjunc** aligner includes a list of discovered exon-exon junction locations and also the complete alignment results for the reads. Table 2 describes the arguments used by the **Subjunc** program.

# Chapter 6

## Read summarization

### 6.1 Introduction

Sequencing reads often need to be assigned to genomic features of interest after they are mapped to the reference genome. This process is often called *read summarization* or *read quantification*. Read summarization is required by a number of downstream analyses such as gene expression analysis and histone modification analysis. The output of read summarization is a count table, in which the number of reads assigned to each feature in each library is recorded.

A particular challenge to the read summarization is how to deal with those reads that overlap more than one feature (eg. an exon) or meta-feature (eg. a gene). Care must be taken to ensure that such reads are not over-counted or under-counted. Here we describe the **featureCounts** program, an efficient and accurate read quantifier. **featureCounts** has the following features:

- It carries out precise and accurate read assignments by taking care of indels, junctions and fusions in the reads.
- It takes less than 4 minutes to summarize 20 million pairs of reads to 26k RefSeq genes using one thread, and uses <20MB of memory (you can run it on a Mac laptop).
- It supports multi-threaded running, making it extremely fast for summarizing large datasets.
- It supports GTF/SAF format annotation and SAM/BAM read data.
- It supports strand-specific read summarization.
- It can perform read summarization at both feature level (eg. exon level) and meta-feature level (eg. gene level).
- It allows users to specify whether reads overlapping with more than one feature should be counted or not.



- It gives users full control on the summarization of paired-end reads, including allowing them to check if both ends are mapped and/or if the fragment length falls within the specified range.
- It can discriminate the features that were overlapped by both ends of the fragment from the features that were overlapped by only one end of the same fragment to get more accurate read assignments.
- It allows users to specify whether chimeric fragments should be counted.
- It automatically detects the read input format (SAM or BAM).
- It automatically re-order paired-end reads if reads belonging to the same pair are not adjacent to each other in input read files.

## 6.2 featureCounts

### 6.2.1 Input data

The data input to **featureCounts** consists of (i) one or more files of aligned reads in either SAM or BAM format and (ii) a list of genomic features in either Gene Transfer Format (GTF) or General Feature Format (GFF) or Simplified Annotation Format (SAF). The read input format (SAM or BAM) is automatically detected and so does not need to be specified by the user. For paired reads, **featureCounts** also automatically sorts reads by name if paired reads are not in consecutive positions in the SAM or BAM file. Both the read alignment and the feature annotation should correspond to the same reference genome, which is a set of reference sequences representing chromosomes or contigs. For each read, the SAM file gives the name of the reference chromosome or contig to which the read mapped, the start position of the read on the chromosome or contig/scaffold, and the so-called CIGAR string giving the detailed alignment information including insertions and deletions and so on relative to the start position.

The genomic features can be specified in either GTF/GFF or SAF format. The SAF format is the simpler and includes only five required columns for each feature (see next section). In either format, the feature identifiers are assumed to be unique, in accordance with commonly used Gene Transfer Format (GTF) refinement of GFF.

**featureCounts** supports strand-specific read counting if strand-specific information is provided. Read mapping results usually include mapping quality scores for mapped reads. Users can optionally specify a minimum mapping quality score that the assigned reads must satisfy.

### 6.2.2 Annotation format

The genomic features can be specified in either GTF/GFF or SAF format. A definition of the GTF format can be found at UCSC website (<http://genome.ucsc.edu/FAQ/FAQformat.html#format4>). The SAF format includes five required columns for each feature: feature

identifier, chromosome name, start position, end position and strand. These five columns provide the minimal sufficient information for read quantification purposes. Extra annotation data are allowed to be added from the sixth column.

A SAF-format annotation file should be a tab-delimited text file. It should also include a header line. An example of a SAF annotation is shown as below:

```
GeneID Chr Start End Strand
497097 chr1 3204563 3207049 -
497097 chr1 3411783 3411982 -
497097 chr1 3660633 3661579 -
100503874 chr1 3637390 3640590 -
100503874 chr1 3648928 3648985 -
100038431 chr1 3670236 3671869 -
...
```

**GeneID** column includes gene identifiers that can be numbers or character strings. Chromosomal names included in the **Chr** column must match the chromosomal names of reference sequences to which the reads were aligned.

### 6.2.3 Single and paired-end reads

Reads may be paired or unpaired. If paired reads are used, then each pair of reads defines a DNA or RNA fragment bookended by the two reads. In this case, **featureCounts** can be instructed to count fragments rather than reads. **featureCounts** automatically sorts reads by name if paired reads are not in consecutive positions in the SAM or BAM file. Users do not need sort their paired reads before providing them to **featureCounts**.

### 6.2.4 Features and meta-features

**featureCounts** is a general-purpose read summarization function, which assigns mapped reads (RNA-seq reads or genomic DNA-seq reads) to genomic features or meta-features. Each feature is an interval (range of positions) on one of the reference sequences. We define a meta-feature to be a set of features representing a biological construct of interest. For example, features often correspond to exons and meta-features to genes. Features sharing the same feature identifier in the GTF or SAF annotation are taken to belong to the same meta-feature. **featureCounts** can summarize reads at either the feature or meta-feature levels.

We recommend to use unique gene identifiers, such as NCBI Entrez gene identifiers, to cluster features into meta-features. Gene names are not recommended to use for this purpose because different genes may have the same names. Unique gene identifiers were often included in many publicly available GTF annotations which can be readily used for summarization. The Bioconductor **Rsubread** package also includes NCBI RefSeq annotations for human and mice. Entrez gene identifiers are used in these annotations.

### 6.2.5 Overlap of reads with features

`featureCounts` performs precise read assignment by comparing mapping location of every base in the read or fragment with the genomic region spanned by each feature. It takes account of any gaps (insertions, deletions, exon-exon junctions or fusions) that are found in the read. It calls a hit if any overlap (1bp or more) is found between the read or fragment and a feature. A hit is called for a meta-feature if the read or fragment overlaps any component feature of the meta-feature.

### 6.2.6 Multiple overlaps

A multi-overlap read or fragment is one that overlaps more than one feature, or more than one meta-feature when summarizing at the meta-feature level. `featureCounts` provides users with the option to either exclude multi-overlap reads or to count them for each feature that is overlapped. The decision whether or not to counting these reads is often determined by the experiment type. We recommend that reads or fragments overlapping more than one gene are not counted for RNA-seq experiments, because any single fragment must originate from only one of the target genes but the identity of the true target gene cannot be confidently determined. On the other hand, we recommend that multi-overlap reads or fragments are counted for most ChIP-seq experiments because epigenetic modifications inferred from these reads may regulate the biological functions of all their overlapping genes.

Note that, when counting at the meta-feature level, reads that overlap multiple features of the same meta-feature are always counted exactly once for that meta-feature, provided there is no overlap with any other meta-feature. For example, an exon-spanning read will be counted only once for the corresponding gene even if it overlaps with more than one exon.

### 6.2.7 Program usage

Table 3 describes the parameters used by the `featureCounts` program.

Table 3: arguments used by the `featureCounts` program included in the SourceForge **Subread** package. Arguments included in parenthesis are the equivalent parameters used by `featureCounts` function in Bioconductor **Rsubread** package.

Arguments	Description
<code>input_files</code> ( <code>files</code> )	Give the names of input read files that include the read mapping results. The program automatically detects the file format (SAM or BAM). Multiple files can be provided at the same time.
<code>-a &lt;input&gt;</code> ( <code>annot.ext</code> , <code>annot.inbuilt</code> )	Give the name of an annotation file.
<code>-o &lt;input&gt;</code>	Give the name of the output file. The output file contains the number of reads assigned to each meta-feature (or each feature if <code>-f</code> is specified). Note that the <code>featureCounts</code> function in <b>Rsubread</b> does not use this parameter. It returns a <code>list</code> object including read summarization results and other data.
<code>-A</code> ( <code>chrAliases</code> )	Give the name of a file that contains aliases of chromosome names. The file should be a comma delimited text file that includes two columns. The first column gives the chromosome names used in the annotation and the second column gives the chromosome names used by reads. This file should not contain header lines. Names included in this file are case sensitive.
<code>-F</code> ( <code>isGTFAnnotationFile</code> )	Specify the format of the annotation file. Acceptable formats include ‘GTF’ and ‘SAF’ (see Section 6.2.2 for details). The C version of <code>featureCounts</code> program uses a GTF format annotation by default, but the R version uses a SAF format annotation by default. The R version also includes in-built annotations.
<code>-t &lt;input&gt;</code> ( <code>GTF.featureType</code> )	Specify the feature type. Only rows which have the matched feature type in the provided GTF annotation file will be included for read counting. ‘exon’ by default.
<code>-g &lt;input&gt;</code> ( <code>GTF.attrType</code> )	Specify the attribute type used to group features (eg. exons) into meta-features (eg. genes), when GTF annotation is provided. ‘gene.id’ by default. This attribute type is usually the gene identifier. This argument is useful for the meta-feature level summarization.
<code>-f</code> ( <code>useMetaFeatures</code> )	If specified, read summarization will be performed at feature level (eg. exon level). Otherwise, it is performed at meta-feature level (eg. gene level).

-O (allowMultiOverlap)	If specified, reads (or fragments if <code>-p</code> is specified) will be allowed to be assigned to more than one matched meta-feature (or feature if <code>-f</code> is specified). Reads/fragments overlapping with more than one meta-feature/feature will be counted more than once. Note that when performing meta-feature level summarization, a read (or fragment) will still be counted once if it overlaps with multiple features belonging to the same meta-feature but does not overlap with other meta-features.
-s < int > (isStrandSpecific)	Indicate if strand-specific read counting should be performed. It has three possible values: 0 (unstranded), 1 (stranded) and 2 (reversely stranded). 0 by default. For paired-end reads, strand of the first read is taken as the strand of the whole fragment and FLAG field of the current read is used to tell if it is the first read in the fragment.
-M	If specified, multi-mapping reads/fragments will be counted (ie. a multi-mapping read will be counted up to N times if it has N reported mapping locations). The program uses the 'NH' tag to find multi-mapping reads.
-Q < int > (minMQS)	The minimum mapping quality score a read must satisfy in order to be counted. For paired-end reads, at least one end should satisfy this criteria. 0 by default.
-T < int > (nthreads)	Number of the threads. 1 by default.
-R	Output read counting result for each read/fragment. For each input read file, read counting results for reads/fragments will be saved to a tab-delimited file that contains four columns including read name, status(assigned or the reason if not assigned), name of target feature/meta-feature and number of hits if the read/fragment is counted multiple times. Name of the file is the same as name of the input read file except a suffix '.featureCounts' is added.
-p (isPairedEnd)	If specified, fragments (or templates) will be counted instead of reads. This option is only applicable for paired-end reads.
-P (checkFragLength)	If specified, the fragment length will be checked when assigning fragments to meta-features or features. This option should be used together with <code>-p</code> (or <code>isPairedEnd</code> in <code>Rsubread featureCounts</code> ). The fragment length thresholds should be specified using <code>-d</code> and <code>-D</code> options.
-d < int > (minFragLength)	Minimum fragment/template length, 50 by default.
-D < int > (maxFragLength)	Maximum fragment/template length, 600 by default.

-B (requireBothEndsMapped)	If specified, only fragments that have both ends successfully aligned will be considered for summarization. This option should be used together with -p (or isPairedEnd in Rsubread featureCounts).
-C (countChimericFragments)	If specified, the chimeric fragments (those fragments that have their two ends aligned to different chromosomes) will NOT be counted. This option should be used together with -p (or isPairedEnd in Rsubread featureCounts).

## 6.3 A quick start for featureCounts in SourceForge Sub-read

You need to provide read mapping results (in either SAM or BAM format) and an annotation file for the read summarization. The example commands below assume your annotation file is in GTF format.

Summarizing single-end reads using 5 threads:

```
featureCounts -T 5 -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_SE.sam
```

Summarizing BAM format single-end read data:

```
featureCounts -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_SE.bam
```

Summarizing paired-end reads and counting fragments (instead of reads):

```
featureCounts -p -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_PE.sam
```

Counting fragments satisfying the fragment length criteria, eg. [50bp, 600bp]:

```
featureCounts -p -P -d 50 -D 600 -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_PE.sam
```

Counting fragments which have both ends successfully aligned without considering the fragment length constraint:

```
featureCounts -p -B -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_PE.sam
```

Excluding chimeric fragments from the fragment counting:

```
featureCounts -p -C -a annotation.gtf -t exon -g gene_id  
-o counts.txt mapping_results_PE.sam
```

## 6.4 A quick start for featureCounts in Bioconductor Rsubread

You need to provide read mapping results (in either SAM or BAM format) and an annotation file for the read summarization. The example commands below assume your annotation file is in GTF format.

Load Rsubread library from you R session:

```
library(Rsubread)
```

Summarizing single-end reads using built-in RefSeq annotation for mouse genome mm9:

```
featureCounts(files="mapping_results_SE.sam",annot.inbuilt="mm9")
```

Summarizing single-end reads using a user-provided GTF annotation file:

```
featureCounts(files="mapping_results_SE.sam",annot.ext="annotation.gtf",
isGTFAnnotationFile=TRUE,GTF.featureType="exon",GTF.attrType="gene_id")
```

Summarizing single-end reads using 5 threads:

```
featureCounts(files="mapping_results_SE.sam",nthreads=5)
```

Summarizing BAM format single-end read data:

```
featureCounts(files="mapping_results_SE.bam")
```

Summarizing paired-end reads and counting fragments (instead of reads):

```
featureCounts(files="mapping_results_PE.sam",isPairedEnd=TRUE)
```

Counting fragments satisfying the fragment length criteria, eg. [50bp, 600bp]:

```
featureCounts(files="mapping_results_PE.sam",isPairedEnd=TRUE,checkFragLength=TRUE,
minFragLength=50,maxFragLength=600)
```

Counting fragments which have both ends successfully aligned without considering the fragment length constraint:

```
featureCounts(files="mapping_results_PE.sam",isPairedEnd=TRUE,requireBothEndsMapped=TRUE)
```

Excluding chimeric fragments from the fragment counting:

```
featureCounts(files="mapping_results_PE.sam",isPairedEnd=TRUE,countChimericFragments=FALSE)
```



# Chapter 7

## SNP calling

### 7.1 Algorithm

SNPs(Single Nucleotide Polymorphisms) are the mutations of single nucleotides in the genome. It has been reported that many diseases were initiated and/or driven by such mutations. Therefore, successful detection of SNPs is very useful in designing better diagnosis and treatments for a variety of diseases such as cancer. SNP detection also is an important subject of many population studies.

Next-gen sequencing technologies provide an unprecedented opportunity to identify SNPs at the highest resolution. However, it is extremely computing-intensive to analyze the data generated from these technologies for the purpose of SNP discovery because of the sheer volume of the data and the large number of chromosomal locations to be considered. To discover SNPs, reads need to be mapped to the reference genome first and then all the read data mapped to a particular site will be used for SNP calling for that site. Discovery of SNPs is often confounded by many sources of errors. Mapping errors and sequencing errors are often the major sources of errors causing incorrect SNP calling. Incorrect alignments of indels, exon-exon junctions and fusions in the reads can also result in wrong placement of blocks of continuous read bases, likely giving rise to consecutive incorrectly reported SNPs.

We have developed a highly accurate and efficient SNP caller, called *exactSNP* [8]. *exactSNP* calls SNPs for individual samples, without requiring control samples to be provided. It tests the statistical significance of SNPs by comparing SNP signals to their background noises. It has been found to be an order of magnitude faster than existing SNP callers.

### 7.2 exactSNP

Below is the command for running `exactSNP` program. The complete list of parameters used by `exactSNP` can be found in Table 4.

```
exactSNP [options] -i input -g reference_genome -o output
```

Table 4: arguments used by the `exactSNP` program included in the SourceForge `Sub-read` package. Arguments included in parenthesis are the equivalent parameters used by `exactSNP` function in Bioconductor `Rsubread` package.

Arguments	Description
<code>-i &lt; file &gt; [-b if BAM]</code> ( <i>readFile</i> )	Specify name of an input file including read mapping results. The format of input file can be SAM or BAM ( <code>-b</code> needs to be specified if a BAM file is provided).
<code>-b</code> ( <i>isBAM</i> )	Indicate the input file provided via <code>-i</code> is in BAM format.
<code>-g &lt; file &gt;</code> ( <i>refGenomeFile</i> )	Specify name of the file including all reference sequences. Only one single FASTA format file should be provided.
<code>-o &lt; file &gt;</code> ( <i>outputFile</i> )	Specify name of the output file. This program outputs a VCF format file that includes discovered SNPs.
<code>-Q &lt; int &gt;</code> ( <i>qvalueCutoff</i> )	Specify the q-value cutoff for SNP calling at sequencing depth of 50X. 12 by default. The corresponding p-value cutoff is $10^{-Q}$ . Note that this program automatically adjusts the q-value cutoff according to the sequencing depth at each chromosomal location.
<code>-f &lt; float &gt;</code> ( <i>minAllelicFraction</i> )	Specify the minimum fraction of mis-matched bases a SNP-containing location must have. Its value must between 0 and 1. 0 by default.
<code>-n &lt; int &gt;</code> ( <i>minAllelicBases</i> )	Specify the minimum number of mis-matched bases a SNP-containing location must have. 1 by default.
<code>-r &lt; int &gt;</code> ( <i>minReads</i> )	Specify the minimum number of mapped reads a SNP-containing location must have (ie. the minimum coverage). 1 by default.
<code>-x &lt; int &gt;</code> ( <i>maxReads</i> )	Specify the maximum number of mapped reads a SNP-containing location could have. 3000 by default. Any location having more than the threshold number of reads will not be considered for SNP calling. This option is useful for removing PCR artefacts.
<code>-s &lt; int &gt;</code> ( <i>minBaseQuality</i> )	Specify the cutoff for base calling quality scores (Phred scores) read bases must satisfy to be used for SNP calling. 13 by default. Read bases that have Phred scores lower than the cutoff value will be excluded from the analysis.
<code>-t &lt; int &gt;</code> ( <i>nTrimmedBases</i> )	Specify the number of bases trimmed off from each end of the read. 3 by default.
<code>-T &lt; int &gt;</code> ( <i>nthreads</i> )	Specify the number of threads. 1 by default.

<p>-a &lt; <i>file</i> &gt; (SNPAnnotationFile)</p>	<p>Specify name of a VCF-format file that includes annotated SNPs. Such annotation files can be downloaded from public databases such as the dbSNP database. Incorporating known SNPs into SNP calling has been found to be helpful. However note that the annotated SNPs may or may not be called for the sample being analyzed.</p>
---	---

# Chapter 8

## Case studies

### 8.1 A Bioconductor R pipeline for analyzing RNA-seq data

Here we illustrate how to use two Bioconductor packages - **Rsubread** and **limma** - to perform a complete RNA-seq analysis, including **Subread** read mapping, **featureCounts** read summarization, **voom** normalization and **limma** differential expression analysis.

**Data and software.** The RNA-seq data used in this case study include four libraries: A\_1, A\_2, B\_1 and B\_2. A\_1 and A\_2 are both Universal Human Reference RNA (UHRR) samples but they underwent separate sample preparation. B\_1 and B\_2 are both Human Brain Reference RNA (HBRR) samples and they also underwent separate sample preparation. Note that these libraries only included reads originating from human chromosome 1 (according to **Subread** aligner). These read data were generated by the SEQC Consortium. We have put into a tar ball these read data and the reference sequence data of chromosome 1 from human genome build GRCh37/hg19, and it can be downloaded from <http://bioinf.wehi.edu.au/RNAseqCaseStudy/data.tar.gz> (283MB).

After downloading the dataset, uncompress it and save it to your current working directory. Launch R and load **Rsubread** and **limma** libraries by issuing the following commands at your R prompt. Version of your R should be 3.0.2 or later. **Rsubread** version should be 1.12.1 or later and **limma** version should be 3.18.0 or later. Note that this case study only runs on Linux/Unix and Mac OS X.

```
library(Rsubread)
library(limma)
```

To install/update **Rsubread** and **limma** packages, issue the following commands at your R prompt:

```
source("http://bioconductor.org/biocLite.R")
biocLite(pkgs=c("Rsubread", "limma"))
```

**Index building.** Build an index for human chromosome 1. This will take  $\sim 3$  minutes. Index files with basename 'chr1' will be generated in your current working directory.

```
buildindex(basename="chr1",reference="hg19_chr1.fa")
```

**Alignment.** Perform read alignment for all four libraries and report uniquely mapped reads only. This will take  $\sim 4$  minutes. SAM files which include the mapping results will be generated in your current working directory.

```
for(i in c("A_1","A_2","B_1","B_2"))  
  align(index="chr1",readfile1=paste(i,"txt",sep="."),output_file=paste(i,"sam",sep="."),  
    tieBreakHamming=TRUE,unique=TRUE,indels=5)
```

**Read summarization.** Summarize mapped reads to RefSeq genes. This will take less than half a minute. Note that the `featureCounts` function contains built-in RefSeq annotations. `featureCounts` returns an R 'List' object that includes a read count table and annotation data. The read count table can be directly fed into `limma` for normalization and differential expression analysis.

```
counts <- featureCounts(files=c("A_1.sam","A_2.sam","B_1.sam","B_2.sam"),annot.inbuilt="hg19")
```

**Filtering.** Calculate RPKM (reads per kilobases of exon per million reads mapped) values for genes and use these values to filter out those genes which failed to achieve a 0.5 RPKM in at least two libraries.

```
counts_rpk <- apply(counts$counts,2,function(x) x*(1000/counts$annotation$Length)*(1e6/sum(x)))  
isexpr <- rowSums(counts_rpk >= 0.5) >= 2  
x <- counts$counts[isexpr,]
```

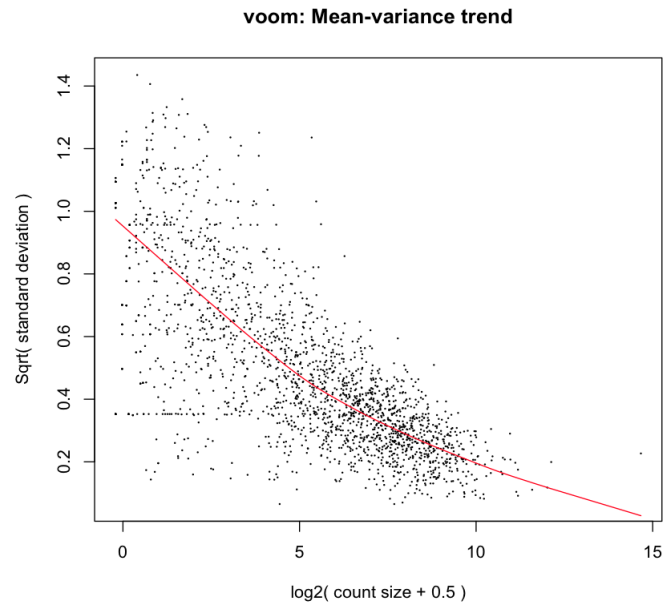
**Design matrix.** The following analyses are very similar to the analyses performed for microarray expression data. Firstly, we create a design matrix:

```
celltype <- factor(c("A","A","B","B"))  
design <- model.matrix(~0+celltype)  
colnames(design) <- levels(celltype)
```

**Normalization.** Then we perform `voom` normalization:

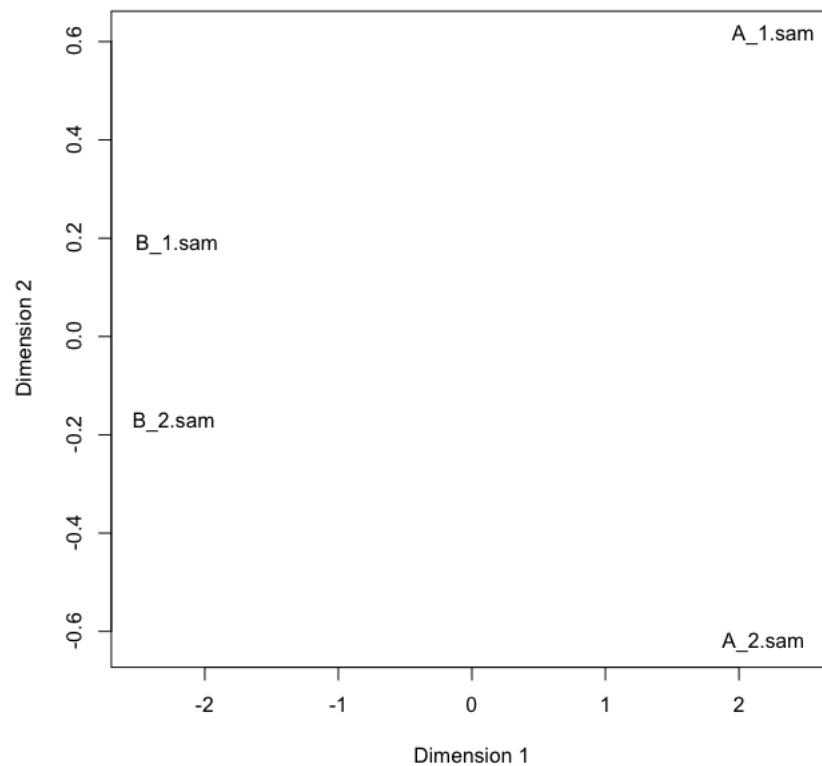
```
y <- voom(x,design,plot=TRUE)
```

The figure below shows the mean-variance relationship estimated by `voom` for the data.



**Sample clustering.** The following multi-dimensional scaling plot shows that sample A libraries are clearly separated from sample B libraries.

```
plotMDS(y,xlim=c(-2.5,2.5))
```



**Linear model fitting and differential expression analysis.** Fit linear models to genes and assess differential expression using the eBayes moderated t statistic. Here we compare A vs B. 556 and 983 genes were found down- and up-regulated in sample A compared to sample B, respectively.

```
fit <- lmFit(y,design)
contr <- makeContrasts(AvsB=A-B,levels=design)
fit.contr <- eBayes(contrasts.fit(fit,contr))
dt <- decideTests(fit.contr)
summary(dt)
      AvsB
-1  556
0   523
1   979
```

List top 10 differentially expressed genes:

```
options(digits=3)
topTable(fit.contr)
```

	ID	logFC	AveExpr	t	P.Value	adj.P.Val	B
1639	2752	-2.39	12.9	-91.5	1.04e-20	1.40e-17	38.0
7	100131754	-1.63	16.0	-89.7	1.36e-20	1.40e-17	36.2
147	22883	-2.24	12.5	-70.3	4.05e-19	2.78e-16	34.4
313	6135	2.23	12.1	67.7	6.81e-19	3.05e-16	33.8
598	4904	2.99	11.5	66.4	8.77e-19	3.05e-16	33.5
136	2023	2.72	13.5	66.4	8.90e-19	3.05e-16	33.3
641	6202	2.40	12.1	64.5	1.32e-18	3.89e-16	33.2
501	23154	-3.73	11.4	-57.6	6.25e-18	1.61e-15	31.3
917	6125	2.01	11.8	50.2	4.27e-17	9.76e-15	29.7
1448	8682	-2.59	11.7	-49.0	5.85e-17	1.20e-14	29.4

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