rnaends: an R package targeted to study the exact RNA ends at the nucleotide resolution

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ABSTRACT

5' and 3' RNA-end sequencing protocols have unlocked new opportunities to study aspects of RNA metabolism such as synthesis, maturation and degradation, by enabling the quantification of exact ends of RNA molecules *in vivo*. From RNA-Seq data that have been generated with one of the specialized protocols, it is possible to identify transcription start sites (TSS) and/or endoribonucleolytic cleavage sites, and even, in some cases, co-translational 5' to 3' degradation dynamics. Furthermore, post-transcriptional addition of ribonucleotides at the 3' end of RNA can be studied at the nucleotide resolution.

While different RNA-end sequencing library protocols can vary, and have their own specificities, the generated RNA-Seq data are very similar and share common processing steps. Most importantly, the major aspect of RNA-end sequencing is that only the 5' or 3' end mapped location is of interest, contrary to conventional RNA sequencing that considers genomic ranges for gene expression analysis. This translates to a simple representation of the quantitative data as a count matrix of RNA-end location on the reference sequences. This representation seems under-exploited and is, to our knowledge, not available in a generic package focused on the analyses on the exact transcriptome ends.

Here, we present the *rnaends* R package which is dedicated to RNA-end sequencing analysis. It offers functions for raw read pre-processing, RNA-ends mapping and quantification, RNA-ends count matrix post-processing, and further count matrix downstream analyses such as TSS identification, fast Fourier transform for signal periodic patterns analysis, or differential proportion of RNA-ends analysis. The use of *rnaends* is illustrated with applications in RNA metabolism studies through selected workflows on published RNA-end datasets: (i) TSS identification, (ii) ribosome translation speed and co-translational degradation, (iii) post-transcriptional modifications analysis and differential proportion analysis.

Keywords: RNA-end sequencing, 5' end, 3' end, R-package, Transcription start site, endoribonucleolytic cleavage site, RNA degradation, post-transcriptional modifications, differential proportions, translation speed profiling

Introduction

Since its first development, high throughput sequencing has been derived into numerous applications in molecular biology, notably for genomics and post-genomics studies. The preparation of sequencing libraries for short read sequencing generally involves fragmentation of the polynucleotide prior to ligation of sequencing adapters. For transcriptome studies, this fragmentation renders the identification of the exact 5’ and 3’ termini of RNA molecules challenging at the nucleotide resolution. Knowledge about the exact ends of RNAs opens various opportunities in the study of RNA metabolism. On the 5’ end, (i) it can determine the exact transcription start site (TSS) positions (and consequently also promoter positions) as in (Prados et al., 2016), or (ii) it can give access to 5’ end degradation intermediates revealing *in vivo* footprint of ribosome occupancy and dynamics (Pelechano et al., 2015; Nersisyan et al., 2020), or (iii) it can locate endoribonucleolytic cleavage sites (Khemici et al., 2015; Broglia et al., 2020)).On the 3’ end, it can also locate endoribonucleolytic cleavage sites (Broglia et al., 2020), and it is also able to reveal the identity (and quantify the abundance) of ribonucleotides added post-transcriptionally to RNAs (Xu et al., 2023). To avoid the loss of precision due to RNAs fragmentation, specialised RNA-Seq library preparation protocols have been developed to obtain reads that correspond only to the exact 5' ends or 3' ends of RNA molecules (Sharma et al., 2010; Sharma & Vogel, 2014).

In eukaryotes, for the mRNA 5’ end identification, many protocols for library preparation rely on the cap protecting the 5' end of messenger RNA from degradation by exoribonuclease, or,for the 3' end studies, on the polyA tail. To analyse such specific data, dedicated software and tools have been developed. For the identification of 5' ends, the CAGE (Murata et al., 2014) and GRO-cap (Core et al., 2014) protocols are recommended (Adiconis et al., 2018) and, for downstream analyses, packages such as *CAGEr* (Haberle et al., 2015) and *CAGEfightR* (Thodberg et al., 2019) are readily available.

In prokaryotes, newly synthesized RNAs harbour a tri-phosphate at their 5' end and a hydroxyl at their 3' end (denoted respectively 5'PPP and 3'OH hereafter). Several protocols adapted for these molecules have been developed for RNA-Seq library preparation such as EMOTE (Redder, 2015), PARE (DiChiara et al., 2016), and Term-seq (Dar et al., 2016). The common principle of these protocols is to ligate a specific adapter (a specific RNA oligonucleotide) to the 5' or 3' termini of RNA molecules as illustrated in Figure 1. Ligation only occurs for RNA ends that exhibit specific biochemical properties such as a mono-phosphate at the 5' end (5'P) or a hydroxyl at the 3' end (3'OH). After ligation of this specific adapter, only RNAs harbouring the adapter are enriched or, alternatively, RNAs that do not harbour the adapter are depleted.

The exact protocol used for the RNA-Seq library preparation will vary depending on the issue addressed by the study of RNA metabolism. However, the sequencing data generated are very similar and share common processing steps to quantify RNA end abundances at the nucleotide resolution. Indeed, once the reads are aligned to the reference genome, only the 5'-most or the 3'-most mapped position is of interest for further analyses. Therefore, unlike conventional high-throughput sequencing analyses, that generally involve taking into account genomic ranges corresponding to mapped reads when quantifying RNAs, RNA-end sequencing quantification only considers reads that map to the same 5'-most or 3'-most position. This reduced, but powerful, representation is, to our knowledge, not readily available and exploited in existing software. Therefore, we are faced with the absence of a generic package focused on the analyses of the exact ends of transcriptomes when studying RNA metabolism. Indeed, in the previously published studies, each research group has developed their own tools and scripts to pre-process and analyse the same type of data to address biological questions related to RNA metabolism.

Over the years, while studying the RNA maturation and decay in *Staphylococcus aureus*, we have developed basic tools as well as more elaborate functions that we have integrated in an R package named *rnaends* presented here. The main principles driving this package are (i) pre-processing of reads prior their alignment to a reference sequence, (ii) quantification of mapped reads on a reference genome at single nucleotide locations (5' end or 3' end) which is given in an

output count table, (iii) downstream studies performed by statistical analyses available in the R environment or in a Bioconductor package, or an *ad hoc* implementation provided by our package.

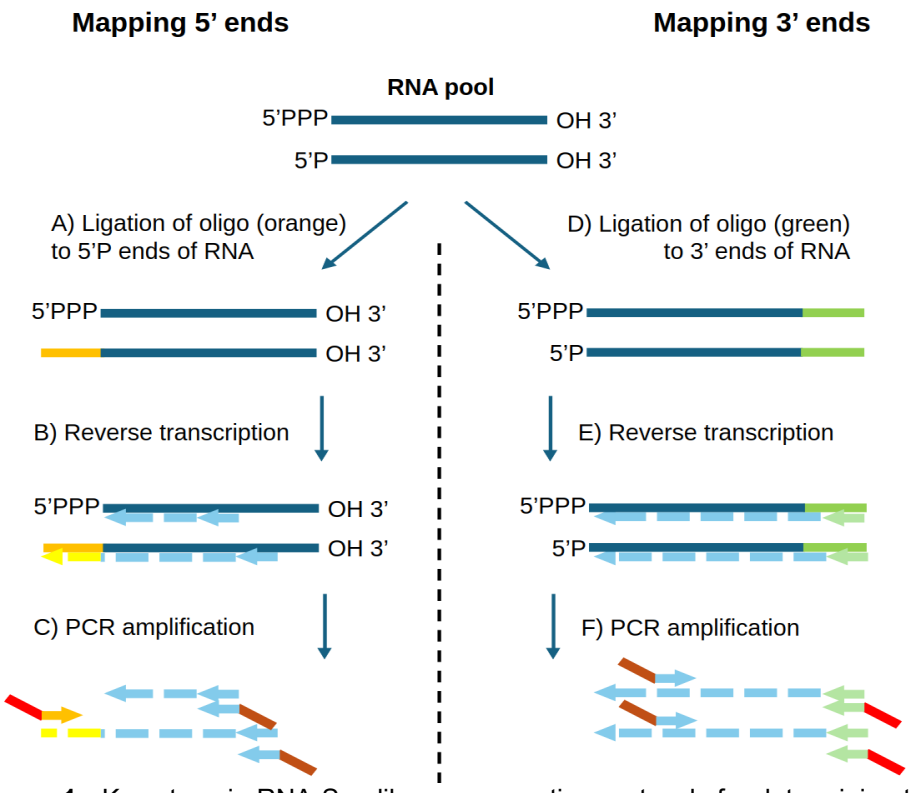
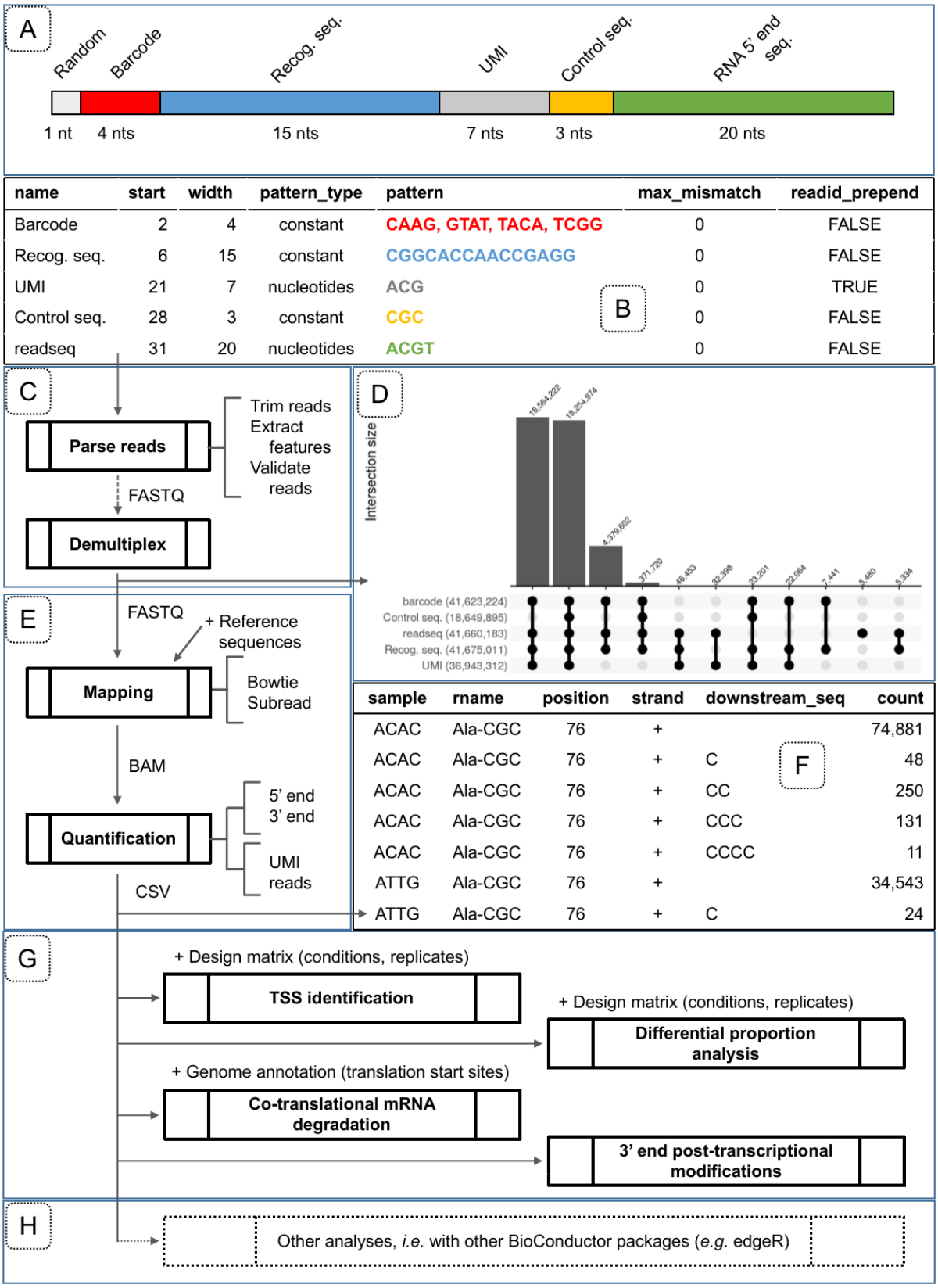


Figure 1 - Key steps in RNA-Seq library preparation protocols for determining the exact RNA-ends. Details vary according to protocol used. Mapping 5' ends (panels A, B and C): A) Ligation of an oligo with known sequence (orange) to 5'P ends of RNA. RNAs with 5'PPP are not accepted as substrate for the ligase. B) Reverse transcription to generate cDNA (light blue). For ligated RNAs, the cDNA will terminate with the reverse complement of the oligo sequence (yellow). C) PCR to amplify the cDNA and add adapter sequences for NGS sequencing (red and brown). Only cDNA from ligated RNAs will be amplified. Sequencing is performed starting from the red adapter sequence. Mapping 3' ends (panels D, E and F): D) Ligation of an oligo with known sequence (green) to 3'OH ends of RNA. E) Reverse transcription to generate cDNA (light blue) that will start with the reverse complement of the oligo sequence (light green). Only ligated RNAs will be reverse transcribed. F) PCR to amplify the cDNA and add adapter sequences for NGS sequencing (red and brown). Only cDNA from ligated RNAs will be amplified. Sequencing is performed starting from the red adapter sequence.

In the following, we present the key *rnaends* R package features that we have organized into different topics corresponding to different phases of typical analysis projects such as those depicted in Figure 2. We first describe the functions developed for the pre-processing of sequencing reads to validate and convert raw sequencing reads into reads mappable to reference sequences (Figure 2B, 2C and 2D). Then, we describe how mapped reads are quantified to obtain a count table representing RNA-end abundances at the nucleotide resolution (Figure 2E and 2F). Finally, we illustrate the use of the *rnaends* package for the investigation of selected questions related to the study of RNA metabolism. Starting with raw read data available from publications, we describe the workflows implemented from read pre-processing to statistical analyses applied to the resulting count table.



name	start	width	pattern_type	pattern	max_mismatch	readid_prepend
Barcode	2	4	constant	CAAG, GTAT, TACA, TCGG	0	FALSE
Recog. seq.	6	15	constant	CGGCACCAACCGAGG	0	FALSE
UMI	21	7	nucleotides	ACG	0	TRUE
Control seq.	28	3	constant	CGC	0	FALSE
readseq	31	20	nucleotides	ACGT	0	FALSE

sample	rname	position	strand	downstream_seq	count
ACAC	Ala-CGC	76	+		74,881
ACAC	Ala-CGC	76	+	C	48
ACAC	Ala-CGC	76	+	CC	250
ACAC	Ala-CGC	76	+	CCC	131
ACAC	Ala-CGC	76	+	CCCC	11
ATTG	Ala-CGC	76	+		34,543
ATTG	Ala-CGC	76	+	C	24

Figure 2 - Overview of the features provided in the *rnaends* package grouped by topics of an RNA-end sequencing project. A) Overview of an example of expected read structure of 5' end sequencing: 1 random nucleotide, then the 4 nucleotides of barcode (here either CAAG, GTAT, TACA or TCGG) identifying 4 multiplexed samples, then the recognition sequence (CGGCACCAACCGAGG), then a UMI (7 random A, C or G nucleotides), then 3 nucleotides of control sequence (CGC), and

eventually the actual 5' end of the RNA (20 nucleotides) which can be mapped onto the reference sequence; B) *read_features* table obtained with the functions *init_read_features* and *add_read_feature* corresponding to the read structure in panel A; C) FASTQ reads pre-processing and validation based on a *read_features* table describing the structure of sequencing reads; D) Distribution of read features validity described in panel A and represented by an upset plot generated after the parsing, validation and demultiplexing of a raw read FASTQ file. Each combination of valid features is displayed in the lower upset plot while the upper bar plot shows the corresponding number of reads; E) Reads alignment on reference sequences and abundance computation at the single nucleotide resolution to obtain: F) a count table representing the abundances (*count*) of RNA 3' ends mapped to a reference sequence (*rname*) at a certain location (*position*) on a *strand* and harbouring a sequence *downstream_seq* corresponding to 3' end additions that occurred post-transcriptionally. In this example of 3' end sequencing, the reads contain UMI extracted in the pre-processing step that are used at this step to remove PCR amplification duplicates introduced in the library preparation; G) *rnaends* provides functions for downstream analysis of the count table for TSS identification, analysis of RNA-ends species proportions (*e.g.* 5'P/5'PPP in WT *vs.* mutant strain), periodic signal analysis such as ribosome translation speed profiling, or the exploration of 3' end modifications; H) *rnaends* count table can also be analysed by other packages.

Material and methods

Development of *rnaends* as an R package

rnaends is implemented in R version 4.4.2. The choice of using only R and R packages available from CRAN or Bioconductor was motivated by the availability of downstream statistical analyses and the broad spectrum of packages offered by the Bioconductor ecosystem (Huber et al., 2015). The aim of the package is also to provide non expert users an entry point for RNA-end sequencing project by providing simple but versatile functions for processing FASTQ files and computing 5' or 3' end count table.

The main functions provided in the package are listed in Table 1, grouped by analysis topic and are detailed in the next subsections. For the pre-processing of reads and the quantification of exact RNA-end abundances steps, we rely on widely used Bioconductor packages that provide FASTQ and BAM files processing to compute the count table. Afterwards, we make use of the *tidyverse* metapackage to further process the count table and integrate some downstream analyses. The choice of *tidyverse* was motivated by the extensive documentation available on-line, and the wide functionalities available for processing and formatting tables (with the included packages *tidyr, dplyr, tibbles)*, as well as their visualisation (with the *ggplot2* package).

The *rnaends* functions are provided in an R package distributed *via* the GitLab platform. Source code is available as a git repository on GitLab at https://gitlab.com/rnaends/rnaends and documentation and tutorial workflows are available on GitLab Pages at https://rnaends.gitlab.io/rnaends (see Data, scripts, code, and supplementary information availability section).

Data

Datasets used in this manuscript and the R notebooks in the on-line documentation make use of publicly available datasets accompanying publications from others (see Data, scripts, code and supplementary information availability section for more details).

For the co-translational degradation analysis, the four RNA-Seq datasets used from (Khemici et al., 2015) are EMOTE assays on two wild type strains and two RNase Y full deletion mutant strains. The library preparation protocol is as follows. Briefly, in this protocol, total RNA is mixed with a synthetic RNA oligonucleotide (specific adapter). In these samples, only 5'P RNAs are ligated with the synthetic oligo which harbours, amongst others, a barcode for multiplexing samples for sequencing and a unique molecule identifier (UMI) of 7 random A, C or G nucleotides.

For the post-transcriptional 3' end addition analysis, the four RNA-Seq datasets used from (Xu et al., 2023) were multiplexed in an RNA-Seq library with other samples. The library preparation protocol is as follows. tRNAs were *in vitro* transcribed from full length tRNA templates harbouring CCA at their 3' end. From these samples, four samples were prepared: two incubated with the MenT1 toxin and two without the toxin. Then samples were then ligated at their 3'OH end with a synthetic oligo harbouring an UMI sequence of length 15 prior a reverse transcription step that incorporates a barcode for multiplexing samples for sequencing.

Table 1 - Main *rnaends* package functions grouped by RNA-end sequencing study steps indicated between parentheses: A) specification of the sequencing read structure expected in a FASTQ file as a *read_features* table (example in Figure 1B), and its use for pre-processing sequencing reads ; B) reads alignment to reference sequences and quantification of the exact 5' or 3' ends as a count table ; C) downstream analyses of the count table for different types of applications integrated in the package.

Function (study step)	Description
init_read_features (A)	Initializes and returns a read_features table with the minimal feature (named readseq) corresponding to the region in the read sequence that is expected to align to a reference sequence
add_read_feature (A)	Adds a feature to the read_features table returned by init_read_features
parse_reads (A)	Parses each read from a FASTQ file to retrieve features, test their validity and extract sequence to align
demultiplex (A)	Demultiplexes a FASTQ file according to barcode sequence reads provided through a read_features object
map_fastq (B)	Performs the mapping of a FASTQ file using either bowtie or subread aligner with appropriate parameters
quantify_5prime (B)	Converts aligned reads on reference sequences from a BAM file to the count table of exact 5' ends
quantify_3prime (B)	Converts aligned reads on reference sequences from a BAM file to the count table of exact 3' ends and unmapped downstream sequences
TSS_betabinomial (C)	Computes a p-value for a single position using a beta-binomial model.
TSS (C)	Performs identification of Transcription Start Sites (TSS) over all positions from a 5' end experiment
differential_proportion (C)	Performs identification of significant differences in proportions over all positions from a 5' or 3' end experiment
metacounts_normalized (C)	Normalizes counts over multiple regions and/or experiments and/or replicates
metacounts_feature (C)	Computes metacounts for a window around a set of locations of a given feature (e.g. 1st nucleotide of all translation start codons)
metacounts_region (C)	Computes metacounts for a set of regions (e.g. start locations and widths of all CDS)
FFT (C)	Formats counts or metacounts for one or multiple regions and calls FFT_profile to performs signal periodicity analysis
FFT_profile (C)	Performs fast Fourier transform signal periodicity analysis on a count or metacount profile
frame_preference (C)	Computes global translation frame preference over a set of regions (e.g. all CDS) from the exact 5' end count table.

Reads parsing, validation, trimming, feature extraction and demultiplexing

As illustrated in Figure 1, RNA-ends sequencing library preparation generally relies on the ligation of a synthetic oligonucleotide to RNAs. Consequently, the ligated oligo sequence will be present in the raw sequencing reads that, therefore, will need to be pre-processed and reformatted prior their alignment on the reference sequences. In *rnaends*, sequencing reads are expected to be

stored in the FASTQ file format, and are processed with the *ShortRead* (Morgan et al., 2009) Bioconductor package version 1.64.0.

Reads corresponding to RNA samples from different conditions or strains might be multiplexed by incorporating an index into the adapter for sequencing. However, further multiplexing is sometimes accomplished by incorporating barcodes into the oligos used for RNA ligation or the PCR primers used for amplification, and/or these oligos may contain functional sequences such as a unique molecule identifier (UMI) to eliminate duplicates resulting from a PCR amplification step that follows the adapter ligation. All these features will be part of the sequencing read itself, and will need to be removed before mapping the read to a reference sequence.

For different published datasets, we identified cases of read pre-processing scenarios and implemented a set of functions to parse read validity, to extract features and to format the reads in order to generate FASTQ files ready for the mapping step. To this end, the user must fill up the *read_features* table (illustrated in Figure 2B) to describe the structure and content of the raw sequencing reads (expected pattern type and location on reads). As processing more complex patterns, such as regular expressions, can be time-consuming, *rnaends* uses more specific patterns to achieve better performances on millions of reads. For instance, patterns can consist of (i) a constant string expected at a certain location on the reads (such as a control sequence to validate that the sequence of a ligated oligo is part of the reads), (ii) a set of allowed constant strings (typically barcodes when multiple samples are multiplexed for sequencing), (iii) a random sequence of allowed nucleotides (typically corresponding to the sequence to be aligned to the reference and/or the UMIs).

Once the expected read features are set up, the user can pre-process the raw reads FASTQ file to perform the feature matching and extraction, and the reads validation (Figure 2A, 2B and 2C). One or more FASTQ files will be obtained containing only valid reads ready to be aligned to the reference sequences, together with a report containing statistics (with number of reads matching each feature) for each sample on the content and quality of the FASTQ file of raw reads.

For an illustration, we used the dataset provided in (Khemici et al., 2015) where the sequencing reads are 50 nucleotides long and structured as follows: the first sequence nucleotide is a random nucleotide followed by a barcode of 4 nucleotides (CAAG, GTAT, TACA or TCGG) identifying 4 multiplexed samples, followed by a recognition sequence (CGGCACCAACCGAGG), then by a UMI (7 random A, C or G nucleotides), and a control sequence (CGC), and finally by the actual 5' end of the RNA which is 20 nucleotides long. Figure 2D is an illustration generated with our package on the results obtained from the report, which contains the presence or absence of each valid feature for each read. For this dataset, we can observe that in about half of the reads (leftmost bar), all features are valid with the exception of the control sequence. This may indicate a problem in the protocol or experiment, and could prove useful to the user who constructed the RNA-Seq library or performed the experiment. Then, almost as many reads carry only valid features and will be retained to be aligned to the reference. Other invalid reads are due to an invalid UMI and control sequence, or to an invalid UMI only. The rest of the invalid features, or their combination, represent smaller fractions of all the reads.

Mapping and quantification

The originality of RNA-end sequencing lies in the fact that only the first (respectively last) mapped position is of interest, as it corresponds to the exact 5' end (respectively 3' end) of the RNA molecule that was ligated to the adapter during library preparation.

For the read alignment, we have integrated the use of *bowtie* (Langmead et al., 2009) and *subread* (Liao et al., 2013) aligners with default appropriate parameters for the following reasons. Sequencing reads are aligned with reference sequences expected in the FASTA format. For alignments that should match nearly perfectly, *Rbowtie* (Langmead et al., 2009) version 1.46.0 is used with parameters *m=1* (for uniquely mapped reads) and *v* set by the user for the maximum number of mismatches authorized in the alignments. For sequencing reads that should align partially due to 3' end addition of ribonucleotides, *Rsubread* (Liao et al., 2019) version 2.20.0 is

used with parameters *unique* and *maxMismatches* set by the user and parameters *output_format="BAM"*, *sortReadsByCoordinates=TRUE*, *type="dna"*, *indels=0*. Alignments are produced or converted to BAM files to reduce space.

After mapping, read alignments can be described using a single nucleotide position in the reference sequence, *i.e.* either the 5' or 3' end of the RNA. However, in the case where ribonucleotides have been added to the 3' end after transcription, users should be interested in accessing the sequence absent from the reference genome. We therefore provide two functions, in the *rnaends* package, , one for processing RNA 5' ends (*quantify_5prime*) and the other for processing RNA 3' ends that might possess post-transcriptional modifications (*quantify_3prime)*. Both functions return the count table from a BAM file in the form of a count table in which mapped reads are counted by reference name, position and strand, and in the case of the 3' end function, the downstream sequence in the read not found in the reference genome is provided. An example illustration is given in Figure 2F.

Of note, BAM alignment files can be obtained through the *rnaends* functions but this is not mandatory: users can make use of any software to produce alignments. Alignments to reference sequences are expected in the BAM file format and are processed with *Rsamtools* (Martin Morgan, 2017) version 2.22.0. The *scanBam* function is used to extract mapped read information and converted to count tables stored as *tibbles* of the *tidyverse* meta-package version 2.0.0.

UMIs are most of the time necessary and incorporated in the reads in order to remove PCR amplification or other bias introduced by the library preparation. If UMIs were present in the sequencing reads and specified during the pre-processing step using the *readid_prepend* parameter in the *add_read_feature* function, they can be used in the quantification to filter library preparation amplification biases, *i.e.* the counts can be reported for mapped reads or for unique UMI mapped reads at a location on the reference. It is also possible to keep the UMI sequences included in the count table for further investigation such as their occurrence distribution in case they are not distributed as one could expect from random sequences (this is for example the case in data from (Khemici et al., 2015), data not shown).

As for the read pre-processing step, a statistics report can be generated for each BAM file produced. These statistics include the total number of reads and the percentage of uniquely mapped reads.

Downstream analyses integrated in the *rnaends* package

Obtaining the exact RNA end count table from raw sequencing reads is generally the required milestone allowing to address the initial questions driving the biological study. Through different studies over the years, we have explored and developed a few downstream analyses of the exact RNA ends count table that could be useful for other analyses. We have therefore integrated them into the *rnaends* package.

Differential abundance between paired samples: Transcription Start Site identification

RNA-end sequencing was initially motivated to address the inability of RNA sequencing protocols to determine precisely the exact 5' end of RNA molecules *in vivo* and their poor performance in identifying and quantifying the preferred transcription start site (TSS) at a genomic location. The strategy in prokaryotes is based on the phosphorylation state of the RNA 5' end. Indeed, only RNAs which 5' ends are tri-phosphorylated correspond to native 5' extremities and therefore to TSSs. Roughly, the library preparation protocol consists in first degrading all 5'P RNAs, and then the RNA is mixed with a synthetic RNA oligo (specific adapter) and split into two pools into which a ligase is added that can only bind the adaptor to 5'P RNAs. One pool serves as control to measure the level of background ligation (noise). The other is treated to convert 5'PPP to 5'P RNAs in order to sequence and quantify all RNA 5' ends that were initially tri-phosphorylated, see the TSS-EMOTE protocol (Prados et al., 2016) for a detailed procedure.

In this type of experimental setup with two paired samples from the same biological replicate, one with the background distribution and the other with some signal, a simple statistical test relying

on a beta-binomial distribution can be used to identify positions for which the signal deviates significantly from the background. This test is implemented in the *TSS_betabinomial* function that takes as input 4 parameters, *i.e.* the total counts in the two samples and the counts at a specific position in the two samples, and returns the *p*-value of the beta-binomial test provided by the *VGAM* package (Yee, 2020) version 1.1. *p*-values obtained from replicates are combined with Fisher's method using the *combine.test* function of *survcomp* (Schröder et al., 2011) version 1.56.0. All positions in an experiment with paired samples and replicates can be tested with the *TSS* function. This function uses the count table accompanied by a design matrix that associates counts to samples and replicates, and returns the *p*-values adjusted for multiple testing with, by default, the False Discovery Rate (Benjamini & Hochberg, 1995).

A tutorial with detailed analysis and code is available in an R notebook provided as supplementary material (see Data, scripts, code, and supplementary information availability section). Given that this approach is simply based on the application of a beta-binomial model between two samples, it can easily be transposed to analyse other experimental designs.

Differential proportion between conditions with replicates

To compare proportion of RNA species at their 5' or 3' end, the statistical framework is different from differential gene expression which compares discrete abundances and for which widely used Bioconductor packages such as *edgeR* (Robinson et al., 2010) and *DESeq2* (Love et al., 2014) are available.

When comparing proportions, it is important, from a statistical perspective, to take into account the sequencing depth, *i.e.* the counts for the different species of RNAs in the samples, and the replicates, in the different experimental conditions or strains. This leads to counts of a fraction over the total counts per condition and replicate at a specific location. The Bioconductor *DSS* package (Park & Wu, 2016) provides well suited procedures for this type of statistical analysis by estimating the local dispersion on counts for a feature over replicates, *i.e.* single nucleotide location in our context, and the global dispersion on counts over all features in the sequencing library. The function provided by *DSS* computes statistics for proportions observed in a DNA region (regardless of the chromosome strand) with counts corresponding to methylated *vs.* non methylated sites with replicates whereas we use it on a single nucleotide location on a specific strand. Thus, in *rnaends*, the use of the *DSS* package (Park & Wu, 2016) version 2.54.0 is integrated in the function *differential_proportion* to perform the statistical test of RNA species proportions difference on a specific location on a given strand. This function uses the count table along with a design matrix that associates counts to samples and replicates, and returns *p*-values and adjusted *p*-values for each strand and position.

Metacounts profile: aggregate counts of multiple regions to amplify or study genome-wide a particular feature

Periodicity of the ribosomal pauses can be revealed by the RNA 5' end degradation intermediates quantification (Pelechano et al., 2015). To study the periodicity of a signal around a given feature, such as the start codon of a protein coding gene, the sequencing depth is generally not sufficient, and one might need to compute a metacount profile from all these features on the chromosome (*i.e.* all start codons) to amplify the signal. The regions from which the counts are to be aggregated can be specified in two ways. First, they can be defined by a fixed window around a set of features (for example 100 residues around the translation start codons), using the *metacounts_feature* function of the package. Second, the set of regions can be specified by their start location and length (for example all the protein coding regions often named CDS), using the *metacounts_region* function. In the latter case, if the regions have different lengths, the length of the metacounts profile is specified by the quantile of the distribution of the region lengths. For instance, a quantile of 0.25 (the default value implying that at least 75% of the regions are longer) will ensure that metacounts at a given position will result from data available in at least 75% of the regions. Smaller regions are filled with zeroes and longer regions are truncated. Once the set of regions of the same length is defined, the core function for metacounts computation is

metacounts_normalized which extracts and normalizes counts on a set of genome regions. Counts are first normalized by region to avoid biases towards highly expressed regions and varying sequencing depths between samples to be merged. This consists in dividing each count in a region by the total counts of the region, *i.e.* converting them to frequencies. Then, the metacount of a given position in the window is obtained by taking the median of frequencies observed at this position in the set of regions to be combined. By default, the *metacounts_normalized* function returns the metacounts for all the samples and regions combined, but it is also possible to compute them for each feature/region and/or sample, for example when sequencing depth is very large and a strong signal is expected in a region.

Signal periodicity

Periodicity in the signal composed of counts in a region or a metacounts profile can be analysed with a fast Fourrier Transform analysis. The *rnaends* package provides the *FFT_profile* function which performs the analysis on a single profile with the the *fft* function of the *stats* R package. *FFT_profile* returns the fast Fourier transform analysis results formatted from an RNA end counts analysis perspective as a table in which each row consists in the signal strength detected for a period measured in nucleotides. The *FFT* function of the package provides a wrapper around the *FFT_profile* function to perform signal periodicity analysis over a set of regions, that can be analysed separately or combined as metacounts.

Frame preference

The *frame_preference* function of the package is specific to protein coding genes and computes the percentages of counts falling into the three possible translation frame. Counts are normalized over regions through a call to *metacounts_region* prior to their percentage computation.

Results

Ribosome translation speed and co-translational degradation

In eukaryotes and in some prokaryotes, degradation of RNA can be achieved by 5' to 3' exoribonucleases (Mathy et al., 2007; Hu et al., 2009; Durand et al., 2015). Such degradation of mRNAs can be blocked by translating ribosomes, and the 5'-3' degradation will follow the last translating ribosome (Hu et al., 2009). Quantification of 5'P RNA ends, *i.e.* degradation intermediates, can therefore reveal an *in vivo* footprint of ribosome occupancy and dynamics on translated mRNAs (Pelechano et al., 2015). When 5' to 3' degradation is co-translational and at least as fast as translation, ribosomal pauses can typically be observed upstream of the start and stop codons, as well as shorter ribosomal pauses along the protein coding sequences (CDS) resulting in a periodicity of three nucleotides (one codon).

To detect such ribosomal pausing events, we implemented three functions based on the work described in (Nersisyan et al., 2020). These functions make use of the 5' end quantification accompanied by a genome annotation table containing start and stop codon locations and potentially other features of interest. We tested our workflow on the raw data published as part of the study of RNase Y in *S. aureus* (Khemici et al., 2015). These data were obtained using the EMOTE protocol (Redder, 2015) in which the total purified RNA, once depleted of rRNA, is mixed with a specific oligonucleotide (specific adapter) and an RNA ligase. As a result, only RNAs with a 5'P end will have a 5' extension that corresponds to the oligonucleotide sequence.

In short, 5' end counts can be considered relative to a reference genomic location as the first nucleotide of the start or stop codon, and all 5' ends that were mapped to a position relative to the chosen location (*e.g.*, position -11 upstream of the start codon) are aggregated over all annotated CDSs to obtain a metacount profile over the user-selected window size around the genomic reference location. This is achieved by applying the *metacounts_feature* function (see Material and methods for details). Figure 3A shows that the positions -11 and -14 relative to the start codon have the highest values for metacounts of 5' ends, which could highlight a pause of the ribosome at the

translation initiation site. After this pause, it can also be seen that the blue bars corresponding to translation frame 2 have higher values than the other two frames. Periodicity analysis can be achieved by applying a fast Fourier transform (FFT) to the metacounts profile computed on all the CDS. In Figure 3B, the strongest signal is observed for a period of approximately three nucleotides. Furthermore, for a given period such as three nucleotides, metacounts from all CDS can be grouped to obtain a frame preference indication by applying the *frame_preference* function. Figure 3C shows that most of the 5' end mRNA degradation intermediates in *S. aureus* align on frame 2 of translation, followed by frame 3, then frame 1.

From these results, we can conclude that in *S. aureus*, a part of the 5' to 3' exonucleolytic RNA degradation occurs simultaneously with the translation. This has been observed in many bacterial species (Huch et al., 2023) and the effect is hypothetically attributed to the 5' exonucleolytic activity of RNase J1 and/or J2 in *S. aureus*. It would be interesting to test this hypothesis through a 5'-end sequencing study in deletion mutants of these enzymes and check whether the signal periodicity disappears in these mutant strains.

The detailed analysis and code is available in a tutorial R notebook provided as supplementary material (see Data, scripts, code, and supplementary information availability section).

Here, we have focused on the start codon as the feature of interest, however any other feature such as operons or unique position like TSSs can be used to centre the 5' end distributions and obtain a metacount profile not necessarily related to the ribosome.

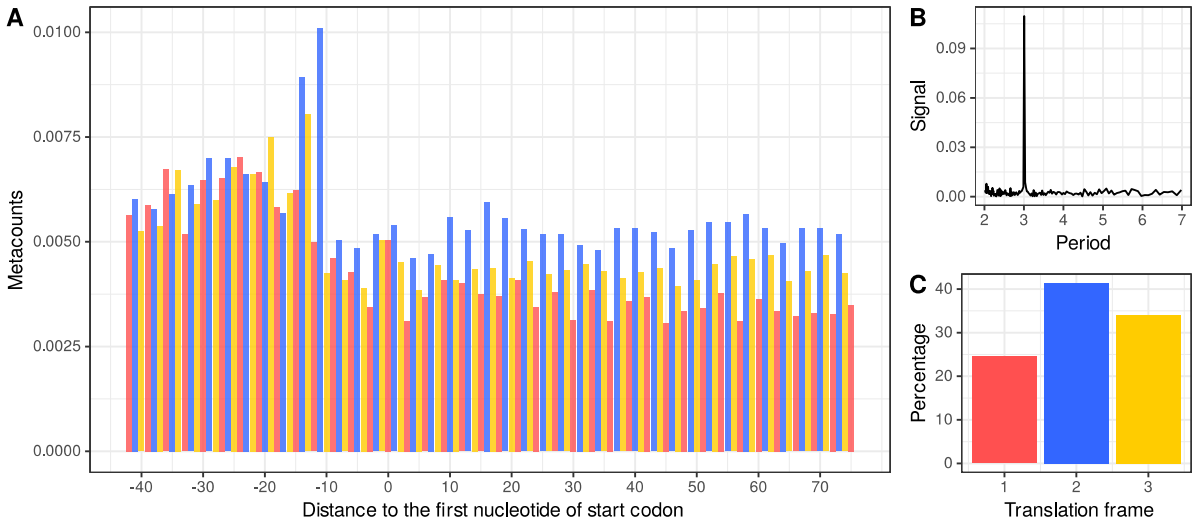


Figure 3 - *S. aureus* mRNA co-translational degradation by an exoRNase. (A) Metacount profile of *S. aureus* CDS start region. Each bar corresponds to the median number of normalized counts of 5' ends mapped at relative distance to the first nucleotide of the start codon. Counts are normalized by the total number of counts in the selected window. (B) Fast Fourier transform of a metacount profile obtained on a window size corresponding to the first quartile of the CDS length distribution. (C) Bar plot representing the percentage of normalized counts falling into each of the three translation frames in all the CDS.

Post-transcriptional modifications and differences in proportions

At the other end of transcripts, *i.e.* at their 3' end, RNAs can be exoribonucleolytically degraded, or non-template ribonucleotides can be added post-transcriptionally. The latter can for example be poly-adenylation of eukaryotic mRNAs, or the addition of CCA at the 3' end of tRNAs that are transcribed from genes without CCA at the 3' end of the genome template. In (Xu et al., 2023), a family of toxin-antitoxins present in *Mycobacterium tuberculosis* has been studied. One of the findings is that the toxins add ribonucleotides to the tRNA 3' ends prior to amino acid loading onto the tRNA, thus preventing its use for translation by the ribosome.

Compared to the 5' end analyses described so far, in which reads should align entirely and with 100% identity to the genome reference, the specificity for this type of analyses is that the reads are constituted by the end of the transcribed gene (up to 3' end of the transcript) and possibly by an additional sequence that does not align to the reference sequence. To be able to align such reads, we make available, in the *rnaends* package, the use of *Rsubread* (Liao et al., 2019) as described in (Xu et al., 2023). In short, the reads are aligned to reference sequences allowing partial alignments (also termed soft-clipping). After mapping, only the locations of the 3' end aligned regions are kept together with the unmapped regions downstream of the 3' end. For quantification, the count table is obtained by regrouping by locations and unmapped downstream sequences.

As an illustration of our implemented workflow, we used one of the raw FASTQ files published in (Xu et al., 2023) corresponding to *in vitro* transcribed tRNAs incubated or not with the MenT1 toxin with two replicates in both experimental conditions. The workflow proceeds as follows. First, raw reads are validated and demultiplexed into FASTQ files ready for alignment to reference tRNA sequences for each condition and replicates. Second, 3' end locations are quantified taking into account UMIs (PCR amplification bias removal) and unmapped 3' end downstream sequences. Third, mapped reads are filtered to keep only full length tRNAs possibly with 3' end addition of cytidines as *in vitro* transcribed tRNAs were incubated with the MenT1 toxin and CTPs.

From this count table, we can study the post-transcriptional modifications on tRNAs made by the MenT1 toxin. This is illustrated in Figure 4 where counts for each tRNA are represented by stacked bar plots. While in the control (without MenT1 treatment), tRNAs do not display 3' end modifications, we can observe that there is a fraction of those having an additional C and, to a lesser extent, multiple Cs as shown in (Xu et al., 2023). Of note, CCC or CCCC additions are present in very low amounts and are negligible.

In addition to what was published in (Xu et al., 2023), *rnaends* can perform a statistical test to decide whether the proportion of native *vs.* modified tRNAs is significantly different in the two conditions and replicates. The statistical framework is different from differential gene expression which compares abundances that are discrete quantities. The goal in this analysis is to compare proportions based on abundances together with replicates. From all the tRNAs of Figure 4, only 5 do not have a significant difference in proportions: the two Met-CAT tRNAs, Pro-TGG, Ser-TGA and Trp-CCA tRNAs. This could be due to the very low counts observed for these tRNAs that would justify to remove them from the main findings. Apart from these, the results show that, at least *in vitro*, the MenT1 toxin does not seem to have preference for specific tRNAs which are modified with similar levels of post-transcriptional additions.

The workflow described here illustrates how to analyse only a subset of the data related to the MenT1 toxin generated by (Xu et al., 2023). Its adaptation to the processing of data generated for the other toxins investigated in this publication should be straightforward.

The detailed analysis and code are available in an R notebook provided as supplementary material (see Data, scripts, code, and supplementary information availability section).

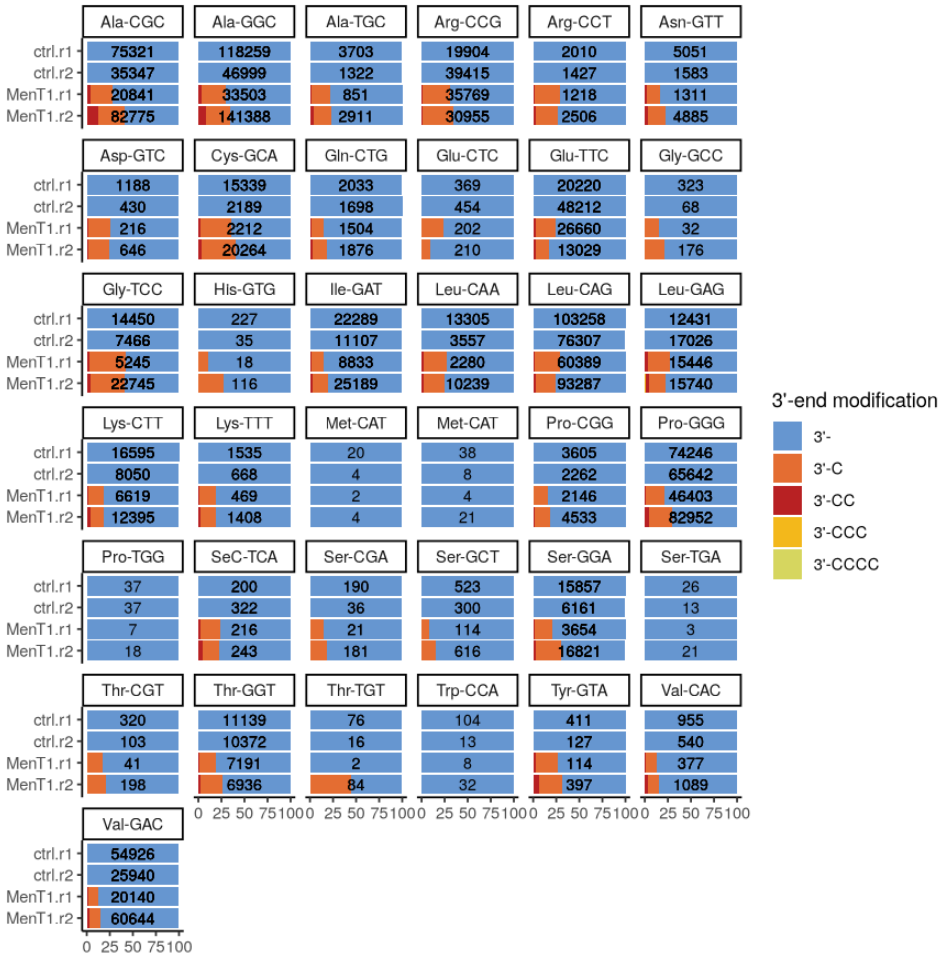


Figure 4 - tRNAs 3' ends modified by the MenT1 toxin were mapped by incubating *in vitro* transcribed tRNA from *Mycobacterium smegmatis* with or without MenT1 toxin in the presence of CTP. The total full length tRNAs (3'-) and full length with C additions are depicted with stacked bar plots for each tRNA represented as percentages of each full length tRNA with or without post-transcriptional additions. The two upper rows correspond to the two control replicates without MenT1 and the two lower rows correspond to the two samples treated with MenT1. The numbers on the bars indicate the number of mapped reads for each tRNA and sample.

Discussion

In this article, we illustrated some of the potential usages of functions implemented in the *rnaends* package through scenarios typical of RNA metabolism studies. Throughout its development, we aimed at making choices to render it as generic and user friendly as possible. The functions and functionalities have been implemented as independently as possible, so that they can be reused in the broadest variety of situations involving the processing RNA-end sequencing reads.

Existing software generally offer some but not all of the functionalities of *rnaends*, or are available in other programming languages or as command line tools. For instance, *MOIRAI* (Hasegawa et al., 2014), *RECLU* (Ohmiya et al., 2014), *CAGEr* (Haberle et al., 2015), *CAGEfightR* (Thodberg et al., 2019), are specific to TSS identification in eukaryotes and to the CAGE protocol. For a more general protocol, *TSSAR* (Amman et al., 2014) is available as Perl scripts that generate R code to process sequencing reads that have already been mapped (SAM or BAM files). It is designed, as *TSSr* (Lu et al., 2021), for TSS identification from exact 5' ends but they do not cover other aspect of exact RNA-end sequencing such as the 3' end or the signal periodicity analysis. Python tools or shell scripts generally focus on specific steps of the analysis: *READemption*

498 (Förstner et al., 2014) is written in Python, and enables reads pre-processing, mapping and
499 differential expression analysis for 'classic' RNA-Seq libraries, *i.e.* not dedicated to the study of the
500 exact 5' or 3' ends of transcripts. *PyRanges* (Stovner & Sætrom, 2020), *Pygenomics* (Tamazian et
501 al., 2023) and *Bioframe* (Open2C et al., 2024) also provide Python modules and tools for the
502 analysis of genomic ranges, whereas our package focuses on exact ends of transcripts. *BigSeqKit*
503 (Piñeiro & Pichel, 2022) targets large volumes of FASTA/FASTQ files but does not perform post-
504 alignment tasks. *UMI-tools* (Smith et al., 2017) implements some functionalities for UMI
505 deduplication. However, it does not allow to perform statistics on the UMI usage distribution and
506 does not perform demultiplexing when barcodes have been embedded in the oligo during library
507 preparation. It should be noted that *QuasR* (Gaidatzis et al., 2015) is an R/Bioconductor package
508 that allows reads pre-processing (but mainly adapter removal, such as 3'AAAA for example),
509 alignment and quantification in a *qCount* object. This latter offers more complex features for
510 genomic intervals, but does not offer the simplicity of considering only 5' or 3' end counts of
511 transcripts. The *BRGenomics* package (DeBerardine, 2023), also available in Bioconductor, is
512 dedicated to the steps following reads mapping and therefore does not offer the pre-processing
513 functions available in *rnaends*. *fivepseq* (Nersisyan et al., 2020), an easy to use software written in
514 Python, performs comprehensive analyses of 5'P degradome datasets in respect of translational
515 features and allows the global and gene-specific translational frame preference and codon-specific
516 ribosome protection patterns. However, it is restricted to 5'end analyses. Overall, although several
517 tools exist, they do not offer the possibility of performing all the analyses from an R environment
518 and/or do not focus on the specific aspects of analysing RNA 5' or 3' end sequencing data, notably
519 by the means of a simple count matrix representation of their single nucleotide locations on the
520 reference sequences.
521 The core of the *rnaends* package exploits the simplicity and versatility of the quantified
522 sequencing reads. These reads can be represented by a table instead of genomic ranges. Indeed,
523 for this type of studies, only the 5' or 3' end location on the chromosome or reference sequence is
524 of interest together with their abundance and strand, as well as potentially post-transcriptionally
525 added ribonucleotides downstream their 3' end. Efforts have been made to rely only on packages
526 where necessary, and thus to integrate into *rnaends* only functions from other packages that
527 already provide the required existing functionality. We hope these choices will allow non-experts to
528 be able to pre-process raw read FASTQ files and map the reads to their reference genome to
529 obtain the count tables corresponding to their experiment.
530 In particular, the *rnaends* package provides the *read_features* table to describe the structure of
531 the sequencing reads prior to their parsing, validation and optionally demultiplexing into separate
532 FASTQ files. The few cases and patterns identified and implemented should provide various
533 possibilities: from simple validation and counting of the raw reads from a FASTQ file, to the
534 demultiplexing into different FASTQ files on the basis of a barcode integrated in the reads during
535 the library preparation. It can also be used to trim reads or extract specific features such as UMIs.
536 Once the RNA end count table has been obtained from the mapped reads, further downstream
537 statistical analyses can be envisaged using the wide range of tests offered in R. This is illustrated in
538 an R notebook for TSS identification in the on-line package documentation. Moreover, if required
539 for analysis purpose, we have also developed dedicated R functions in *rnaends* as described when
540 investigating degradation intermediates in *S. aureus* (computation of metacount profiles,
541 periodicity signal analysis) to highlight that a part of the 5' to 3' exonucleolytic RNA degradation
542 occurs simultaneously with the translation. However, for more in-depth analyses of ribosome
543 profiling, such as quantification for di- or tri-nucleotides or even amino acids, we recommend the
544 use of *fivepseq* (Nersisyan et al., 2020). Post-transcriptional 3' end modifications are also simply
545 represented in the count table by adding the various sequences observed downstream the mapped
546 3' end into an additional column specific to this kind of study. One can then explore the diverse 3'
547 end modifications of RNAs. It is also possible to compute statistics as illustrated with the tRNA
548 modified or not by the MenT1 toxin.
549 On the other hand, our package can interoperate with packages providing some more elaborate
550 data analysis such as differential expression with *edgeR* (Robinson et al., 2010) or *DESeq2* (Love

et al., 2014). We set up an example to illustrate the simplicity of this integration through the identification of the cleavage sites of the endoribonuclease Y (RNase Y) in *Streptococcus pyogenes* based on the study of (Broglia et al., 2020) in a dedicated R notebook in the on-line package documentation. In particular, the authors have performed 5' end sequencing on a wild-type strain and an RNase Y deletion mutant strain. Using the raw data provided with their publication, we show that differential expression analysis can be conducted using the data table structure generated by our package.

The *rnaends* package is under continuous development and features will be added in the future. The code documentation and the R notebooks should allow experts as well as less experienced programmers to pre-process and analyse RNA-ends sequencing libraries.

Acknowledgements

The authors are thankful to Julien Prados (University of Geneva, Switzerland) who initiated the use of R for EMOTE data analysis and made his source code public and for his helpful comments.

Funding

TC was supported by an MESR (Ministère de l'Enseignement Supérieur et de la Recherche) fellowship.

Conflict of interest disclosure

The authors declare that they comply with the PCI rule of having no financial conflicts of interest in relation to the content of the article.

Data, scripts, code, and supplementary information availability

The *rnaends* package is available on GitLab at (https://rnaends.gitlab.io/rnaends) and its version 1.0.0 has been deposited on Zenodo at https://doi.org/10.5281/zenodo.15076101 (Caetano et al., 2025). All RNA-Seq datasets used in this article are available in public repositories: RNA-Seq dataset used for TSS identification from (Prados et al., 2016) in GEO repository with accession number GSE85110. RNA-Seq dataset used for RNase Y endoribonucleolytic cleavage sites identification from (Broglia et al., 2020) in SRA NCBI repository with accession number SRP149896. RNA-Seq dataset used in degradation intermediates analysis from (Khemici et al., 2015) in GEO repository with accession number GSE68811. RNA-Seq dataset used in 3' end post-transcriptional modifications from (Xu et al., 2023) in European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB62085.

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