

KisSplice
De-novo calling alternative splicing events
from RNA-seq data
User's guide, version 2.4.0

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1 KisSplice, at a glance

KisSplice is dedicated to *de-novo* calling of alternative splicing events from one or several RNA-seq datasets. In addition to splicing events, KisSplice detects small indels (1, 2, 4 or 5 nucleotides), and provides a list of potential inexact tandem repeats and SNPs. Data from different conditions can be co-assembled with KisSplice .

2 KisSplice software package

2.1 CeCILL licence

This software is governed by the CeCILL licence. Details are mentioned in the `COPYING` file.

2.2 Reference

If you use KisSplice in a published work, please cite the following reference:

Gustavo AT Sacomoto, Janice Kielbassa, Rayan Chikhi, Raluca Uricaru, Pavlos Antoniou, Marie-France Sagot, Pierre Peterlongo* and Vincent Lacroix*, *KISSPLICE: de-novo calling alternative splicing events from RNA-seq data*, **BMC Bioinformatics** **2012**, 13(Suppl 6):S5

2.3 Mailing list

To be informed about new releases, bugs or updates, please subscribe to the mailing list `kisssplice-users@lists.gforge.inria.fr`.

To do so, please subscribe at : `http://lists.gforge.inria.fr/cgi-bin/mailman/listinfo/kisssplice-users`. Do not hesitate to write to the list for any comment or questions on KisSplice.

2.4 Requirements

KisSplice uses the CMake building tool (at least 2.8.5 CMake version is required). If it is not directly available on your system, you can download it from `http://www.cmake.org/cmake/resources/software.html`. KisSplice relies on zlib that is included in most systems, however it is also available from `http://zlib.net`.

2.5 Installation

KisSplice is written in C/C++ and is running on Mac OS X and Linux 64 bits platforms. A version of python 2.7 is needed (not compatible with Python 3).

In order to install KisSplice, you need to:

1. Uncompress the archive file:

```
tar zxvf kisssplice-x.y.z.tar.gz
```

2. Go into the corresponding directory:

```
cd kissplice-x.y.z
```

3. Launch cmake:

```
cmake .
```

If **zlib** library is present in your system in an uncommon path, launch cmake with the following options:

```
cmake . -DCMAKE_LIBRARY_PATH=/path/to/zlib -DCMAKE_INCLUDE_PATH=/path/to/zlib
```

4. Compile the package:

```
make
```

5. Run the functional and integration tests:

```
make test
```

6. Install the package on your system:

```
sudo make install
```

If you want to install the package in a particular directory `/path_to_install/`, then replace the step 3 by:

```
cmake . -DCMAKE_INSTALL_PREFIX=/path_to_install/
```

If you do not install the software, the program is available in the repertory **bin**.

3 Usage

3.1 Testing KisSplice in a couple of minutes

The example that will be presented only deals with alternative splicing events. However, other types of outputs are very similar and their interpretation can be easily inferred from this case.

However, please note that **KisSplice** may output 2 files for SNPs events, one for **Type0a** and another for **Type0b**. **Type0a** indicates that there is a single SNP within the sequence, while **Type0b** would indicate the presence of multiple SNPs, i.e. several SNPs separated by less than k nt, or a pattern created by paralogous genes.

NB : SNPs and **Type0a**/**Type0b** are not output by default. The **-s** option must be set to 1 or 2.

The `sample_example` directory contains two files containing simulated reads from two transcripts of *D. melanogaster*; `reads1.fa` and `reads2.fa`. There is a single splicing event (exon skipping).

Once you installed `KisSplice`, you can run it with defaults parameters (`k=41`) on the two fasta files present in the `sample_example` directory of the release:

```
bin/kissplice -r sample_example/reads1.fa -r sample_example/reads2.fa
less results/results_reads1_reads2_k25_coherents_type_1.fa
```

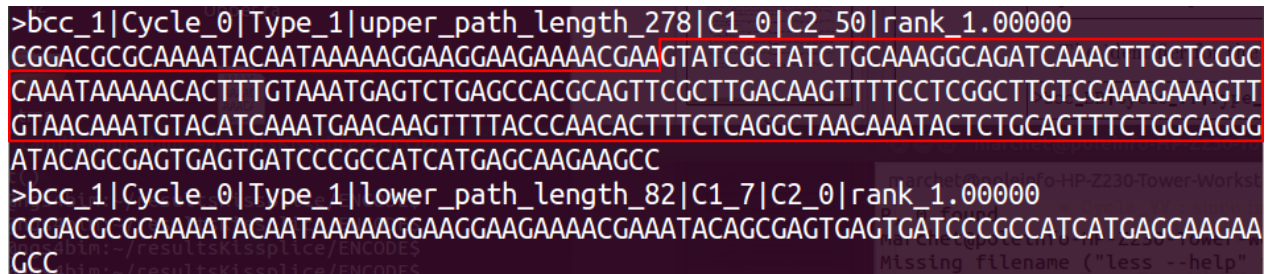
Using several `-r` can help you to co-assemble datasets. For our example, the output files can be found in the results directory:

`results_reads1_reads2_k41_coherents_type_1.fa` which contains the alternative splicing event (Fig. 1). The other files are empty.

We can look more in detail the results. Please see also the output section (4.2, page 9)

The output is pairwise, representing the variation between two sequences. Here it is an exon skipping : there is an inclusion in the path called "upper path" which is not in the path called "lower path". There are also the counts of the two isoforms:

- inclusion isoform : 0 (for condition 1, `reads1.fa` file) , 50 (for condition 2, `reads2.fa` file)
- exclusion isoform : 7 , 0



```
>bcc_1|Cycle_0|Type_1|upper_path_length_278|C1_0|C2_50|rank_1.00000
CGGACGCGCAAATACAATAAAAGGAAGGAAGAAAACGAAATATCGCTATCTGCAAAGGCAGATCAAACCTTGCTCGGC
CAAATAAAACACTTTGTAAATGAGTCTGAGCCACGCAGTTCGCTTGACAAGTTTCTCGGCTTGCCAAAGAAAGTT
GTAACAAATGTACATCAAATGAACAAGTTTTACCCAACACTTTCTCAGGCTAACAAATACTCTGCAGTTTCTGGCAGGG
ATACAGCGAGTGAGTGATCCCGCCATCATGAGCAAGAAGCC
>bcc_1|Cycle_0|Type_1|lower_path_length_82|C1_7|C2_0|rank_1.00000
CGGACGCGCAAATACAATAAAAGGAAGGAAGAAAACGAAATACAGCGAGTGAGTGATCCCGCCATCATGAGCAAGAA
GCC
```

Figure 1: Content of the file `results_reads1_reads2_k41_coherents_type_1.fa`, the alternative splicing event found. In red is the variable part in the inclusion isoform.

The splicing event is condition-specific with the inclusion isoform covered by 52 reads in condition 2 and the exclusion isoform covered by 9 reads in condition 1.

You may want to map the sequences against a reference genome if you have one to see what the output corresponds to. This can be done using `blat`, `GMAP` or any other RNA/DNA alignment software.

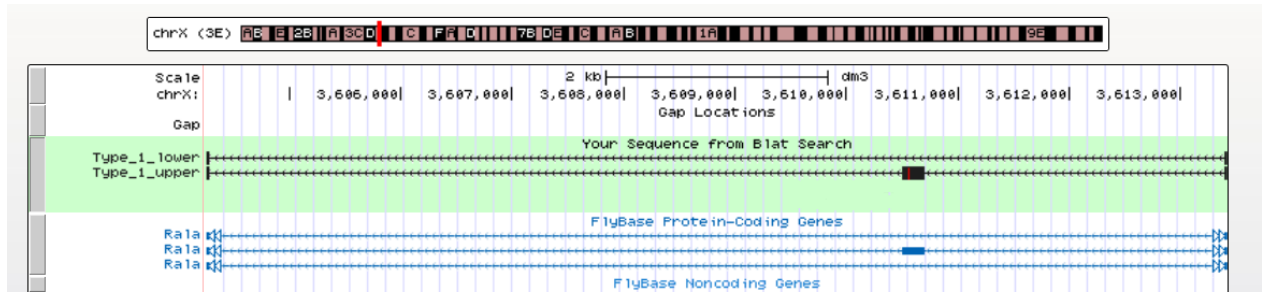


Figure 2: Using the reference genome, it is possible to see that the type 1 event is an exon skipping (figure obtained using the UCSC web platform)

3.2 Common Troubleshooting

Before running **KisSplice** on a large dataset, (10 conditions with 100M reads each), which takes time and memory, we advise to first run **KisSplice** on a subset of your dataset (2 conditions with 10M reads each) and get familiar with the output.

If you encounter problems running **KisSplice**, this is often due to the stack size limit. Please try increasing your stack size before running **KisSplice**. The best way to do this is setting the stack size to unlimited, if your operating system accepts this, executing the following command:

```
> ulimit -s unlimited
```

If your operating system does not accept the previous command, you can increase your stack size to the highest value possible. To find this value, execute:

```
> ulimit -H -s
```

And then, to set the stack size, execute:

```
> ulimit -s <highest_value>
```

If the problem persists, please do not hesitate to contact us.

3.3 Options

Type **kissplice -h** to see the options and parameters:

```
usage: kissplice [-h] [-r READFILES] [-k KVAL] [-b BVAL] [-l LLMAX] [-m LL_MIN]
[-M UL_MAX] [-g GRAPH_PREFIX] [-o OUT_DIR] [-d PATH_TO_TMP] [-t NBPROCS]
[-s OUTPUT_SNPS] [-v] [-u] [-c MIN_COV] [-C MIN_RELATIVE_COV] [-z GENOME_SIZE]
[-e MIN_EDIT_DIST] [-y MAX_CYCLES] [--mismatches MISM] [--counts COUNTSMETHOD]
[--min_overlap MINOVERLAP] [--timeout TIMEOUT] [--version] [--output-context]
[--output-path] [--output-branch-count] [--keep-bccs] [--experimental]
[--max-memory MAX_MEMORY] [--keep-counts]
```

kisSplice - local assembly of SNPs, indels and AS events

optional arguments:

-h, --help Show this help message and exit

-r READFILES Input fasta/q read files or compressed (.gz) fasta/q files (mutiple, such as "-r file1 -r file2...")

-k KVAL k-mer size (default=41)

-b BVAL Maximum number of branching nodes (default 5)

-l LLMAX Maximal length of the shorter path (default: 2k+1)

-m LL_MIN Minimum length of the shorter path (default 2k-8)

-M UL_MAX Maximum length of the longest path (default 1000000), skipped exons longer than UL_MAX are not reported

-g GRAPH_PREFIX Path and prefix to pre-built de Bruijn graph (suffixed by .edges/.nodes) if jointly used with -r, graph used to find bubbles and reads used for quantification

-o OUT_DIR Path to store the results and the summary log file (default = ./results)

-d PATH_TO_TMP Specific directory (absolute path) where to build temporary files (default temporary directory otherwise)

-t NBPROCS Number of cores (must be <= number of physical cores)

-s OUTPUT_SNPS 0, 1 or 2. Changes which types of SNPs will be output. If 0 (default), will not output SNPs. If 1, will output Type0a-SNPs. If 2, will output Type0a and Type0b SNPs (warning: this option may increase a lot the running time. You might also want to try the experimental algorithm here)

-v Verbose mode

-u Keep the nodes/edges file for unfinished bccs

-c MIN_COV An integer, k-mers present strictly less than this number of times in the dataset will be discarded (default 2)

-C MIN_RELATIVE_COV A percentage from [0,1), edges with relative coverage below this number are removed (default 0.05)

-z GENOME_SIZE Estimated genome/transcriptome size (default = 1G)

-e MIN_EDIT_DIST Edit distance threshold, if the two sequences (paths) of a bubble have edit distance smaller than this threshold, the bubble is classified as an inexact repeat (default 3)

-y MAX_CYCLES Maximal number of bubbles enumeration in each bcc. If exceeded, no bubble is output for the bcc (default 100M)

--mismatches MISM Maximal number of substitutions authorized between a read and a fragment (for quantification only), default 0. If you increase the mismatch and use --counts think of increasing min_overlap too.

--counts COUNTSMETHOD 0,1 or 2 . Changes how the counts will be reported. If 0 (default): total counts, if 1: counts on junctions, if 2: all counts. see User guide for more information

--min_overlap MINOVERLAP Set how many nt must overlap a junction to be counted by --counts option. Default=3. See User guide for more information

--timeout TIMEOUT Max amount of time (in seconds) spent for enumerating bubbles in each bcc. If exceeded, no bubble is output for the bcc (default 100000)

--version Show program's version number and exit

```

--output-context      Will output the maximum non-ambiguous context of a bubble
--output-path         Will output the id of the nodes composing the two paths of
                      the bubbles.
--output-branch-count Will output the number of branching nodes in each path.
--keep-bccs           Keep the node/edges files for all bccs.
--experimental        Uses a new experimental algorithm that searches for bubbles
                      by listing all paths.
--max-memory MAX_MEMORY If you use the experimental algorithm, you must provide the
                      maximum size of the process's virtual memory (address space) in megabytes (
                      default unlimited). WARNING: this option does not work on Mac operating systems
.
--keep-counts         Keep the .counts file after the sequencing-errors-removal
                      step.

```

3.4 Counts option

KisSplice can use several method to quantify the paths found in the DBG. The quantification depends on the `min_overlap` parameters. A read is used (and counted) if and only if it overlaps (by at least `min_overlap nt`) the variable part of the path. We can distinguish various parts in the paths of an event (please see Fig. 3):

- AS: junction between the left part of the path (A in green) and the variable part of the longer path (S in red). A read is in AS if it has more than `min_overlap nt` in S and A
- SB: junction between the right part of the path (B in blue), and the variable part of the longer path (S in red). A read is in SB if it has more than `min_overlap nt` in S and B.
- S: the variable part (in red), the difference between the upper and lower path. A read is in S if it has less than `min_overlap nt` in A or B.
- AB: junction between A and B (left, resp. right part of the path) in the shorter path. A read is in AB if it has more than `min_overlap nt` in A and B.
- ASSB: the junction AS and SB with S. A read is counted in ASSB if it has more than `min_overlap nt` in A and B (and so if fully in S).

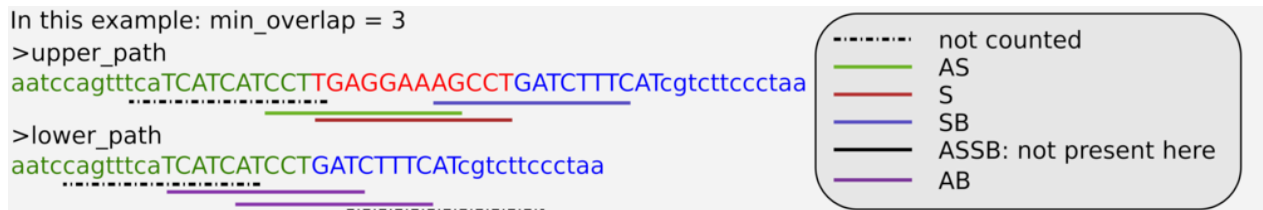


Figure 3: Example of quantification. In lower case, the context obtained using the `-output-context` parameter.

The `-counts` options can take three values: 0,1,2. It does not affect the quantification itself, but changes the output.

0. the total count is reported (default behaviour) as CX
1. the counts are written for ASX, SBX, ASSBX, ABX
2. all the counts are reported (ASX, SBX, SX, ASSBX and ABX)

Here X indicates the read file used for the quantification. It is possible to retrieve the total count C using: $C = (AS - ASSB) + (SB - ASSB) + S + ASSB$

3.5 Paired-end reads

For now, paired-end reads are not supported. We recommend that you use them as two input files :

```
kissplice -r condition1/1 -r condition1/2
```

4 I/O

4.1 Input

KisSplice may be used either directly from one or several sets of reads, or from a bi-directed de-Bruijn graph.

In the first case, one or several fasta/fastq files containing the reads have to be provided. In the second case, the de-Bruijn graph alone has to be provided (in dot format). In the input fasta/q files, each read should be written on exactly two lines for fasta (one for the fasta identifier and one for the sequence) and four lines for fastq.

The input files should have one of the following extensions: `fq`, `fastq`, `txt` or `fasta`, `fa`. KisSplice also handles compressed reads (`.fa.gz`)

To have an example of the input files, run KisSplice on the test data (provided in the directory `sample_example`), consisting of two read files in fasta format. You will find the constructed de-Bruijn graph built by KisSplice in the KisSplice directory.

4.2 Output

If it was not provided, a de-Bruijn graph is built for the N fasta files (read) given as input; the following three files are created in the results directory (for $k = XX$):

- `graph_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX.nodes` for the nodes of the de-Bruijn graph
- `graph_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX.edges` for its edges.
- `graph_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX.solid_kmers_binary_with_cou` a binary file relevant for the option `-C`

- `graph_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX.counts` for the k – *mers* count, created only if `KisSplice` was run with option `-C` coupled with `--keep-counts`.

In the node file, the 1st column is the node ID, the 2nd its (forward) sequence.

In the file describing the edges, the 1st and 2nd column are the IDs of the nodes that are connected by an edge, the 3rd column codes for the direction of the edge (FF = from the forward sequence of a node to the forward sequence of the other node, RR= from reverse to reverse, FR and RF). Note that if node A is connected with node B by an edge with direction “FF” (“FR”), node B is connected to node A by an edge directed “RR” (“RF”) and vice versa.

Moreover, six fasta files for the results are created:

1. `results_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX_type_0a.fa`: Single SNPs or sequencing substitution errors
2. `results_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX_type_0b.fa`: Multiple SNPs or sequencing substitution errors
3. `results_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX_type_1.fa`: alternative splicing events
4. `results_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX_type_2.fa`: inexact tandem repeats
5. `results_NameReadFile1_NameReadFile2_..._NameReadFileN_kXX_type_3.fa`: short indels
6. `results_NameReadFile1_..._NameReadFileN_kXX_type_4 .fa`: all others, composed by a shorter path of length $> 2k$ not being a SNP

If the read files (option `-r`) are provided as input files, the fasta files are checked for read coherency, which means that each nucleotide of each sequence has to be covered by at least one read. If `KisSplice` is used with option `-g` (only the de-bruijn graph is provided), a check for read coherency is not possible due to missing information about read coverage.

Since the read coherency step may take a long time, `KisSplice` preemptively outputs these six fasta files before checking for read coherency in the directory `results_without_read_coherency` inside the results directory. This enables the user to access the results even before the read coherency module finishes.

`KisSplice` also creates a summary log file in the results directory named `kissplice_log_summary_(time)_(date)_(random_integer)` in case the user wants to view a summary of the execution. This is not meant to be a detailed output. If you need a detailed output, please run `KisSplice` in verbose mode (parameter `-v`) and redirect the output and error stream to a file.

Fasta files are organized as follows, each 4-lines groups are an event

```
> identifier_upper_path
sequence upper path
> identifier_lower_path
sequence lower path
```

The identifier for the upper path is formatted as follows :

```
>bcc_BB|Cycle_YY|Type_ZZ|upper_path_Length_UU|C1_cov1|C2_cov2[...]|CN_covN|rank_RR
```

- **bcc_BB** : Bi-connected component BB the event belongs to
- **Cycle_YY** : since in each bcc, several cycles may exist, this attribute indicates the ID of the cycle (here: cycle YY) that generated the bubble
- **Type_ZZ**: with ZZ={0a, 0b, 1, 2, 3, 4}, the type also corresponds to the type given by the file name
- **upper_path_Length_UU** : length (in nucleotides) of the sequence of the upper path of the bubble
- **Cn**: with n={1, ..., N}: coverage of the path using reads from the read file n; coverage is the raw count of reads mapping the path with at least k (i.e. the value specified for the $k - mer$ length) nucleotides.
- **rank_RR** : This piece of information used to give an indication of if the alleles/the variants were differentially expressed between two conditions. It was an indication of the strength of the association between the allele/the variant and a condition but this was not a statistical test. We now provide a reliable indicator based on statistical analysis in our package kissDE (see <http://kisssplice.prabi.fr/tools/kissDE/>).

The identifier for the lower path provides virtually the same information, but concerning the lower (shorter) path of the bubble. In order to facilitate the further post-processing of these files, the bcc, cycle, type and rank are repeated, although this information is redundant.

If **KisSplice** is run with option **-r** (read files), the events in the output files are sorted with respect to their rank (this is not possible with option **-g** due to missing information about read coverage).

4.3 Uncoherent Bubbles

In order for a bubble to be considered as coherent, each nt has to be covered by at least one read. Uncoherent bubbles are output in a separate file. In principle, uncoherent bubbles should correspond to artefacts of the DBG (we lose information when we move from reads to $k - mers$). In practice, real events which have a low coverage may produce uncoherent bubbles. Hence, it may be worth to mine this file if you are interested in unfrequent events.

5 Performances

5.1 Pre-treatment

KisSplice can be run on raw data. However, as any assembler, it performs better if the data is pre-treated. Common pre-treatment includes polyA tail removal and quality filter. These

filters are not included in **KisSplice** distribution but can easily be found. For instance, FASTX Toolkit can be used to trim the reads, i.e. removing the last bases for which quality is below a threshold (we commonly use 20). Reads shorter than 20 nt are then removed.

5.2 Job calibration

The first time you run **KisSplice**, you may be interested in getting quickly initial results. We recommend to run it on a subset of your data (say 10%). It will give you an idea of the results and also an estimation of the running time for the full dataset.

We also suggest to use a higher k value than the default one during your first jobs (we recommend $k > \frac{\text{length of the read}}{2}$). Increasing the value of k will solve more issues due to repeats, though it will be followed by a loss of sensitivity. For a very first run such a loss can be a good compromise, with the production of more specific results.

You might be wondering about the disk space you need to have available for a job. **KisSplice** writes in several files and temporary files, and we currently consider that you must have two to three times the size of your data in free space to run **KisSplice** under proper conditions. To resolve disk space issues, we recommend to use **-d** option followed by a directory able to contain between two and three times the size of the input data.

If you want to use parallelisation option in your job, our advice is to put **-t** at 4 or 6 processors, more is not really efficient.

5.3 Relative coverage

The option **-C** edits the De-Bruijn graph constructed by removing edges non covered. It is a local filter. For a specific node, if one of its outgoing edges is covered less than **C** (in percentage) it is removed. For a small value of **C**, this option allows to remove artificial edges due to overlapping of two k -mers (not supported per the reads) or sequencing errors. Increasing **-C** will reduce the run time as there will be less things to enumerate, but the price is to lose some events with a rare isoform.

5.4 Big datasets

For some datasets, enumerating all bubbles is very long. Hence, we set a maximum amount of time (100000s by default) that the algorithm spends in each BCC (biconnected component). If after this time, the BCC is not finished, the algorithm stops and moves to the next BCC. Running **KisSplice** with **-u** option enables to output these unfinished BCCs for further inspection (for instance using Cytoscape).

If you intend to work on rather big datasets (> 1G reads), have a look at your **.e** log file in the end of the run. If you read "Timeout reached", it means the maximum amount of time has been reached, some bccs may have been lost then. We recommend to run again **KisSplice** with a higher **-C** (**-C** 0.05 is a good input) to simplify the graph (while not losing

too much information as there are already many reads) and retrieve all events, or to increase the timeout (option `-timeout`).

5.5 Genome-size option

The `-z` option is used for a rough estimation of the graph size. It is an estimation of unique k -mers contained into the dataset. A good evaluation of this parameter leads to an optimal graph construction step in term of memory consumption and time. On one hand, if the value is too low, the construction will take fewer memory but will take more time. On the other hand, if the value is too high, it will use a lot of memory. By default `z` is set to a billion (1 000 000 000) which is a good trade-off for most datasets.

6 Further questions

Please visit our at FAQ <http://kissplice.prabi.fr/FAQ/>.