Quantification of Splice Junctions in RNA-seq Data by *sjcount* v3.0

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Contents

1 Synopsis

The purpose of *sjcount* is to provide a fast utility for counting splice junctions in BAM files. It is the annotation-agnostic version of bam2ssj. This document describes the version **v3.0** of *sjcount*. The older versions of *sjcount* (v1.0, v2.0) is also included in the package under the name *deprecated*.

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2 Changes since previous version

The has been a substantial change between v2.0 and v3.0.

1. The utility now counts and reports reads with multi-splits
2. Accordingly, the output format has changed to account for multi-splits
3. A simpler and more efficient data structure is now used to store and parse multi-splits
4. Test routines are now added to check the quality and integrity of the output as compared to the output of a pearl script with easily controlled syntax

3 Installation and usage

See README.md file for installation instructions. The program *sjcount* is used from the command line with the following keys

```
sjcount_v3 -bam bam_file [-ssj junctions_output] [-ssc boundary_output]
       [-read1 0|1] [-read2 0|1] [-unstranded] [-nbins number_of_bins]
       [-lim number_of_lines] [-quiet]
```

where

- **bam_file** is a sorted input BAM file with a header
- **junctions_output** is the output file with junction counts
- **boundary_output** is the output file with boundary counts
- **read1** 0/1, reverse complement read1 no/yes (default=yes)
- **read2** 0/1, reverse complement read2 no/yes (default=no)
- **unstranded**, force strand=0
- **nbins** number of offset bins, (default=1)
- **maxnh** the max value of the NH tag, (default=none)
- **lim** stop after reading these many lines, (default=no limit)
- **quiet** – suppress verbose output **NOTE: use -quiet if you redirect stderr to a file!**

The output consists of two files. First, a tab-delimited file containing multi-split counts is produced as follows

```
chr1_100_200_+              1       34      1
chr1_100_200_+              1       36      1
chr1_100_200_+              1       37      6
chr1_100_200_+              1       38      3
chr1_100_200_300_400_+      2       49      1
chr1_100_400_+              1       33      1
...                         ...     ...     ...
```

where the first column contains the coordinates of the split alignment (including multi-splits, see below). The second column contains the number of splits. The third column contains the offset defined as the distance within the short read sequence of the latest split (defined below). The last column is the respective count, i.e., the number of split-mapped reads with the given combination of alignment coordinates and offset.

For instance, `chr1_100_200_+` denotes an alignment that was split once between positions 100 and 200 on the '+' strand, while `chr1_100_200_300_400_+` denotes an alignment that was split twice, first between positions 100 and 200, and then between positions 300 and 400. The coordinates are 1-based and always refer to terminal *exonic* nucleotides. The strand is denoted by '+' and '-' for stranded data or by '.' for unstranded data.

The second output is also a tab-delimited file which contains the counts of read alignments that *overlap* exon boundaries (exon boundaries are defined by splice junctions in the previous file). In this version all alignments that overlap an exon boundary by at least one nucleotide are counted (in older versions only continuous alignments were counted. This second file is optional and is needed to compute the completeness of splicing index [2, 3].

4 Method

4.1 Definitions

By definition, we say that we observe a *splice junction* each time we see an 'N' symbol in the CIGAR attribute of the alignment. If the CIGAR attribute contains several N's, then we have a *multi-split* or n-split, where n is the number of N's in CIGAR. In this terms, each 1-split defines one splice junction while each n-split defines n splice junctions.

Each multi-split is counted according to the number of splits so that, for example, the alignment `chr1_100_200_300_400_500_600_+` is counted once as a 3-split, two times as a 2-split (`chr1_100_200_300_400_+` and `chr1_300_400_500_600_+`), and three times as single-split (`chr1_100_200_+`, `chr_300_400_+`, and `chr1_500_600_+`). The positions of splits are decided entirely by the mapper which produced the alignment.

As an example, consider the multi-split alignment shown in Figure 1 below. In the output file it will be counted in three lines: in `chr1_31_52_+` as having 1

```
      10        20        30        40        50        60        70        80
      |         |         |         |         |         |         |         |
      12345678 90123456789012345678901234567890123456789012345678901234567890123456789012

chr1   AGTCTAGG*GACGGCATAGGAGGTGAGCATTTGTGTACGCAGATCTACAAAACATGTGTCACGGATAGGATCG
Query     CTAGGAGACGG**TAGGAG.....................ATCTA*AAAACAT.............GATa
                             |<-----   SJ1  ----->|           |<--- SJ2 --->|
```

The corresponding SAM line is:

```
Query   123   chr1  14    255    5M1I5M2D6M2ON5M1D7M13N3M1S 1234
```

Figure 1: An example alignment and its CIGAR attribute

split, in `chr1_64_78_+` as having 1 split, and in `chr1_31_52_64_78_+` as having 2 splits. One may want to subset the output to regular splice junctions by requiring the second column be equal to one.

4.2 Offset-specific counts

Artifacts may arise from combining counts that come from different starting positions of the alignment. We define the *offset* to be the distance (*in the query sequence!*) from the first alignment position to the corresponding 'N'. For instance, the junction SJ_1 in Figure 1 has offset 17, while the junction SJ_1 has offset 29. The offset of the multi-split is defined to be the offset of it's last N, i.e., 29 in this case. Since the offset is defined as a position in the query sequence, its value cannot exceed the read length.

Some offsets may give artifactually large read counts corresponding to CPR artifacts [1]. In Figure 2 we show six split reads supporting the same splice junction with offsets 14 (Q1), 12 (Q2–Q4), and 8 (Q5–Q6). Note that offsets appear decreasing when sequentially processing lines a sorted BAM file.

Offset-specific counts are generated as follows. We initialize and keep *nbins* separate counters for each n-split. For each instance of n-split, we increment

```
       10        20        30        40        50        60        70        80
       |         |         |         |         |         |         |         |
       12345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012

chr1   AGTCTAGGGACGGCATAGGAGGTGAGCATTTGTGTACGCAGATCTACAAAACATGTGTCACGGATAGGATCG

Q1             GGACGGCATAGGAG...................ATCT
Q2               ACGGCATAGGAG...................ATCTAC
Q3               ACGGCATAGGAG...................ATCTAC
Q4               ACGGCATAGGAG...................ATCTAC
Q5                   CATAGGAG...................ATCTACAAAA
Q6                   CATAGGAG...................ATCTACAAAA
```

Figure 2: Split-mapped reads support the same splice junction with different offsets

the counter corresponding to its offset. If the offset is larger than or equal to $nbins$ then it is set to be equal to $nbins - 1$.

For example, in the default settings we have $nbins = 1$. This means that the bin number will be $1 - 1 = 0$ for all supporting reads, regardless of their offset ($t = 14$ for Q1, $t = 12$ for Q2–Q4, and $t = 8$ for Q5–Q6 in Figure 2). Therefore, there is only one counter to increment, and the result will be so called "collapsed" counts. The output corresponding to Figure 2 will then be

```
Ref_31_52_+     1       0       6
```

By contrast, if we set $nbins$ equal to read length, there will be a separate counter for each offset and the output corresponding to Figure 2 will be

```
Ref_31_52_+     1       8       2
Ref_31_52_+     1       12      3
Ref_31_52_+     1       14      1
```

4.3 Aggregation

One single number is usually reported for each splice junction as an endpoint. Normally, the user wants to know how many reads aligned to a certain split regardless of the offset. This number is equal to the sum of counts for the given alignment over all values of offset. In other words, the total number of counts is obtained from offset-specific counts by aggregation using the function $f(x_1, \ldots, x_n) = x_1 + \cdots + x_n$.

Offset-specific counts are quite useful when aggregating by using different functions. For example, the number of *staggered* counts is the result of aggregation using $f(x_1, \ldots, x_n) = \theta(x_1) + \cdots + \theta(x_n)$, where $\theta(x) = 1$ for $x > 0$ and $\theta(x) = 0$ for $x \leq 0$. Another useful function is *entropy*, which is obtained from the offset-specific counts by aggregation with

$$f(x_1, \ldots, x_n) = \log_2(\sum_{i=1}^{n} x_i) - \frac{\sum_{i=1}^{n} x_i \log_2(x_i)}{\sum_{i=1}^{n} x_i}.$$

The entropy and the number of staggered reads can be used to filter out artefactual read counts. Note that *sjcount* only reports offset-specific counts, while the aggregation is left to the user. These aggregation functions are implemented in the splicing pipeline package https://github.com/pervouchine/ipsa.

4.4 Reads overlapping exon boundaries

By definition, we say that an alignment overlaps an exon boundary, if the terminal exonic nucleotide and the following first intronic nucleotide is also aligned. For example, consider exon boundaries defined by the alignment Q1 in Figure 2. In this example, Q7 and Q8 overlap the exon boundary at position 31, while

```
       10        20        30        40        50        60        70        80
       |         |         |         |         |         |         |         |
       12345678901234567890123456789012345678901234567890123456789012345678901234567890123456789012

chr1   AGTCTAGGGACGGCATAGGAGGTGAGCATTTGTGTACGCAGATCTACAAAACATGTGTCACGGATAGGATCG

Q1             GGACGGCATAGGAG...................ATCT
Q7              GACGGCATAGGAGGTGA
Q8               ACGGCATAGGAGGTGAG
Q9                CGGCATAGGA-GTGAGC
Q9                 GGCAT.....................................TGTGTCACGGA
```

Figure 3: Reads overlapping exon boundaries

Q1, Q9, and Q10 do not (note that Q1 defines exon boundary, but it would be incorrect to count it as contributing to intron retention).

5 Benchmark

In principle there is not too much to benchmark in *sjcount* because it only does the job of counting. We nevertheless test the performance of *sjcount* with respect to a naïve counting routine implemented in `perl`. In order to initiate benchmark, call `'make test'` in *sjcount* directory. It will download a small BAM file, run *sjcount*, and prompt of the outputs are different. Offset is also defined in this case as the position of the alignned exon boundary in the query sequence. For example, the offset of Q7 in Figure 3 is equal to 13. The position of exon boundary is denoted by `chr_pos_str`, e.g., `chr1_31_+` and `chr1_52_+` as in Figure 3. The number of splis is equal to 0.

References

[1] B. Kakaradov, H. Y. Xiong, L. J. Lee, N. Jojic, and B. J. Frey. Challenges in estimating percent inclusion of alternatively spliced junctions from RNA-seq data. *BMC Bioinformatics*, 13 Suppl 6:S11, 2012.

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