

# Filtering read alignments in BAM format: user guide

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## Abstract

This note documents "filterBAM", a program designed to clean alignments stored in BAM format. The filter is based on filterPSL, a perl script written by Prof. Mario Stanke as part of the AUGUSTUS software suite Stanke et al. (2008). Both filterPSL and filterBam are designed for the cleaning of alignment data that will subsequently be applied to the gene prediction problem. The filter produces also output that might later be used for transcriptome quantification. The code should be modifiable rather easily, if it is to be applied to a different type of application (see the reference manual). filterBam is written in C++ and makes use of the Bamtools API of Barnett et al. (2011).

## 1 Installation

In this section we describe the system requirements for installing, compiling and running filterBam on a Linux terminal.

### 1.1 Requirements

Table 1 and 2 below list the software packages and libraries required for compiling filterBam. As the filter works with sorted BAM files, it might be useful to have both **samtools** and **bamtools** utility kits installed, so both software packages have been included in the tables.

### 1.2 Compilation

In this section we briefly describe the compilation process of filterBam.

1. Download or checkout the latest version of filterBam from **to-specify**.
2. Create and export an environment variable **BAMTOOLS** that points to the folder where **bamtools** has been installed. For example, if **bamtools** is under **/home/myAccount/**, then do the following: **\$BAMTOOLS=/home/myAccount;** and then **\$export BAMTOOLS**.
3. Move to the directory where filterBam has been installed and type **make**. This should compile the program **filterBam.cc** and the source of this document, **filterBam.tex**.
4. The binary will be stored in **filterBam/bin** and the documentation in **filterBam/doc**.

| Software Dependencies |         |   |
|-----------------------|---------|---|
| Name                  | version | Available at  |
| Bamtools              | 2.1.0   | <a href="https://github.com/pezmaster31/bamtools">https://github.com/pezmaster31/bamtools</a> |
| Samtools (optional)   | 0.1.18  | <a href="http://samtools.sourceforge.net/">http://samtools.sourceforge.net/</a>               |

Table 1: List of software required by filterBam

| Library Dependencies |            |   |                            |
|----------------------|------------|---|----------------------------|
| Names                | version    | Available at  | Notes                      |
| zlib                 | >= 1.2.2.1 | < <a href="http://www.zlib.net">http://www.zlib.net</a> > | For support of BGZF format |

Table 2: List of libraries required by filterBam

## 2 A couple of examples

In this section we show how the filter works through the application of a couple of examples. The first example documents the operation of the filter for single alignments, while the second example describes the operation of the filter in paired-alignment mode. The filter accepts a **help** option to display its functionalities.

### 2.1 Dummy data

We have generated two dummy data sets to show filterBam in operation. One file illustrates how the cleaning of single alignments works, while the other file shows how paired alignments are processed. The file `example_single.bam` is a BAM file consists of a set of alignments that were specifically tailored to show the functionalities of the filter when treating inputs as single alignments. In a similar way, the file `example_paired.bam` was constructed with alignments that are product of paired-reads, so that the functionalities of the filter under this mode of operation are shown. Dummy data are stored under the folder `filterBam/data`.

### 2.2 Filtering of single alignments

The filter allows screening out alignment under a set of different criteria: coverage level, percentage of identity and insert gaps, with default values specified in Table 3. Running the filter on `example_paired.bam` with default options, that is:

```
> filterBam --in data/example_1.bam --out data/example_1.f.bam
```

will lead to the following output:

```
-----
Summary of filtered alignments:
-----
```

```
unmapped : 11
percent identity: 5
coverage : 0
-----
```

```
Cmd line:
```

```
filterBam --in data/example_1.bam --out data/example_1.f.bam
-----
```

```
Elapsed time: 0 seconds.
```

The source file, `example_1.bam` stores 35 alignments, whereas the filtered output has 19 alignments. As the output shows, 16 alignments were cleaned, 11 because they were not mapped and 5 because of the percent identity criteria.

Parameters of the filter might be modified. A run with `minCover= 70` will lead to a very different output. The options **best** and **uniq** are both mutually exclusive, because they make the filter let pass only alignments that have a minimum thresholding score.

## 2.3 Filtering of paired alignments

RNA-seq libraries might contain paired-reads, which provide additional information by means of the distance kept between the end of one read and the start of the other; something termed as the insert length. In fact, aligners such as Bowtie and GSNAP allow the alignment of paired reads by simply plugging-in both sets of reads as inputs. Nevertheless, sets of single reads that have been aligned independently might also be used to extract pairedness information: if you have two sets of aligned single reads, simply look pairs of alignments that might be potential pair-mates. filterBam does precisely that, within a single BAM file.

An example of how to clean paired-alignments. The option `--paired<value>` must be set:

```
> ./filterBam --in ../data/example_paired.bam --out
../data/example_paired.f.bam --paired
processed line 1
-----
Summary of filtered alignments:
-----
unmapped : 2
percent identity: 3
coverage : 0
not paired : 2
quantiles of unspliced insert lengths: [insertlen.size()=0]
-----
Cmd line:
./filterBam --in ../data/example_paired.bam --out
../data/example_paired.f.bam --paired
-----
Elapsed time: 0 seconds.
```

In this case the file

## 2.4 Retrieving additional information

For paired alignments, and in fact for paired reads, an important source of information is the distance kept between reads. When operating in paired-alignment mode, filterBam preserves the information of pairedness coverage between mate pairs. This feature is activated with the `--pairBed<value>`

**Warning:** At present time, the routine for collecting pairedness coverage information slows down quite a lot the execution time, so use it your own risk. Nevertheless, a faster version of this feature should be available relatively soon.

## 2.5 Other

This software has been tested on a a Dell (x86\_64) computer with Ubuntu 10.04 (lucid). Compilation of the code was done with GNU's C++ compiler, gcc version 4.4.3.

# 3 Technical specifications

Other relevant issues that might be well documenting go here.

## 3.1 Input data

The filter should work fine for data coming from 454 and Illumina technologies but not for colorspace data generated by SOLiD technology.

## 4 About Samtools and Bamtools

We introduce some examples of how to use Samtools and Bamtools to make life easier when working with BAM files. In particular we concentrate on the issue of sorting files by query name, as this is the requirement for filterBam. Attention should be paid to the fact that SAM and BAM files contain a header, so any sorting routine must consider the following: to momentarily put aside the header, do the sorting, and then insert the header at the top of the sorted file.

### 4.1 Samtools

Samtools is a set of utilities for manipulating SAM/BAM files. After downloading and compiling (follow the instructions of **Samtools**), a binary with the name **Samtools** will be created. Some useful commands in **Samtools** are the following:

- Convert from SAM to BAM  
`samtools view -bS input.sam -o output.bam`
- Convert from BAM to SAM  
`samtools view -h input.bam > output.sam`
- Sort BAM file  
`samtools sort input.bam out`
- Help `samtools --help`

Samtools comes as well with a C written API so that one can program routines.

### 4.2 Bamtools

Bamtools is a C++ wrapper API of the more well-known **Samtools** software; in addition, it includes a binary file with utilities that might be useful to manipulate BAM files. Some of the commands I have found most useful are:

- Count number of alignments in a BAM file  
`bamtools count -in input.bam`
- Sorting files by query name [WARNING: it seems that this sorting does not behave well with characters s.a. ":" ]  
`bamtools sort -byname -in input.bam -out output.bam`
- Help  
`bamtools --help`

# Filtering read alignments in BAM format: reference manual

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## Abstract

This note describes the detailed operation of the filter and lists the main classes that were used to implement it. In this way, people in the future might improve or reuse the filterBam code for other type of applications.

## 5 Introduction

RNA-seq data has become an important source of information for tasks such as differential expression analysis, transcript quantification and gene prediction. Given that this new technology produces millions of such short-reads ( $\sim 30bp$ ), bespoke methods and tools are required to process such big amounts of information. For example, a single run of an RNAseq experiment will produce millions, if not, hundreds of millions of short reads [Ref: Wiki].

After sequencing and generation of an RNAseq dataset, a later step consists of utilising the short reads to obtain an approximate version of the transcriptome, typically via the alignment of the reads to a reference genome. Such alignments can be carried out with tools such as BLAT, Bowtie, GSNAP, among others. In particular, Bowtie and GSNAP are specifically designed to align reads as short as 50 and 14 bp, respectively. Very recently, the introduction of the Sequence AlignMent Format, or SAM, by Li et al. (2009), has meant that many of the aforementioned alignment tools now produce outputs in SAM format.

filterBam is a C++ code that cleans alignment files stored in BAM format, which is the binary version of SAM. The software package is based on filterPSL, a Perl routine written by Prof. Dr. Mario Stanke for the processing of alignment records stored in PSL format. filterPSL is part of a set of Perl scripts that accompany the distribution of the annotation software, AUGUSTUS [Ref]. filterPSL is mainly used as a preprocessing step for cleaning alignments obtained with softwares such as BLAT, and filterBam is supposed to supersede it by doing the same task but on RNAseq alignment data stored in a BAM file.

## 6 Main features

In a nutshell, assuming a BAM file given as input, filterBam by default cleans all those alignments that are either unmapped or do not satisfy any of the following conditions:

1. do not comply with a minimum coverage;
2. do not have a minimum value of percentage identity, or
3. (optionally), do not satisfy a minimum value of base inserts.

Table 3 above summarises the main features of the filter.

After this basic set of filters has been applied, the alignments are processed according to whether they originated from single- or paired- RNAseq reads. Single alignments are cleaned by dropping out all those that do not satisfy a score value that depends on the coverage and the percentage identity of the aligned read, i.e.  $score(coverage, percId)$ . Paired alignments are mated to other alignments according to the distance and insert length from their associated reads; the filter then drops out all those pairs of alignments that do not satisfy

| filterBam         |                          |                |               |
|-------------------|--------------------------|----------------|---------------|
| Action            | Feature                  | Option         | Default value |
| Every alignment   |                          |                |               |
| Screens out       | unmapped                 | –              | –             |
|                   | coverage level           | minCover       | 80%           |
|                   | pctge identity           | minId          | 92%           |
|                   | insert gaps              | insertLimit    | 10bp          |
| Single alignments |                          |                |               |
| Screens out       | best                     | –              | nore          |
|                   | unique                   | uniqThresh     | 0.96          |
| Paired alignments |                          |                |               |
| Screens out       | best                     |                |               |
|                   | uniq                     | uniqThresh     | 0.96          |
| Writes to file    | common target genes      | commonGeneFile | false         |
|                   | pairedness coverage info | pairBedFile    | false         |

Table 3: Main features of filterBam.

a score value that, once again, depends on coverage and percentage identity, ( $score(coverage, percId)$ ).

The subsequent sections of this document describe in a step-wise manner how the filtering of single- and paired-alignments is done. The basic set of filters is described in Section 7. Then the filtering of single alignments is explained in Section 8, and finally the filtering of paired alignments is explained in Section 9.

## 7 Basic filters

Figure ?? below shows the schematics of the operation of the filter for single alignments. In the subsequent, we will assume an input BAM file is constituted by a series of records  $i = \{1, \dots, N\}$ , each containing the information of an alignment. See (Li et al., 2009) for further reference. The filter first checks whether alignment  $i$  is mapped or not, and this is easily done by means of verifying the bit  $0 \times 4$  of the alignment FLAG (SAM field number 2). As the specification suggests, this bit is the only source of reliable information to determine whether a read is mapped or not Li et al. (2009). This verification is achieved by using the `isMapped` method of BamTools. Unmapped reads are dropped, while mapped reads continue further processing. A counter keeps track of the number of unmapped reads that were dropped.

As a second step, alignments that passed the mapping test are appended with two additional but temporary string-tags. Tag ‘co’ and tag ‘pi’ are added to the binary alignment by the `addTag` method of BamTools. ‘co’ stands for *coverage* and is a measure of the amount of reads located at a given genomic position. ‘pi’ stands for *percentage identity* and is a measure of the number of basis that correctly identify a genomic position. Estimation of the coverage is done according to Equation 2, whilst estimation of the percentage identity is done following Equation 3, both in the Appendix.

If the estimated coverage value for the read in alignment  $i$  is less than that of the specified `minCover`, the alignment will be dropped and a counter keeping track of such types of events will be updated. In a similar way, if the value of `percId` for the read of alignment  $i$ , is less than that specified by `minId`, the alignment

will be dropped and the corresponding counter will be updated. Default values for **minCover** and **percId** are shown in Table 3 respectively, but might be modified by using the options **--minCover<value>** and **--minId<value>**.

## 8 Single alignments

Continuing with Figure ??, we assume either options **best** or **uniq** are selected, but not option **paired**. The core issue to understand in the operation of the single-alignment mode filter, is that batches of alignments belonging to a common query QNAME<sub>1</sub> will be processed independently from alignments belonging to a different query name QNAME<sub>2</sub>.

Alignments that passed the mapping test are appended with two additional but temporary string-tags. Tag *co* and tag *pi* are added to the binary alignment by means of the **addTag** method of BamTools. Tag *co* stands for *coverage* and is a measure of the amount of reads located at a given genomic position. Meanwhile *pi* stands for *percentage identity* and is a measure of the number of basis that correctly identify a genomic position. Whereas estimation of the coverage is done according to Equation 2, estimation of the percentage identity is done according to Equation 3, both in the Appendix. Table 4 below shows a series of alignments with the *co* and *pi* tags added.

If the estimated coverage value of alignment *i* is less than that of **minCover**, the alignment will be dropped and a counter keeping track of such types of drops will be updated. In a similar way, if the value of **percId** for alignment *i*, is less than that specified by **minId**, the read will be dropped and the corresponding counter will be updated. Default values for **minCover**= 80 and **percId**= 92 might be modified by using the options **--minCover<value>** and **--minId<value>**, respectively.

An optional value, the number of inserts to the base reference (**baseInsert**), is computed optionally if the '**noIntrons**' option is used. The number of insertions to the reference is computed through the application of Equation 4. This filter depends on the **insertLimit** value that has been specified, and which by default has a value of 10. The **insertLimit** parameter might be modified by applying the **--insertLimit<value>** option.

### 8.1 Uniq and Best criteria

Further cleaning can be achieved by means of selecting the mutually exclusive **unique** or **best** options. If such is the case, Figure 1 shows how an alignment record continues throughout the process path. Options **best** and **unique** stand for the filter selecting the *best* group of alignments, or the single-top alignment (i.e. **unique**), in terms of a cost function, which in this case is given defining the expression

$$\text{score} = \text{percId} + \text{coverage}. \quad (1)$$

Thus, after an alignment has passed through the mapping, coverage, percentage identity and intron-gap filters, the information from coverage and percentage identity is be combined into the figure **score**. Such value is added to the alignment as the tag *sc*.

After a group of alignments belonging to the same query has been scored, the group is sorted by such score value; as illustrated in Tables 4 and 5 below.

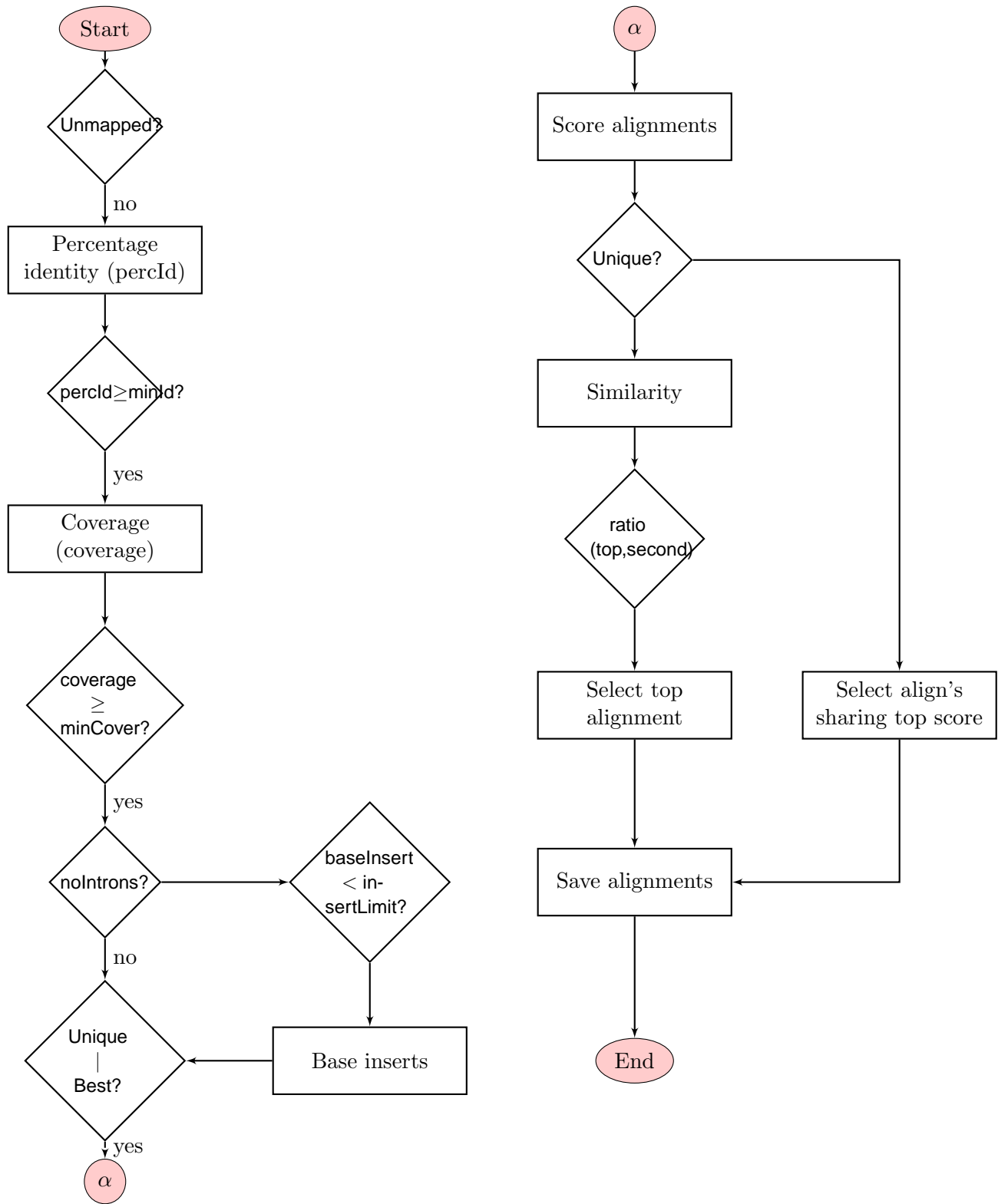


Figure 1: Flow diagram of the operation of the single-read filter



| QNAME | RNAME | startPOS | endPOS   | pi | co  | sc  |
|-------|-------|----------|----------|----|-----|-----|
| r2/1  | chr17 | 27698729 | 27698778 | 98 | 100 | 198 |
| r2/1  | chr17 | 20320140 | 20320189 | 94 | 100 | 194 |
| r2/1  | chr19 | 1364     | 1413     | 98 | 100 | 198 |
| r2/1  | chr17 | 8038458  | 8038507  | 96 | 100 | 196 |
| r2/1  | chr17 | 24524223 | 24524271 | 94 | 100 | 194 |
| r2/1  | chr17 | 30676704 | 30676750 | 96 | 96  | 192 |
| r2/1  | chr17 | 16894327 | 16894376 | 94 | 100 | 194 |
| r2/1  | chr17 | 5031882  | 5031931  | 96 | 100 | 196 |
| r2/1  | chr18 | 0        | 49       | 98 | 100 | 198 |

Table 4: SAM alignments with added tags: percId, coverage and score

| QNAME | RNAME | startPOS | endPOS   | pi | co  | sc  |
|-------|-------|----------|----------|----|-----|-----|
| r2/1  | chr17 | 27698729 | 27698778 | 98 | 100 | 198 |
| r2/1  | chr19 | 1364     | 1413     | 98 | 100 | 198 |
| r2/1  | chr18 | 0        | 49       | 98 | 100 | 198 |
| r2/1  | chr17 | 8038458  | 8038507  | 96 | 100 | 196 |
| r2/1  | chr17 | 5031882  | 5031931  | 96 | 100 | 196 |
| r2/1  | chr17 | 20320140 | 20320189 | 94 | 100 | 194 |
| r2/1  | chr17 | 24524223 | 24524271 | 94 | 100 | 194 |
| r2/1  | chr17 | 16894327 | 16894376 | 94 | 100 | 194 |
| r2/1  | chr17 | 30676704 | 30676750 | 96 | 96  | 192 |

Table 5: SAM alignments after sorting by score

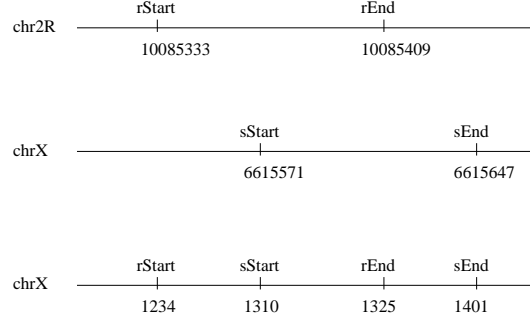


Figure 2: The similarity function checks whether mate paired reads are overlapping or not.

The difference between the **uniq** and **best** criteria is that the former will select only the top-scored alignment and will write it into file. Table 5 shows the same set of alignments as in Table 4, but after ranking by score. According to the **best** criterion, only the alignments sharing the optimal score will be preserved, while the rest of suboptimal alignments will be dropped. In the example of Table 5, the alignments sharing the score=198 will be preserved while the rest will be discarded.

The option **uniq** is based also on the sorting of alignments by its score. The main difference however is that only one alignment is preserved, provided that it is one of those sharing the top score, but also provided that it is eligible to be preserved. However, in case a group of alignments happen to share the same score, filterBam checks whether such alignments are similar; whereby similarity refers to two reads to have been aligned on overlapping positions.

## 8.2 Similarity function

A function that tests whether alignments (or two alignment pairs) are similar has been included within filterBam. Testing for similarity is required given that by handling separately spliced and unspliced alignments, there is the possibility that very similar alignments are reported, *an unspliced read going approximately up to an intron and a spliced read with a few base pairs on one exon*. Such type of cases should not be considered ambiguous when **uniq** is specified.

Figure 2 below shows two scenarios. In scenario one, a pair of reads were aligned to overlapping ranges of the reference genome; both reads are deemed similar. In scenario two, the reads are aligned to non-contiguous ranges of the reference genome, thus are considered not-similar.

Thus to finalise, a top-scored alignment will be let pass by the filter, if and only if, the second ranked alignment is not all too-similar to the top alignment.

## 9 Paired alignments

This section describes the filtering of paired alignments. This feature is enabled by selecting the option **paired**. By doing so, filterBam will compare a set of alignments belonging to a common query and determine which alignments are paired with which others. Such matching is done by examining the distance and insert length between candidate pairs. A more thorough explanation follows, nevertheless it is worth pointing out that before alignments are processed as paired alignments, they are subjected to the basic filters described in Section

| Mate 1      | Mate 2      | Strand 1 | Strand 2 | dist | insLen | score |
|-------------|-------------|----------|----------|------|--------|-------|
| rs1/1 (70)  | rs1/1 (71)  | false    | false    | –    | –      | –     |
| rs1/1 (70)  | rs1/2 (138) | false    | true     | 57   | 77     | 3.8   |
| rs1/1 (70)  | rs1/2 (201) | false    | true     | 120  | 138    | 3.875 |
| rs1/1 (70)  | rs1/2 (499) | false    | false    | –    | –      | –     |
| rs1/1 (71)  | rs1/2 (138) | false    | true     | 58   | 76     | 3.55  |
| rs1/1 (71)  | rs1/2 (201) | false    | true     | 121  | 137    | 3.625 |
| rs1/1 (71)  | rs1/2 (499) | false    | false    | –    | –      | –     |
| rs1/2 (138) | rs1/2 (201) | true     | true     | –    | –      | –     |
| rs1/2 (138) | rs1/2 (499) | true     | false    | –    | –      | –     |
| rs1/2 (201) | rs1/2 (499) | true     | false    | –    | –      | –     |

Table 6: Candidate mate pairs in the example presented in Section 9

7. A flow chart of the operation of filterBam for paired alignments is shown in Figure 3 below.

### 9.1 Mate pairs

Figure 4 shows a diagram in which four reads have been aligned: rs.1 (71), rs.2 (72), rs.2 (139) and rs.1 (202); with starting positions between parentheses. Bare in mind that query names have been made to coincide, in order to facilitate the understanding of the matching process. We recall that filterBam accepts inputs with '/1', '/2' suffix when the option **paired** has been selected.

Within a group of alignments, presented in the example as queries *rs*, filterBam defines a list of candidate mate pairs, as shown in Table X below. If one pair of alignments belongs to different mates 1,2 and come from different strands +,-, then their distance and insert-length is computed. If a pair of alignments has  $dist \geq 0$  and  $insLen \leq maxInsertLimit$ , the alignments are considered a valid mate-pair.

### 9.2 Uniq and Best criteria

Figure 3 shows the flow chart of operation of filterBam for paired alignments. As it can be seen, the filter operates under very similar tenets to those of the filter for single alignments, the main difference being that under the **paired** option, alignments are processed in pairs. Thus after scoring of the alignments of forming of the mate-pairs, the **uniq** selects the top-ranked pair of mates; where the rank is given by a function that makes use of the *coverage* and *percId* in very similar terms to those of Equation 1. Analogously as well, the option **best**, lets pass the set of mate-pairs that share the maximum score. It is important to remark that alignments that were not paired are dropped.

## 10 Coverage, percent of identity and insert length

The coverage is computed as the sum of the alignment matches (sequence matches or mismatches) and the insertions to the reference. Both figures, alignment matches and insertions to the reference, correspond to CIGAR string operations *M* and *I*, respectively. Thus the following is done

$$coverage = \frac{\sum CIGAR(M, I)}{qLength} \quad (2)$$

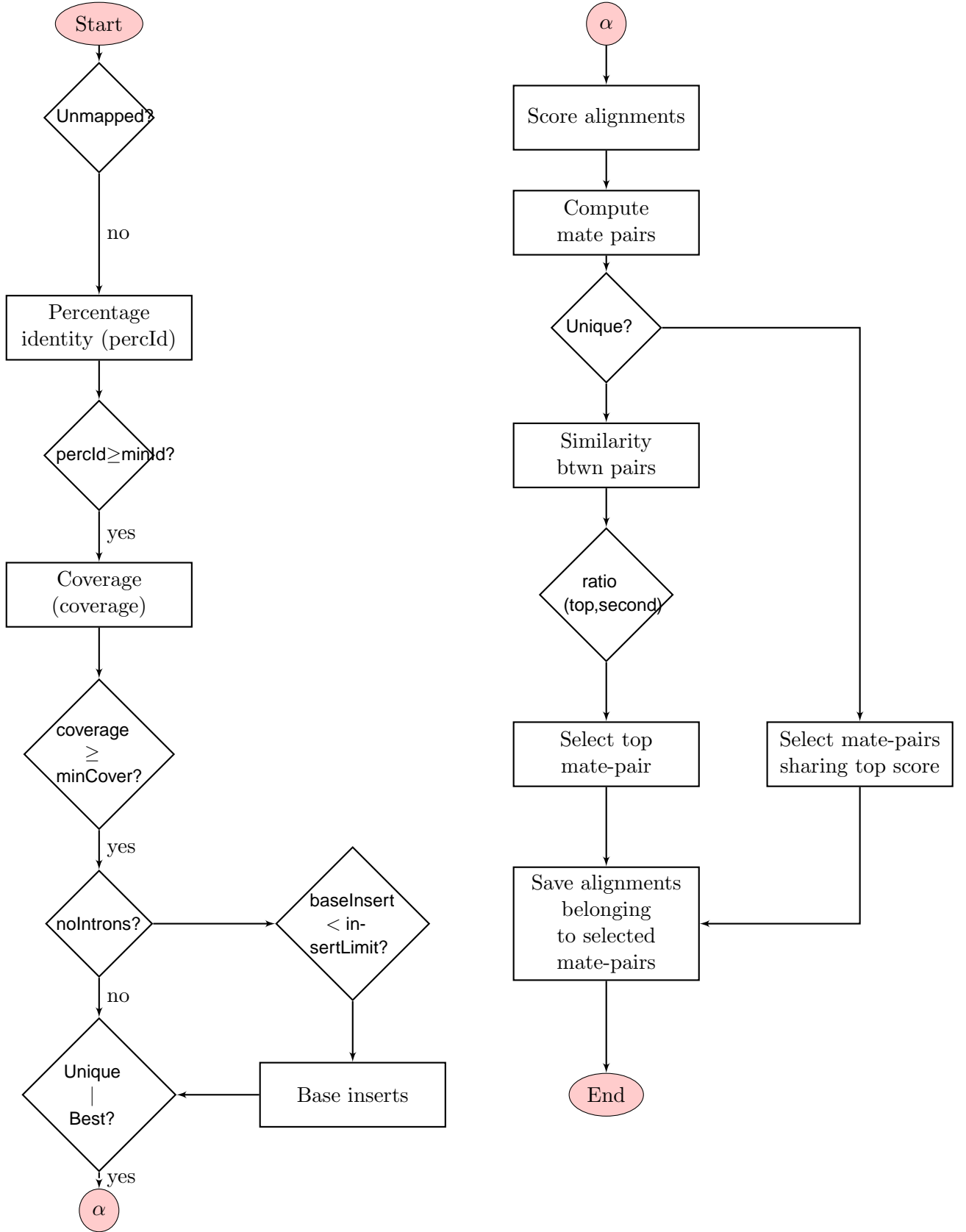


Figure 3: Flow diagram of the operation of the single-read filter

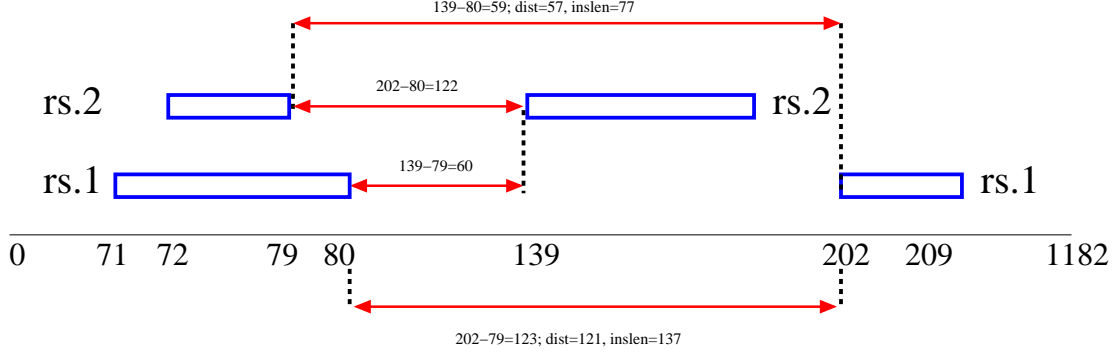


Figure 4: Paired reads

An approximation to the percentage of identity is given by computing the query length and subtracting the so-called edit distance to the reference (tag “NM” in SAM jargon), i.e.

$$\text{percId} = \frac{qLength - \text{Tag}(NM)}{qLength} \quad (3)$$

The length of inserts is estimated by summing CIGAR operations “M” and “I”, which correspond to alignment matches and deletions from the reference. In other words, we do the following

$$\text{InsertSize} = \frac{\sum \text{CIGAR}(D, I)}{qLength} \quad (4)$$

## References

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