Overlap encodings

Hervé Pagès

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Contents

1 Introduction

In the context of an RNA-seq experiment, encoding the overlaps between the aligned reads and the transcripts can be used for detecting those overlaps that are "splice compatible", that is, compatible with the splicing of the transcript.

Various tools are provided in the *GenomicAlignments* package for working with *overlap encodings*. In this vignette, we illustrate the use of these tools on the single-end and paired-end reads of an RNA-seq experiment.

2 Load reads from a BAM file

2.1 Load single-end reads from a BAM file

BAM file `untreated1_chr4.bam` (located in the *pasillaBamSubset* data package) contains single-end reads from the "Pasilla" experiment and aligned against the dm3 genome (see `?untreated1_chr4` in the *pasillaBamSubset* package for more information about those reads):

```
> library(pasillaBamSubset)
> untreated1_chr4()

[1] "/home/biocbuild/bbs-3.23-bioc/R/site-library/pasillaBamSubset/extdata/untreated1_chr4.bam"
```

We use the `readGAlignments` function defined in the *GenomicAlignments* package to load the reads into a *GAlignments* object. It's probably a good idea to get rid of the PCR or optical duplicates (flag bit 0x400 in the SAM format, see the SAM Spec [1] for the details),

[1] http://samtools.sourceforge.net/

as well as reads not passing quality controls (flag bit 0x200 in the SAM format). We do this by creating a *ScanBamParam* object that we pass to `readGAlignments` (see `?ScanBamParam` in the *Rsamtools* package for the details). Note that we also use `use.names=TRUE` in order to load the *query names* (aka *query template names*, see QNAME field in the SAM Spec) from the BAM file (`readGAlignments` will use them to set the names of the returned object):

```
> library(GenomicAlignments)
> flag0 <- scanBamFlag(isDuplicate=FALSE, isNotPassingQualityControls=FALSE)
> param0 <- ScanBamParam(flag=flag0)
> U1.GAL <- readGAlignments(untreated1_chr4(), use.names=TRUE, param=param0)
> head(U1.GAL)

GAlignments object with 6 alignments and 0 metadata columns:
                    seqnames strand       cigar    qwidth     start       end     width     njunc
                       <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer>
  SRR031729.3941844     chr4      -         75M        75       892       966        75         0
  SRR031728.3674563     chr4      -         75M        75       919       993        75         0
  SRR031729.8532600     chr4      +         75M        75       924       998        75         0
  SRR031729.2779333     chr4      +         75M        75       936      1010        75         0
  SRR031728.2826481     chr4      +         75M        75       949      1023        75         0
  SRR031728.2919098     chr4      -         75M        75       967      1041        75         0
  -------
  seqinfo: 8 sequences from an unspecified genome
```

Because the aligner used to align those reads can report more than 1 alignment per *original query* (i.e. per read stored in the input file, typically a FASTQ file), we shouldn't expect the names of `U1.GAL` to be unique:

```
> U1.GAL_names_is_dup <- duplicated(names(U1.GAL))
> table(U1.GAL_names_is_dup)

U1.GAL_names_is_dup
 FALSE   TRUE
190770  13585
```

Storing the *query names* in a factor will be useful as we will see later in this document:

```
> U1.uqnames <- unique(names(U1.GAL))
> U1.GAL_qnames <- factor(names(U1.GAL), levels=U1.uqnames)
```

Note that we explicitely provide the levels of the factor to enforce their order. Otherwise `factor()` would put them in lexicographic order which is not advisable because it depends on the locale in use.

Another object that will be useful to keep near at hand is the mapping between each *query name* and its first occurence in `U1.GAL_qnames`:

```
> U1.GAL_dup2unq <- match(U1.GAL_qnames, U1.GAL_qnames)
```

Our reads can have up to 2 *skipped regions* (a *skipped region* corresponds to an N operation in the CIGAR):

```
> head(unique(cigar(U1.GAL)))

[1] "75M"          "35M6727N40M"  "22M6727N53M"  "13M6727N62M"  "26M292N49M"   "62M21227N13M"
```

```
> table(njunc(U1.GAL))

     0      1      2
184039  20169    147
```

Also, the following table indicates that indels were not allowed/supported during the alignment process (no I or D CIGAR operations):

```
> colSums(cigarOpTable(cigar(U1.GAL)))

     M      I      D      N      S      H      P      =      X
224818      0      0  20463      0      0      0      0      0
```

2.2 Load paired-end reads from a BAM file

BAM file `untreated3_chr4.bam` (located in the *pasillaBamSubset* data package) contains paired-end reads from the "Pasilla" experiment and aligned against the dm3 genome (see `?untreated3_chr4` in the *pasillaBamSubset* package for more information about those reads). We use the `readGAlignmentPairs` function to load them into a *GAlignmentPairs* object:

```
> U3.galp <- readGAlignmentPairs(untreated3_chr4(), use.names=TRUE, param=param0)
> head(U3.galp)

GAlignmentPairs object with 6 pairs, strandMode=1, and 0 metadata columns:
                    seqnames strand :    ranges --    ranges
                       <Rle>  <Rle> : <IRanges> -- <IRanges>
  SRR031715.1138209     chr4      + :   169-205 --   326-362
   SRR031714.756385     chr4      + :   943-979 -- 1086-1122
  SRR031714.2355189     chr4      + :   944-980 -- 1119-1155
  SRR031714.5054563     chr4      + :   946-982 --  986-1022
  SRR031715.1722593     chr4      + :  966-1002 -- 1108-1144
  SRR031715.2202469     chr4      + :  966-1002 -- 1114-1150
  -------
  seqinfo: 8 sequences from an unspecified genome
```

The `show` method for *GAlignmentPairs* objects displays two ranges columns, one for the *first* alignment in the pair (the left column), and one for the *last* alignment in the pair (the right column). The `strand` column corresponds to the strand of the *first* alignment.

```
> head(first(U3.galp))

GAlignments object with 6 alignments and 0 metadata columns:
                    seqnames strand       cigar    qwidth     start       end     width     njunc
                       <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer>
  SRR031715.1138209     chr4      +         37M        37       169       205        37         0
   SRR031714.756385     chr4      +         37M        37       943       979        37         0
  SRR031714.2355189     chr4      +         37M        37       944       980        37         0
  SRR031714.5054563     chr4      +         37M        37       946       982        37         0
  SRR031715.1722593     chr4      +         37M        37       966      1002        37         0
  SRR031715.2202469     chr4      +         37M        37       966      1002        37         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> head(last(U3.galp))
```

```
GAlignments object with 6 alignments and 0 metadata columns:
                     seqnames strand       cigar    qwidth     start       end     width     njunc
                        <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer>
  SRR031715.1138209     chr4      -         37M        37       326       362        37         0
   SRR031714.756385     chr4      -         37M        37      1086      1122        37         0
  SRR031714.2355189     chr4      -         37M        37      1119      1155        37         0
  SRR031714.5054563     chr4      -         37M        37       986      1022        37         0
  SRR031715.1722593     chr4      -         37M        37      1108      1144        37         0
  SRR031715.2202469     chr4      -         37M        37      1114      1150        37         0
  -------
  seqinfo: 8 sequences from an unspecified genome
```

According to the SAM format specifications, the aligner is expected to mark each alignment pair as *proper* or not (flag bit 0x2 in the SAM format). The SAM Spec only says that a pair is *proper* if the *first* and *last* alignments in the pair are "properly aligned according to the aligner". So the exact criteria used for setting this flag is left to the aligner.

We use `isProperPair` to extract this flag from the *GAlignmentPairs* object:

```
> table(isProperPair(U3.galp))

FALSE  TRUE
29581 45828
```

Even though we could do *overlap encodings* with the full object, we keep only the *proper* pairs for our downstream analysis:

```
> U3.GALP <- U3.galp[isProperPair(U3.galp)]
```

Because the aligner used to align those reads can report more than 1 alignment per *original query template* (i.e. per pair of sequences stored in the input files, typically 1 FASTQ file for the *first* ends and 1 FASTQ file for the *last* ends), we shouldn't expect the names of `U3.GALP` to be unique:

```
> U3.GALP_names_is_dup <- duplicated(names(U3.GALP))
> table(U3.GALP_names_is_dup)

U3.GALP_names_is_dup
FALSE  TRUE
43659  2169
```

Storing the *query template names* in a factor will be useful:

```
> U3.uqnames <- unique(names(U3.GALP))
> U3.GALP_qnames <- factor(names(U3.GALP), levels=U3.uqnames)
```

as well as having the mapping between each *query template name* and its first occurence in `U3.GALP_qnames`:

```
> U3.GALP_dup2unq <- match(U3.GALP_qnames, U3.GALP_qnames)
```

Our reads can have up to 1 *skipped region* per end:

```
> head(unique(cigar(first(U3.GALP))))
[1] "37M"       "6M58N31M"  "25M56N12M" "19M62N18M" "29M222N8M" "9M222N28M"
> head(unique(cigar(last(U3.GALP))))
[1] "37M"         "19M58N18M"   "12M58N25M"   "27M2339N10M" "29M2339N8M"  "9M222N28M"
> table(njunc(first(U3.GALP)), njunc(last(U3.GALP)))

       0     1
  0 44510   596
  1   637    85
```

Like for our single-end reads, the following tables indicate that indels were not allowed/supported during the alignment process:

```
> colSums(cigarOpTable(cigar(first(U3.GALP))))
    M     I     D     N     S     H     P     =     X
46550     0     0   722     0     0     0     0     0
> colSums(cigarOpTable(cigar(last(U3.GALP))))
    M     I     D     N     S     H     P     =     X
46509     0     0   681     0     0     0     0     0
```

3 Find all the overlaps between the reads and transcripts

3.1 Load the transcripts from a *TxDb* object

In order to compute overlaps between reads and transcripts, we need access to the genomic positions of a set of known transcripts and their exons. It is essential that the reference genome of this set of transcripts and exons be **exactly** the same as the reference genome used to align the reads.

We could use the `makeTxDbFromUCSC` function defined in the *GenomicFeatures* package to make a *TxDb* object containing the dm3 transcripts and their exons retrieved from the UCSC Genome Browser[2]. The Bioconductor project however provides a few annotation packages containing *TxDb* objects for the most commonly studied organisms (those data packages are sometimes called the *TxDb* packages). One of them is the *TxDb.Dmelanogaster.UCSC.dm3.ensGene* package. It contains a *TxDb* object that was made by pointing the `makeTxDbFromUCSC` function to the dm3 genome and *Ensembl Genes* track [3]. We can use it here:

```
> library(TxDb.Dmelanogaster.UCSC.dm3.ensGene)
> TxDb.Dmelanogaster.UCSC.dm3.ensGene

TxDb object:
# Db type: TxDb
# Supporting package: GenomicFeatures
```

[2] http://genome.ucsc.edu/cgi-bin/hgGateway

[3] See http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=276880911&g=ensGene for a description of this track.

```
# Data source: UCSC
# Genome: dm3
# Organism: Drosophila melanogaster
# Taxonomy ID: 7227
# UCSC Table: ensGene
# Resource URL: http://genome.ucsc.edu/
# Type of Gene ID: Ensembl gene ID
# Full dataset: yes
# miRBase build ID: NA
# transcript_nrow: 29173
# exon_nrow: 76920
# cds_nrow: 62135
# Db created by: GenomicFeatures package from Bioconductor
# Creation time: 2015-10-07 18:15:53 +0000 (Wed, 07 Oct 2015)
# GenomicFeatures version at creation time: 1.21.30
# RSQLite version at creation time: 1.0.0
# DBSCHEMAVERSION: 1.1
```

```
> txdb <- TxDb.Dmelanogaster.UCSC.dm3.ensGene
```

We extract the exons grouped by transcript in a *GRangesList* object:

```
> exbytx <- exonsBy(txdb, by="tx", use.names=TRUE)
> length(exbytx)  # nb of transcripts
```

```
[1] 29173
```

We check that all the exons in any given transcript belong to the same chromosome and strand. Knowing that our set of transcripts is free of this sort of trans-splicing events typically allows some significant simplifications during the downstream analysis [4]. A quick and easy way to check this is to take advantage of the fact that `seqnames` and `strand` return *RleList* objects. So we can extract the number of Rle runs for each transcript and make sure it's always 1:

```
> table(elementNROWS(runLength(seqnames(exbytx))))
```

```
    1
29173
```

```
> table(elementNROWS(runLength(strand(exbytx))))
```

```
    1
29173
```

Therefore the strand of any given transcript is unambiguously defined and can be extracted with:

```
> exbytx_strand <- unlist(runValue(strand(exbytx)), use.names=FALSE)
```

We will also need the mapping between the transcripts and their gene. We start by using `transcripts` to extract this information from our *TxDb* object `txdb`, and then we construct a named factor that represents the mapping:

[4] Dealing with trans-splicing events is not covered in this document.

```
> tx <- transcripts(txdb, columns=c("tx_name", "gene_id"))
> head(tx)

GRanges object with 6 ranges and 2 metadata columns:
      seqnames      ranges strand |     tx_name         gene_id
         <Rle>   <IRanges>  <Rle> | <character> <CharacterList>
  [1]    chr2L   7529-9484      + | FBtr0300689     FBgn0031208
  [2]    chr2L   7529-9484      + | FBtr0300690     FBgn0031208
  [3]    chr2L   7529-9484      + | FBtr0330654     FBgn0031208
  [4]    chr2L 21952-24237      + | FBtr0309810     FBgn0263584
  [5]    chr2L 66584-71390      + | FBtr0306539     FBgn0067779
  [6]    chr2L 67043-71081      + | FBtr0306536     FBgn0067779
  -------
  seqinfo: 15 sequences (1 circular) from dm3 genome

> df <- mcols(tx)
> exbytx2gene <- as.character(df$gene_id)
> exbytx2gene <- factor(exbytx2gene, levels=unique(exbytx2gene))
> names(exbytx2gene) <- df$tx_name
> exbytx2gene <- exbytx2gene[names(exbytx)]
> head(exbytx2gene)

FBtr0300689 FBtr0300690 FBtr0330654 FBtr0309810 FBtr0306539 FBtr0306536
FBgn0031208 FBgn0031208 FBgn0031208 FBgn0263584 FBgn0067779 FBgn0067779
15682 Levels: FBgn0031208 FBgn0263584 FBgn0067779 FBgn0031213 FBgn0031214 FBgn0031216 ... FBgn0264003

> nlevels(exbytx2gene)  # nb of genes

[1] 15682
```

3.2 Single-end overlaps

3.2.1 Find the single-end overlaps

We are ready to compute the overlaps with the `findOverlaps` function. Note that the strand of the queries produced by the RNA-seq experiment is typically unknown so we use `ignore.strand=TRUE`:

```
> U1.OV00 <- findOverlaps(U1.GAL, exbytx, ignore.strand=TRUE)
```

`U1.OV00` is a *Hits* object that contains 1 element per overlap. Its length gives the number of overlaps:

```
> length(U1.OV00)

[1] 563552
```

3.2.2 Tabulate the single-end overlaps

We will repeatedly use the 2 following little helper functions to "tabulate" the overlaps in a given *Hits* object (e.g. `U1.OV00`), i.e. to count the number of overlaps for each element in the query or for each element in the subject:

Number of transcripts for each alignment in `U1.GAL`:

```
> U1.GAL_ntx <- countQueryHits(U1.OV00)
> mcols(U1.GAL)$ntx <- U1.GAL_ntx
> head(U1.GAL)

GAlignments object with 6 alignments and 1 metadata column:
                    seqnames strand       cigar    qwidth     start       end     width     njunc |
                       <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer> |
  SRR031729.3941844     chr4      -         75M        75       892       966        75         0 |
  SRR031728.3674563     chr4      -         75M        75       919       993        75         0 |
  SRR031729.8532600     chr4      +         75M        75       924       998        75         0 |
  SRR031729.2779333     chr4      +         75M        75       936      1010        75         0 |
  SRR031728.2826481     chr4      +         75M        75       949      1023        75         0 |
  SRR031728.2919098     chr4      -         75M        75       967      1041        75         0 |
                         ntx
                    <integer>
  SRR031729.3941844         0
  SRR031728.3674563         0
  SRR031729.8532600         0
  SRR031729.2779333         0
  SRR031728.2826481         0
  SRR031728.2919098         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U1.GAL_ntx)

U1.GAL_ntx
    0     1     2     3     4     5     6     7     8     9    10    11    12
47076  9493 26146 82427  5291 14530  8158   610  1952  2099   492  4945  1136

> mean(U1.GAL_ntx >= 1)

[1] 0.7696362
```

76% of the alignments in `U1.GAL` have an overlap with at least 1 transcript in `exbytx`.

Note that `countOverlaps` can be used directly on `U1.GAL` and `exbytx` for computing `U1.GAL_ntx`:

```
> U1.GAL_ntx_again <- countOverlaps(U1.GAL, exbytx, ignore.strand=TRUE)
> stopifnot(identical(unname(U1.GAL_ntx_again), U1.GAL_ntx))
```

Because `U1.GAL` can (and actually does) contain more than 1 alignment per *original query* (aka read), we also count the number of transcripts for each read:

```
> U1.OV10 <- remapHits(U1.OV00, Lnodes.remapping=U1.GAL_qnames)
> U1.uqnames_ntx <- countQueryHits(U1.OV10)
> names(U1.uqnames_ntx) <- U1.uqnames
> table(U1.uqnames_ntx)

U1.uqnames_ntx
    0     1     2     3     4     5     6     7     8     9    10    11    12
39503  9298 18394 82346  5278 14536  9208   610  2930  2099   488  4944  1136

> mean(U1.uqnames_ntx >= 1)

[1] 0.7929287
```

78.4% of the reads have an overlap with at least 1 transcript in `exbytx`.

Number of reads for each transcript:

```
> U1.exbytx_nOV10 <- countSubjectHits(U1.OV10)
> names(U1.exbytx_nOV10) <- names(exbytx)
> mean(U1.exbytx_nOV10 >= 50)

[1] 0.009015185
```

Only 0.869% of the transcripts in `exbytx` have an overlap with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_nOV10, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0112904 FBtr0289951 FBtr0089243 FBtr0333672 FBtr0089186
      40654       40529       40529       11735       11661       11656       10087       10084
FBtr0089187 FBtr0089172
      10084        6749
```

3.3 Paired-end overlaps

3.3.1 Find the paired-end overlaps

Like with our single-end overlaps, we call `findOverlaps` with `ignore.strand=TRUE`:

```
> U3.OV00 <- findOverlaps(U3.GALP, exbytx, ignore.strand=TRUE)
```

Like `U1.OV00`, `U3.OV00` is a *Hits* object. Its length gives the number of paired-end overlaps:

```
> length(U3.OV00)

[1] 113827
```

3.3.2 Tabulate the paired-end overlaps

Number of transcripts for each alignment pair in `U3.GALP`:

```
> U3.GALP_ntx <- countQueryHits(U3.OV00)
> mcols(U3.GALP)$ntx <- U3.GALP_ntx
> head(U3.GALP)

GAlignmentPairs object with 6 pairs, strandMode=1, and 1 metadata column:
                    seqnames strand :    ranges --    ranges |       ntx
                       <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer>
  SRR031715.1138209     chr4      + :   169-205 --   326-362 |         0
   SRR031714.756385     chr4      + :   943-979 -- 1086-1122 |         0
  SRR031714.5054563     chr4      + :   946-982 --  986-1022 |         0
  SRR031715.1722593     chr4      + :  966-1002 -- 1108-1144 |         0
  SRR031715.2202469     chr4      + :  966-1002 -- 1114-1150 |         0
  SRR031714.3544437     chr4      - : 1087-1123 --   963-999 |         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_ntx)
```

```
U3.GALP_ntx
    0     1     2     3     4     5     6     7     8     9    10    11    12
12950  2080  5854 17025  1078  3083  2021    70   338   370    59   803    97
```

```
> mean(U3.GALP_ntx >= 1)
```

```
[1] 0.7174217
```

71% of the alignment pairs in `U3.GALP` have an overlap with at least 1 transcript in `exbytx`.

Note that `countOverlaps` can be used directly on `U3.GALP` and `exbytx` for computing `U3.GALP_ntx`:

```
> U3.GALP_ntx_again <- countOverlaps(U3.GALP, exbytx, ignore.strand=TRUE)
> stopifnot(identical(unname(U3.GALP_ntx_again), U3.GALP_ntx))
```

Because `U3.GALP` can (and actually does) contain more than 1 alignment pair per *original query template*, we also count the number of transcripts for each template:

```
> U3.OV10 <- remapHits(U3.OV00, Lnodes.remapping=U3.GALP_qnames)
> U3.uqnames_ntx <- countQueryHits(U3.OV10)
> names(U3.uqnames_ntx) <- U3.uqnames
> table(U3.uqnames_ntx)
```

```
U3.uqnames_ntx
    0     1     2     3     4     5     6     7     8     9    10    11    12
11851  2061  4289 17025  1193  3084  2271    70   486   370    59   803    97
```

```
> mean(U3.uqnames_ntx >= 1)
```

```
[1] 0.7285554
```

72.3% of the templates have an overlap with at least 1 transcript in `exbytx`.

Number of templates for each transcript:

```
> U3.exbytx_nOV10 <- countSubjectHits(U3.OV10)
> names(U3.exbytx_nOV10) <- names(exbytx)
> mean(U3.exbytx_nOV10 >= 50)
```

```
[1] 0.00712988
```

Only 0.756% of the transcripts in `exbytx` have an overlap with at least 50 templates.

Top 10 transcripts:

```
> head(sort(U3.exbytx_nOV10, decreasing=TRUE), n=10)
```

```
FBtr0308296 FBtr0089175 FBtr0089176 FBtr0112904 FBtr0089243 FBtr0289951 FBtr0333672 FBtr0089186
       7574        7573        7572        2750        2732        2732        2260        2260
FBtr0089187 FBtr0310542
       2260        1698
```

4 Encode the overlaps between the reads and transcripts

4.1 Single-end encodings

The *overlap encodings* are strand sensitive so we will compute them twice, once for the "original alignments" (i.e. the alignments of the *original queries*), and once again for the "flipped alignments" (i.e. the alignments of the "flipped *original queries*"). We extract the ranges of the "original" and "flipped" alignments in 2 *GRangesList* objects with:

```
> U1.grl <- grglist(U1.GAL, order.as.in.query=TRUE)
> U1.grlf <- flipQuery(U1.grl)  # flipped
```

and encode their overlaps with the transcripts:

```
> U1.ovencA <- encodeOverlaps(U1.grl, exbytx, hits=U1.OV00)
> U1.ovencB <- encodeOverlaps(U1.grlf, exbytx, hits=U1.OV00)
```

`U1.ovencA` and `U1.ovencB` are 2 *OverlapsEncodings* objects of the same length as *Hits* object `U1.OV00`. For each hit in `U1.OV00`, we have 2 corresponding encodings, one in `U1.ovencA` and one in `U1.ovencB`, but only one of them encodes a hit between alignment ranges and exon ranges that are on the same strand. We use the `selectEncodingWithCompatibleStrand` function to merge them into a single *OverlapsEncodings* of the same length. For each hit in `U1.OV00`, this selects the encoding corresponding to alignment ranges and exon ranges with compatible strand:

```
> U1.grl_strand <- unlist(runValue(strand(U1.grl)), use.names=FALSE)
> U1.ovenc <- selectEncodingWithCompatibleStrand(U1.ovencA, U1.ovencB,
+                                                U1.grl_strand, exbytx_strand,
+                                                hits=U1.OV00)
> U1.ovenc

OverlapEncodings object of length 563552 with 0 metadata columns:
             Loffset   Roffset encoding flippedQuery
           <integer> <integer> <factor>    <logical>
       [1]         0         3     1:i:         TRUE
       [2]         4         0     1:k:        FALSE
       [3]         4         0     1:k:         TRUE
       [4]         4         0     1:k:         TRUE
       [5]         4         0     1:k:         TRUE
       ...       ...       ...      ...          ...
  [563548]        22         0     1:i:         TRUE
  [563549]        23         0     1:i:         TRUE
  [563550]        24         0     1:i:         TRUE
  [563551]        24         0     1:i:         TRUE
  [563552]        23         0     1:i:         TRUE
```

As a convenience, the 2 above calls to `encodeOverlaps` + merging step can be replaced by a single call to `encodeOverlaps` on `U1.grl` (or `U1.grlf`) with `flip.query.if.wrong.strand=TRUE`:

```
> U1.ovenc_again <- encodeOverlaps(U1.grl, exbytx, hits=U1.OV00, flip.query.if.wrong.strand=TRUE)
> stopifnot(identical(U1.ovenc_again, U1.ovenc))
```

Unique encodings in `U1.ovenc`:

```
> U1.unique_encodings <- levels(U1.ovenc)
> length(U1.unique_encodings)

[1] 120

> head(U1.unique_encodings)

[1] "1:c:" "1:e:" "1:f:" "1:h:" "1:i:" "1:j:"

> U1.ovenc_table <- table(encoding(U1.ovenc))
> tail(sort(U1.ovenc_table))

    1:f:   1:k:c:     1:k:     1:c: 2:jm:af:     1:i:
    1555     1889     8800     9523    72929   455176
```

Encodings are sort of cryptic but utilities are provided to extract specific meaning from them. Use of these utilities is covered later in this document.

4.2 Paired-end encodings

Let's encode the overlaps in `U3.OV00`:

```
> U3.grl <- grglist(U3.GALP)
> U3.ovenc <- encodeOverlaps(U3.grl, exbytx, hits=U3.OV00, flip.query.if.wrong.strand=TRUE)
> U3.ovenc

OverlapEncodings object of length 113827 with 0 metadata columns:
             Loffset   Roffset   encoding flippedQuery
           <integer> <integer>   <factor>    <logical>
       [1]         4         0 1--1:i--k:         TRUE
       [2]         4         0 1--1:i--i:         TRUE
       [3]         4         0 1--1:i--k:        FALSE
       [4]         4         0 1--1:i--k:        FALSE
       [5]         4         0 1--1:a--c:         TRUE
       ...       ...       ...        ...          ...
  [113823]        22         0 1--1:i--i:         TRUE
  [113824]        23         0 1--1:i--i:         TRUE
  [113825]        24         0 1--1:i--i:         TRUE
  [113826]        24         0 1--1:i--i:         TRUE
  [113827]        23         0 1--1:i--i:         TRUE
```

Unique encodings in `U3.ovenc`:

```
> U3.unique_encodings <- levels(U3.ovenc)
> length(U3.unique_encodings)

[1] 123

> head(U3.unique_encodings)

[1] "1--1:a--c:" "1--1:a--i:" "1--1:a--j:" "1--1:a--k:" "1--1:b--i:" "1--1:b--k:"

> U3.ovenc_table <- table(encoding(U3.ovenc))
> tail(sort(U3.ovenc_table))
```

```
1--1:i--m:       1--1:i--k:       1--1:c--i: 1--2:i--jm:a--af: 2--1:jm--m:af--i:
       852              1485             1714              2480              2700
1--1:i--i:
    100084
```

5 Detect "splice compatible" overlaps

We are interested in a particular type of overlap where the read overlaps the transcript in a "splice compatible" way, that is, in a way that is compatible with the splicing of the transcript. The `isCompatibleWithSplicing` function can be used on an *OverlapEncodings* object to detect this type of overlap. Note that `isCompatibleWithSplicing` can also be used on a character vector or factor.

5.1 Detect "splice compatible" single-end overlaps

5.1.1 "Splice compatible" single-end encodings

`U1.ovenc` contains 7 unique encodings compatible with the splicing of the transcript:

```
> sort(U1.ovenc_table[isCompatibleWithSplicing(U1.unique_encodings)])

     2:jm:ag:      2:gm:af: 3:jmm:agm:aaf:          1:j:          1:f:      2:jm:af:
           32            79            488          1538          1555         72929
        1:i:
      455176
```

Encodings `"1:i:"` (455176 occurences in `U1.ovenc`), `"2:jm:af:"` (72929 occurences in `U1.ovenc`), and `"3:jmm:agm:aaf:"` (488 occurences in `U1.ovenc`), correspond to the following overlaps:

- `"1:i:"`

```
- read (no skipped region):          oooooooo
- transcript:              ...   >>>>>>>>>>>>>   ...
```

- `"2:jm:af:"`

```
- read (1 skipped region):           ooooo---ooo
- transcript:              ...   >>>>>>>>>   >>>>>>>>>   ...
```

- `"3:jmm:agm:aaf:"`

```
- read (2 skipped regions):             oo---ooooo---o
- transcript:              ...   >>>>>>>>   >>>>>   >>>>>>>   ...
```

For clarity, only the exons involved in the overlap are represented. The transcript can of course have more upstream and downstream exons, which is denoted by the ... on the left side (5' end) and right side (3' end) of each drawing. Note that the exons represented in the 2nd and 3rd drawings are consecutive and adjacent in the processed transcript.

Encodings `"1:f:"` and `"1:j:"` are variations of the situation described by encoding `"1:i:"`. For `"1:f:"`, the first aligned base of the read (or "flipped" read) is aligned with the first base of the exon. For `"1:j:"`, the last aligned base of the read (or "flipped" read) is aligned with the last base of the exon:

- `"1:f:"`

```
      - read (no skipped region):       oooooooo
      - transcript:              ...   >>>>>>>>>>>>>   ...
```

- `"1:j:"`

```
      - read (no skipped region):             oooooooo
      - transcript:              ...   >>>>>>>>>>>>>   ...
```

```
> U1.OV00_is_comp <- isCompatibleWithSplicing(U1.ovenc)
> table(U1.OV00_is_comp)  # 531797 "splice compatible" overlaps

U1.OV00_is_comp
 FALSE   TRUE
 31755 531797
```

Finally, let's extract the "splice compatible" overlaps from `U1.OV00`:

```
> U1.compOV00 <- U1.OV00[U1.OV00_is_comp]
```

Note that high-level convenience wrapper `findCompatibleOverlaps` can be used for computing the "splice compatible" overlaps directly between a *GAlignments* object (containing reads) and a *GRangesList* object (containing transcripts):

```
> U1.compOV00_again <- findCompatibleOverlaps(U1.GAL, exbytx)
> stopifnot(identical(U1.compOV00_again, U1.compOV00))
```

5.1.2 Tabulate the "splice compatible" single-end overlaps

Number of "splice compatible" transcripts for each alignment in `U1.GAL`:

```
> U1.GAL_ncomptx <- countQueryHits(U1.compOV00)
> mcols(U1.GAL)$ncomptx <- U1.GAL_ncomptx
> head(U1.GAL)

GAlignments object with 6 alignments and 2 metadata columns:
                    seqnames strand       cigar    qwidth     start       end     width     njunc |
                       <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer> |
  SRR031729.3941844     chr4      -         75M        75       892       966        75         0 |
  SRR031728.3674563     chr4      -         75M        75       919       993        75         0 |
  SRR031729.8532600     chr4      +         75M        75       924       998        75         0 |
  SRR031729.2779333     chr4      +         75M        75       936      1010        75         0 |
  SRR031728.2826481     chr4      +         75M        75       949      1023        75         0 |
  SRR031728.2919098     chr4      -         75M        75       967      1041        75         0 |
                         ntx   ncomptx
                    <integer> <integer>
  SRR031729.3941844         0         0
  SRR031728.3674563         0         0
  SRR031729.8532600         0         0
  SRR031729.2779333         0         0
  SRR031728.2826481         0         0
  SRR031728.2919098         0         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U1.GAL_ncomptx)
```

```
U1.GAL_ncomptx
    0     1     2     3     4     5     6     7     8     9    10    11    12
51101  9848 33697 72987  5034 14021  7516   581  1789  2015   530  4389   847
```

```
> mean(U1.GAL_ncomptx >= 1)
```

```
[1] 0.7499401
```

75% of the alignments in `U1.GAL` are "splice compatible" with at least 1 transcript in `exbytx`.

Note that high-level convenience wrapper `countCompatibleOverlaps` can be used directly on `U1.GAL` and `exbytx` for computing `U1.GAL_ncomptx`:

```
> U1.GAL_ncomptx_again <- countCompatibleOverlaps(U1.GAL, exbytx)
> stopifnot(identical(U1.GAL_ncomptx_again, U1.GAL_ncomptx))
```

Number of "splice compatible" transcripts for each read:

```
> U1.compOV10 <- remapHits(U1.compOV00, Lnodes.remapping=U1.GAL_qnames)
> U1.uqnames_ncomptx <- countQueryHits(U1.compOV10)
> names(U1.uqnames_ncomptx) <- U1.uqnames
> table(U1.uqnames_ncomptx)
```

```
U1.uqnames_ncomptx
    0     1     2     3     4     5     6     7     8     9    10    11    12
42886  9711 26075 72989  5413 14044  8584   581  2706  2015   530  4389   847
```

```
> mean(U1.uqnames_ncomptx >= 1)
```

```
[1] 0.7751953
```

77.5% of the reads are "splice compatible" with at least 1 transcript in `exbytx`.

Number of "splice compatible" reads for each transcript:

```
> U1.exbytx_ncompOV10 <- countSubjectHits(U1.compOV10)
> names(U1.exbytx_ncompOV10) <- names(exbytx)
> mean(U1.exbytx_ncompOV10 >= 50)
```

```
[1] 0.008706681
```

Only 0.87% of the transcripts in `exbytx` are "splice compatible" with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_ncompOV10, decreasing=TRUE), n=10)
```

```
FBtr0308296 FBtr0089175 FBtr0089176 FBtr0089243 FBtr0289951 FBtr0112904 FBtr0089186 FBtr0089187
      40309       40158       33490       11365       11332       11284       10018        9627
FBtr0333672 FBtr0089172
       9568        6599
```

Note that this "top 10" is slightly different from the "top 10" we obtained earlier when we counted **all** the overlaps.

5.2 Detect "splice compatible" paired-end overlaps

5.2.1 "Splice compatible" paired-end encodings

WARNING: For paired-end encodings, `isCompatibleWithSplicing` considers that the encoding is "splice compatible" if its 2 halves are "splice compatible". This can produce false positives if for example the right end of the alignment is located upstream of the left end in transcript space. The paired-end read could not come from this transcript. To eliminate these false positives, one would need to look at the position of the left and right ends in transcript space. This can be done with `extractQueryStartInTranscript`.

`U3.ovenc` contains 13 unique paired-end encodings compatible with the splicing of the transcript:

```
> sort(U3.ovenc_table[isCompatibleWithSplicing(U3.unique_encodings)])

         1--2:f--jm:a--af:                 1--1:f--j:          2--1:jm--m:af--j:
                         3                         12                         21
         2--1:jm--m:af--f:            1--1:j--m:a--i:        2--2:jm--jm:af--af:
                        24                         51                         64
2--2:jm--mm:af--jm:aa--af:            1--1:i--m:a--i:                1--1:i--j:
                       153                        287                        403
                1--1:f--i:          1--2:i--jm:a--af:          2--1:jm--m:af--i:
                       617                       2480                       2700
                1--1:i--i:
                    100084
```

Paired-end encodings `"1--1:i-` (100084 occurences in `U3.ovenc`), `"2--1:jm--m:a` (2700 occurences in `U3.ovenc`), `"1--2:i--jm:a` (2480 occurences in `U3.ovenc`), `"1--1:i--m:` (287 occurences in `U3.ovenc`), and `"2--2:jm--mm:af--jm:` (153 occurences in `U3.ovenc`), correspond to the following paired-end overlaps:

- `"1--1:i-`

  ```
  - paired-end read (no skipped region on the first end, no skipped region
    on the last end):          oooo   oooo
  - transcript:       ...   >>>>>>>>>>>>>>>>   ...
  ```

- `"2--1:jm--m:a`

  ```
  - paired-end read (1 skipped region on the first end, no skipped region
    on the last end):            ooo---o    oooo
  - transcript:       ...   >>>>>>>>   >>>>>>>>>>>   ...
  ```

- `"1--2:i--jm:a`

  ```
  - paired-end read (no skipped region on the first end, 1 skipped region
    on the last end):         oooo      oo---oo
  - transcript:       ...   >>>>>>>>>>>>>>   >>>>>>>>>  ...
  ```

- `"1--1:i--m:`

  ```
  - paired-end read (no skipped region on the first end, no skipped region
    on the last end):          oooo      oooo
  - transcript:       ...   >>>>>>>>>   >>>>>>>   ...
  ```

- `"2--2:jm--mm:af--jm:`

```
      - paired-end read (1 skipped region on the first end, 1 skipped region
        on the last end):          ooo---o    oo---oo
      - transcript:       ...   >>>>>>   >>>>>>>   >>>>>   ...
```

Note: switch use of "first" and "last" above if the read was "flipped".

```
> U3.OV00_is_comp <- isCompatibleWithSplicing(U3.ovenc)
> table(U3.OV00_is_comp)  # 106835 "splice compatible" paired-end overlaps

U3.OV00_is_comp
 FALSE   TRUE
  6928 106899
```

Finally, let's extract the "splice compatible" paired-end overlaps from `U3.OV00`:

```
> U3.compOV00 <- U3.OV00[U3.OV00_is_comp]
```

Note that, like with our single-end reads, high-level convenience wrapper `findCompatibleOverlaps` can be used for computing the "splice compatible" paired-end overlaps directly between a *GAlignmentPairs* object (containing paired-end reads) and a *GRangesList* object (containing transcripts):

```
> U3.compOV00_again <- findCompatibleOverlaps(U3.GALP, exbytx)
> stopifnot(identical(U3.compOV00_again, U3.compOV00))
```

5.2.2 Tabulate the "splice compatible" paired-end overlaps

Number of "splice compatible" transcripts for each alignment pair in `U3.GALP`:

```
> U3.GALP_ncomptx <- countQueryHits(U3.compOV00)
> mcols(U3.GALP)$ncomptx <- U3.GALP_ncomptx
> head(U3.GALP)

GAlignmentPairs object with 6 pairs, strandMode=1, and 2 metadata columns:
                    seqnames strand :    ranges --    ranges |       ntx   ncomptx
                       <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer> <integer>
  SRR031715.1138209     chr4      + :   169-205 --   326-362 |         0         0
   SRR031714.756385     chr4      + :   943-979 -- 1086-1122 |         0         0
  SRR031714.5054563     chr4      + :   946-982 --  986-1022 |         0         0
  SRR031715.1722593     chr4      + :  966-1002 -- 1108-1144 |         0         0
  SRR031715.2202469     chr4      + :  966-1002 -- 1114-1150 |         0         0
  SRR031714.3544437     chr4      - : 1087-1123 --   963-999 |         0         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_ncomptx)

U3.GALP_ncomptx
    0     1     2     3     4     5     6     7     8     9    10    11    12
13884  2029  8094 14337  1099  2954  1865    84   296   332    89   699    66

> mean(U3.GALP_ncomptx >= 1)

[1] 0.6970411
```

69.7% of the alignment pairs in `U3.GALP` are "splice compatible" with at least 1 transcript in `exbytx`.

Note that high-level convenience wrapper `countCompatibleOverlaps` can be used directly on `U3.GALP` and `exbytx` for computing `U3.GALP_ncomptx`:

```
> U3.GALP_ncomptx_again <- countCompatibleOverlaps(U3.GALP, exbytx)
> stopifnot(identical(U3.GALP_ncomptx_again, U3.GALP_ncomptx))
```

Number of "splice compatible" transcripts for each template:

```
> U3.compOV10 <- remapHits(U3.compOV00, Lnodes.remapping=U3.GALP_qnames)
> U3.uqnames_ncomptx <- countQueryHits(U3.compOV10)
> names(U3.uqnames_ncomptx) <- U3.uqnames
> table(U3.uqnames_ncomptx)

U3.uqnames_ncomptx
    0     1     2     3     4     5     6     7     8     9    10    11    12
12769  2027  6534 14337  1210  2954  2114    84   444   332    89   699    66

> mean(U3.uqnames_ncomptx >= 1)

[1] 0.7075288
```

70.7% of the templates are "splice compatible" with at least 1 transcript in `exbytx`.

Number of "splice compatible" templates for each transcript:

```
> U3.exbytx_ncompOV10 <- countSubjectHits(U3.compOV10)
> names(U3.exbytx_ncompOV10) <- names(exbytx)
> mean(U3.exbytx_ncompOV10 >= 50)

[1] 0.007061324
```

Only 0.7% of the transcripts in `exbytx` are "splice compatible" with at least 50 templates.

Top 10 transcripts:

```
> head(sort(U3.exbytx_ncompOV10, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0289951 FBtr0089243 FBtr0112904 FBtr0089187 FBtr0089186
       7425        7419        5227        2686        2684        2640        2257        2250
FBtr0333672 FBtr0310542
       2206        1650
```

Note that this "top 10" is slightly different from the "top 10" we obtained earlier when we counted **all** the paired-end overlaps.

6 Compute the *reference query sequences* and project them on the transcriptome

6.1 Compute the *reference query sequences*

The *reference query sequences* are the query sequences **after** alignment, by opposition to the *original query sequences* (aka "true" or "real" query sequences) which are the query sequences **before** alignment.

The *reference query sequences* can easily be computed by extracting the nucleotides mapped to each read from the reference genome. This of course requires that we have access to the reference genome used by the aligner. In Bioconductor, the full genome sequence for the dm3 assembly is stored in the *BSgenome.Dmelanogaster.UCSC.dm3* data package [5]:

```
> library(BSgenome.Dmelanogaster.UCSC.dm3)
> Dmelanogaster

| BSgenome object for Fly
| - organism: Drosophila melanogaster
| - provider: UCSC
| - genome: dm3
| - release date: Apr. 2006
| - 15 sequence(s):
|     chr2L     chr2R     chr3L     chr3R     chr4      chrX      chrU      chrM      chr2LHet
|     chr2RHet  chr3LHet  chr3RHet  chrXHet   chrYHet   chrUextra
|
| Tips: call 'seqnames()' on the object to get all the sequence names, call 'seqinfo()' to get the
| full sequence info, use the '$' or '[[' operator to access a given sequence, see '?BSgenome' for
| more information.
```

To extract the portions of the reference genome corresponding to the ranges in `U1.grl`, we can use the `extractTranscriptSeqs` function defined in the *GenomicFeatures* package:

```
> library(GenomicFeatures)
> U1.GAL_rqseq <- extractTranscriptSeqs(Dmelanogaster, U1.grl)
> head(U1.GAL_rqseq)

DNAStringSet object of length 6:
    width seq                                                                 names
[1]    75 GGACAACCTAGCCAGGAAAGGGGCAGAGAACCC...GCCCGAACCATTCTGTGGTGTTGGTCACCACAG SRR031729.3941844
[2]    75 CAACAACATCCCGGGAAATGAGCTAGCGGACAA...GAAAGGGGCAGAGAACCCTCTAATTGGGCCCGA SRR031728.3674563
[3]    75 CCCAATTAGAGGGTTCTCTGCCCCTTTCCTGGC...CGCTAGCTCATTTCCCGGGATGTTGTTGTGTCC SRR031729.8532600
[4]    75 GTTCTCTGCCCCTTTCCTGGCTAGGTTGTCCGC...TCCCGGGATGTTGTTGTGTCCCGGGACCCACCT SRR031729.2779333
[5]    75 TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCC...TTGTGTCCCGGGACCCACCTTATTGTGAGTTTG SRR031728.2826481
[6]    75 CAAACTTGGAGCTGTCAACAAACTCACAATAAG...GGGACACAACAACATCCCGGGAAATGAGCTAGC SRR031728.2919098
```

When reads are paired-end, we need to extract separately the ranges corresponding to their *first* ends (aka *first* segments in BAM jargon) and those corresponding to their *last* ends (aka *last* segments in BAM jargon):

[5] See http://bioconductor.org/packages/release/data/annotation/ for the full list of annotation packages available in the current release of Bioconductor.

```
> U3.grl_first <- grglist(first(U3.GALP, real.strand=TRUE), order.as.in.query=TRUE)
> U3.grl_last <- grglist(last(U3.GALP, real.strand=TRUE), order.as.in.query=TRUE)
```

Then we extract the portions of the reference genome corresponding to the ranges in *GRangesList* objects `U3.grl_first` and `U3.grl_last`:

```
> U3.GALP_rqseq1 <- extractTranscriptSeqs(Dmelanogaster, U3.grl_first)
> U3.GALP_rqseq2 <- extractTranscriptSeqs(Dmelanogaster, U3.grl_last)
```

6.2 Project the single-end alignments on the transcriptome

The `extractQueryStartInTranscript` function computes for each overlap the position of the *query start* in the transcript:

```
> U1.OV00_qstart <- extractQueryStartInTranscript(U1.grl, exbytx,
+                                                 hits=U1.OV00, ovenc=U1.ovenc)
> head(subset(U1.OV00_qstart, U1.OV00_is_comp))
```

```
   startInTranscript firstSpannedExonRank startInFirstSpannedExon
1                100                    1                     100
8               4229                    5                     137
9               4229                    5                     137
10              4207                    5                     115
11              4207                    5                     115
12              4187                    5                      95
```

`U1.OV00_qstart` is a data frame with 1 row per overlap and 3 columns:

1. `startInTranscript`: the 1-based start position of the read with respect to the transcript. Position 1 always corresponds to the first base on the 5' end of the transcript sequence.
2. `firstSpannedExonRank`: the rank of the first exon spanned by the read, that is, the rank of the exon found at position `startInTranscript` in the transcript.
3. `startInFirstSpannedExon`: the 1-based start position of the read with respect to the first exon spanned by the read.

Having this information allows us for example to compare the read and transcript nucleotide sequences for each "splice compatible" overlap. If we use the *reference query sequence* instead of the *original query sequence* for this comparison, then it should match **exactly** the sequence found at the *query start* in the transcript.

Let's start by using `extractTranscriptSeqs` again to extract the transcript sequences (aka transcriptome) from the dm3 reference genome:

```
> txseq <- extractTranscriptSeqs(Dmelanogaster, exbytx)
```

For each "splice compatible" overlap, the read sequence in `U1.GAL_rqseq` must be an *exact* substring of the transcript sequence in `exbytx_seq`:

```
> U1.OV00_rqseq <- U1.GAL_rqseq[queryHits(U1.OV00)]
> U1.OV00_rqseq[flippedQuery(U1.ovenc)] <- reverseComplement(U1.OV00_rqseq[flippedQuery(U1.ovenc)])
> U1.OV00_txseq <- txseq[subjectHits(U1.OV00)]
```

```
> stopifnot(all(
+     U1.OV00_rqseq[U1.OV00_is_comp] ==
+         narrow(U1.OV00_txseq[U1.OV00_is_comp],
+                start=U1.OV00_qstart$startInTranscript[U1.OV00_is_comp],
+                width=width(U1.OV00_rqseq)[U1.OV00_is_comp])
+ ))
```

Because of this relationship between the *reference query sequence* and the transcript sequence of a "splice compatible" overlap, and because of the relationship between the *original query sequences* and the *reference query sequences*, then the edit distance reported in the NM tag is actually the edit distance between the *original query* and the transcript of a "splice compatible" overlap.

6.3 Project the paired-end alignments on the transcriptome

For a paired-end read, the *query start* is the start of its "left end".

```
> U3.OV00_Lqstart <- extractQueryStartInTranscript(U3.grl, exbytx,
+                                                  hits=U3.OV00, ovenc=U3.ovenc)
> head(subset(U3.OV00_Lqstart, U3.OV00_is_comp))
```

```
   startInTranscript firstSpannedExonRank startInFirstSpannedExon
2               4118                    5                      26
7               3940                    4                      31
8               3940                    4                      31
9               3692                    3                     320
10              3692                    3                     320
11              3690                    3                     318
```

Note that `extractQueryStartInTranscript` can be called with `for.query.right.end=TRUE` if we want this information for the "right ends" of the reads:

```
> U3.OV00_Rqstart <- extractQueryStartInTranscript(U3.grl, exbytx,
+                                                  hits=U3.OV00, ovenc=U3.ovenc,
+                                                  for.query.right.end=TRUE)
> head(subset(U3.OV00_Rqstart, U3.OV00_is_comp))
```

```
   startInTranscript firstSpannedExonRank startInFirstSpannedExon
2               4267                    5                     175
7               3948                    4                      39
8               3948                    4                      39
9               3849                    3                     477
10              3849                    3                     477
11              3831                    3                     459
```

Like with single-end reads, having this information allows us for example to compare the read and transcript nucleotide sequences for each "splice compatible" overlap. If we use the *reference query sequence* instead of the *original query sequence* for this comparison, then it should match **exactly** the sequences of the "left" and "right" ends of the read in the transcript.

Let's assign the "left and right reference query sequences" to each overlap:

```
> U3.OV00_Lrqseq <- U3.GALP_rqseq1[queryHits(U3.OV00)]
> U3.OV00_Rrqseq <- U3.GALP_rqseq2[queryHits(U3.OV00)]
```

For the single-end reads, the sequence associated with a "flipped query" just needed to be "reverse complemented". For paired-end reads, we also need to swap the 2 sequences in the pair:

```
> flip_idx <- which(flippedQuery(U3.ovenc))
> tmp <- U3.OV00_Lrqseq[flip_idx]
> U3.OV00_Lrqseq[flip_idx] <- reverseComplement(U3.OV00_Rrqseq[flip_idx])
> U3.OV00_Rrqseq[flip_idx] <- reverseComplement(tmp)
```

Let's assign the transcript sequence to each overlap:

```
> U3.OV00_txseq <- txseq[subjectHits(U3.OV00)]
```

For each "splice compatible" overlap, we expect the "left and right reference query sequences" of the read to be *exact* substrings of the transcript sequence. Let's check the "left reference query sequences":

```
> stopifnot(all(
+     U3.OV00_Lrqseq[U3.OV00_is_comp] ==
+         narrow(U3.OV00_txseq[U3.OV00_is_comp],
+                start=U3.OV00_Lqstart$startInTranscript[U3.OV00_is_comp],
+                width=width(U3.OV00_Lrqseq)[U3.OV00_is_comp])
+ ))
```

and the "right reference query sequences":

```
> stopifnot(all(
+     U3.OV00_Rrqseq[U3.OV00_is_comp] ==
+         narrow(U3.OV00_txseq[U3.OV00_is_comp],
+                start=U3.OV00_Rqstart$startInTranscript[U3.OV00_is_comp],
+                width=width(U3.OV00_Rrqseq)[U3.OV00_is_comp])
+ ))
```

7 Align the reads to the transcriptome

Aligning the reads to the reference genome is not the most efficient nor accurate way to count the number of "splice compatible" overlaps per *original query*. Supporting junction reads (i.e. reads that align with at least 1 skipped region in their CIGAR) introduces a significant computational cost during the alignment process. Then, as we've seen in the previous sections, each alignment produced by the aligner needs to be broken into a set of ranges (based on its CIGAR) and those ranges compared to the ranges of the exons grouped by transcript.

A more straightforward and accurate approach is to align the reads directly to the transcriptome, and without allowing the typical skipped region that the aligner needs to introduce when aligning a junction read to the reference genome. With this approach, a "hit" between a read and a transcript is necessarily compatible with the splicing of the transcript.

In case of a "hit", we'll say that the read and the transcript are "string-based compatible" (to differentiate from our previous notion of "splice compatible" overlaps that we will call "encoding-based compatible" in this section).

7.1 Align the single-end reads to the transcriptome

7.1.1 Find the "hits"

The single-end reads are in `U1.oqseq`, the transcriptome is in `exbytx_seq`.

Since indels were not allowed/supported during the alignment of the reads to the reference genome, we don't need to allow/support them either for aligning the reads to the transcriptome. Also since our goal is to find (and count) "splice compatible" overlaps between reads and transcripts, we don't need to keep track of the details of the alignments between the reads and the transcripts. Finally, since BAM file `untreated1_chr4.bam` is not the full output of the aligner but the subset obtained by keeping only the alignments located on chr4, we don't need to align `U1.oqseq` to the full transcriptome, but only to the subset of `exbytx_seq` made of the transcripts located on chr4.

With those simplifications in mind, we write the following function that we will use to find the "hits" between the reads and the transcriptome:

```
> ### A wrapper to vwhichPDict() that supports IUPAC ambiguity codes in 'qseq'
> ### and 'txseq', and treats them as such.
> findSequenceHits <- function(qseq, txseq, which.txseq=NULL, max.mismatch=0)
+ {
+     .asHits <- function(x, pattern_length)
+     {
+         query_hits <- unlist(x)
+         if (is.null(query_hits))
+             query_hits <- integer(0)
+         subject_hits <- rep.int(seq_len(length(x)), elementNROWS(x))
+         Hits(query_hits, subject_hits, pattern_length, length(x),
+              sort.by.query=TRUE)
+     }
+
+     .isHitInTranscriptBounds <- function(hits, qseq, txseq)
+     {
+         sapply(seq_len(length(hits)),
+             function(i) {
+                 pattern <- qseq[[queryHits(hits)[i]]]
+                 subject <- txseq[[subjectHits(hits)[i]]]
+                 v <- matchPattern(pattern, subject,
+                                   max.mismatch=max.mismatch, fixed=FALSE)
+                 any(1L <= start(v) & end(v) <= length(subject))
+             })
+     }
+
+     if (!is.null(which.txseq)) {
+         txseq0 <- txseq
+         txseq <- txseq[which.txseq]
+     }
+
```

```
+     names(qseq) <- NULL
+     other <- alphabetFrequency(qseq, baseOnly=TRUE)[ , "other"]
+     is_clean <- other == 0L  # "clean" means "no IUPAC ambiguity code"
+
+     ## Find hits for "clean" original queries.
+     qseq0 <- qseq[is_clean]
+     pdict0 <- PDict(qseq0, max.mismatch=max.mismatch)
+     m0 <- vwhichPDict(pdict0, txseq,
+                       max.mismatch=max.mismatch, fixed="pattern")
+     hits0 <- .asHits(m0, length(qseq0))
+     hits0@nLnode <- length(qseq)
+     hits0@from <- which(is_clean)[hits0@from]
+
+     ## Find hits for non "clean" original queries.
+     qseq1 <- qseq[!is_clean]
+     m1 <- vwhichPDict(qseq1, txseq,
+                       max.mismatch=max.mismatch, fixed=FALSE)
+     hits1 <- .asHits(m1, length(qseq1))
+     hits1@nLnode <- length(qseq)
+     hits1@from <- which(!is_clean)[hits1@from]
+
+     ## Combine the hits.
+     query_hits <- c(queryHits(hits0), queryHits(hits1))
+     subject_hits <- c(subjectHits(hits0), subjectHits(hits1))
+
+     if (!is.null(which.txseq)) {
+         ## Remap the hits.
+         txseq <- txseq0
+         subject_hits <- which.txseq[subject_hits]
+         hits0@nRnode <- length(txseq)
+     }
+
+     ## Order the hits.
+     oo <- orderIntegerPairs(query_hits, subject_hits)
+     hits0@from <- query_hits[oo]
+     hits0@to <- subject_hits[oo]
+
+     if (max.mismatch != 0L) {
+         ## Keep only "in bounds" hits.
+         is_in_bounds <- .isHitInTranscriptBounds(hits0, qseq, txseq)
+         hits0 <- hits0[is_in_bounds]
+     }
+     hits0
+ }
```

Let's compute the index of the transcripts in `exbytx_seq` located on chr4 (`findSequenceHits` will restrict the search to those transcripts):

```
> chr4tx <- transcripts(txdb, vals=list(tx_chrom="chr4"))
> chr4txnames <- mcols(chr4tx)$tx_name
> which.txseq <- match(chr4txnames, names(txseq))
```

We know that the aligner tolerated up to 6 mismatches per read. The 3 following commands find the "hits" for each *original query*, then find the "hits" for each "flipped *original query*", and finally merge all the "hits" (note that the 3 commands take about 1 hour to complete on a modern laptop):

```
> U1.sbcompHITSa <- findSequenceHits(U1.oqseq, txseq,
+                                    which.txseq=which.txseq, max.mismatch=6)
> U1.sbcompHITSb <- findSequenceHits(reverseComplement(U1.oqseq), txseq,
+                                    which.txseq=which.txseq, max.mismatch=6)
> U1.sbcompHITS <- union(U1.sbcompHITSa, U1.sbcompHITSb)
```

7.1.2 Tabulate the "hits"

Number of "string-based compatible" transcripts for each read:

```
> U1.uqnames_nsbcomptx <- countQueryHits(U1.sbcompHITS)
> names(U1.uqnames_nsbcomptx) <- U1.uqnames
> table(U1.uqnames_nsbcomptx)

U1.uqnames_nsbcomptx
    0     1     2     3     4     5     6     7     8     9    10    11    12
40555 10080 25299 74609  5207 14265  8643   610  3410  2056   534  4588   914

> mean(U1.uqnames_nsbcomptx >= 1)

[1] 0.7874142
```

77.7% of the reads are "string-based compatible" with at least 1 transcript in `exbytx`.

Number of "string-based compatible" reads for each transcript:

```
> U1.exbytx_nsbcompHITS <- countSubjectHits(U1.sbcompHITS)
> names(U1.exbytx_nsbcompHITS) <- names(exbytx)
> mean(U1.exbytx_nsbcompHITS >= 50)

[1] 0.008809516
```

Only 0.865% of the transcripts in `exbytx` are "string-based compatible" with at least 50 reads.

Top 10 transcripts:

```
> head(sort(U1.exbytx_nsbcompHITS, decreasing=TRUE), n=10)

FBtr0308296 FBtr0089175 FBtr0089176 FBtr0089243 FBtr0289951 FBtr0112904 FBtr0089186 FBtr0333672
      40548       40389       34275       11605       11579       11548       10059        9742
FBtr0089187 FBtr0089172
       9666        6704
```

7.1.3 A closer look at the "hits"

[WORK IN PROGRESS, might be removed or replaced soon...]

Any "encoding-based compatible" overlap is of course "string-based compatible":

```
> stopifnot(length(setdiff(U1.compOV10, U1.sbcompHITS)) == 0)
```

but the reverse is not true:

```
> length(setdiff(U1.sbcompHITS, U1.compOV10))
[1] 13549
```

7.2 Align the paired-end reads to the transcriptome

[COMING SOON...]

8 Detect "almost splice compatible" overlaps

In many aspects, "splice compatible" overlaps can be seen as perfect. We are now insterested in a less perfect type of overlap where the read overlaps the transcript in a way that *would* be "splice compatible" if 1 or more exons were removed from the transcript. In that case we say that the overlap is "almost splice compatible" with the transcript. The `isCompatibleWithSkippedExons` function can be used on an *OverlapEncodings* object to detect this type of overlap. Note that `isCompatibleWithSkippedExons` can also be used on a character vector of factor.

8.1 Detect "almost splice compatible" single-end overlaps

8.1.1 "Almost splice compatible" single-end encodings

`U1.ovenc` contains 7 unique encodings "almost splice compatible" with the splicing of the transcript:

```
> sort(U1.ovenc_table[isCompatibleWithSkippedExons(U1.unique_encodings)])
   2:jm:am:am:am:af: 2:jm:am:am:am:am:af:             2:gm:am:af:       2:jm:am:am:am:af:
                   1                     1                       4                       7
 3:jmm:agm:aam:aam:aaf:      3:jmm:agm:aam:aaf:          2:jm:am:am:af:             2:jm:am:af:
                   9                    21                     144                    1015
```

Encodings `"2:jm:am:af:"` (1015 occurences in `U1.ovenc`), `"2:jm:am:am:af:"` (144 occurences in `U1.ovenc`), and `"3:jmm:agm:aam:aaf:"` (21 occurences in `U1.ovenc`), correspond to the following overlaps:

- `"2:jm:am:af:"`

```
- read (1 skipped region):        ooooo----------ooo
- transcript:             ...   >>>>>>>   >>>>   >>>>>>>>   ...
```

- `"2:jm:am:am:af:"`

```
- read (1 skipped region):        ooooo------------------ooo
- transcript:             ...   >>>>>>>   >>>>   >>>>>   >>>>>>>>   ...
```

- `"3:jmm:agm:aam:aaf:"`

```
- read (2 skipped regions):          oo---oooo-----------oo
- transcript:             ...   >>>>>>>   >>>>   >>>>>   >>>>>>>>   ...
```

```
> U1.OV00_is_acomp <- isCompatibleWithSkippedExons(U1.ovenc)
> table(U1.OV00_is_acomp)  # 1202 "almost splice compatible" overlaps
```

```
U1.OV00_is_acomp
 FALSE   TRUE
562350   1202
```

Finally, let's extract the "almost splice compatible" overlaps from `U1.OV00`:

```
> U1.acompOV00 <- U1.OV00[U1.OV00_is_acomp]
```

8.1.2 Tabulate the "almost splice compatible" single-end overlaps

Number of "almost splice compatible" transcripts for each alignment in `U1.GAL`:

```
> U1.GAL_nacomptx <- countQueryHits(U1.acompOV00)
> mcols(U1.GAL)$nacomptx <- U1.GAL_nacomptx
> head(U1.GAL)

GAlignments object with 6 alignments and 3 metadata columns:
                    seqnames strand       cigar    qwidth     start       end     width     njunc |
                       <Rle>  <Rle> <character> <integer> <integer> <integer> <integer> <integer> |
  SRR031729.3941844     chr4      -         75M        75       892       966        75         0 |
  SRR031728.3674563     chr4      -         75M        75       919       993        75         0 |
  SRR031729.8532600     chr4      +         75M        75       924       998        75         0 |
  SRR031729.2779333     chr4      +         75M        75       936      1010        75         0 |
  SRR031728.2826481     chr4      +         75M        75       949      1023        75         0 |
  SRR031728.2919098     chr4      -         75M        75       967      1041        75         0 |
                         ntx   ncomptx  nacomptx
                    <integer> <integer> <integer>
  SRR031729.3941844         0         0         0
  SRR031728.3674563         0         0         0
  SRR031729.8532600         0         0         0
  SRR031729.2779333         0         0         0
  SRR031728.2826481         0         0         0
  SRR031728.2919098         0         0         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U1.GAL_nacomptx)

U1.GAL_nacomptx
     0      1      2      3      4      5      6      7      8      9     10     11     12
203800    283    101    107     19     24      2      3      1      3      4      4      4

> mean(U1.GAL_nacomptx >= 1)

[1] 0.002715862
```

Only 0.27% of the alignments in `U1.GAL` are "almost splice compatible" with at least 1 transcript in `exbytx`.

Number of "almost splice compatible" alignments for each transcript:

```
> U1.exbytx_nacompOV00 <- countSubjectHits(U1.acompOV00)
> names(U1.exbytx_nacompOV00) <- names(exbytx)
> table(U1.exbytx_nacompOV00)
```

```
U1.exbytx_nacompOV00
    0     1     2     3     4     5     6     7     8     9    10    12    13    14    17    18
29039    50     8    15    12     2     3     7     5     7     3     2     1     1     1     2
   20    21    32    34    44    55    59    77   170
    1     3     2     1     3     2     1     1     1
```

```
> mean(U1.exbytx_nacompOV00 >= 50)
```

```
[1] 0.0001713914
```

Only 0.017% of the transcripts in `exbytx` are "almost splice compatible" with at least 50 alignments in `U1.GAL`.

Finally note that the "query start in transcript" values returned by `extractQueryStartInTranscript` are also defined for "almost splice compatible" overlaps:

```
> head(subset(U1.OV00_qstart, U1.OV00_is_acomp))
```

```
       startInTranscript firstSpannedExonRank startInFirstSpannedExon
144226               133                    1                     133
144227               133                    1                     133
144240               151                    1                     151
144241               151                    1                     151
146615               757                    7                      39
146616               689                    8                      39
```

8.2 Detect "almost splice compatible" paired-end overlaps

8.2.1 "Almost splice compatible" paired-end encodings

`U3.ovenc` contains 5 unique paired-end encodings "almost splice compatible" with the splicing of the transcript:

```
> sort(U3.ovenc_table[isCompatibleWithSkippedExons(U3.unique_encodings)])
```

```
    2--1:jm--m:am--m:am--m:af--i:     1--2:i--jm:a--am:a--am:a--af:
                               1                                 5
2--2:jm--mm:am--mm:af--jm:aa--af:           1--2:i--jm:a--am:a--af:
                               9                                53
          2--1:jm--m:am--m:af--i:
                              73
```

Paired-end encodings `"2--1:jm--m:am--m` (73 occurences in `U3.ovenc`), `"1--2:i--jm:a--am` (53 occurences in `U3.ovenc`), and `"2--2:jm--mm:am--mm:af--j` (9 occurences in `U3.ovenc`), correspond to the following paired-end overlaps:

- `"2--1:jm--m:am--m`

  - paired-end read (1 skipped region on the first end, no skipped region on the last end): ooo----------o oooo
  - transcript: ... >>>>> >>>> >>>>>>>>> ...

- `"1--2:i--jm:a--am`

  - paired-end read (no skipped region on the first end, 1 skipped region on the last end): oooo oo---------oo

```
    - transcript:       ...   >>>>>>>>>>>   >>>   >>>>>>   ...
```

- `"2--2:jm--mm:am--mm:af--j`

```
    - paired-end read (1 skipped region on the first end, 1 skipped region
      on the last end):           o----------ooo   oo---oo
    - transcript:       ...   >>>>>   >>>>   >>>>>>>>   >>>>>>   ...
```

Note: switch use of "first" and "last" above if the read was "flipped".

```
> U3.OV00_is_acomp <- isCompatibleWithSkippedExons(U3.ovenc)
> table(U3.OV00_is_acomp)  # 141 "almost splice compatible" paired-end overlaps

U3.OV00_is_acomp
 FALSE   TRUE
113686    141
```

Finally, let's extract the "almost splice compatible" paired-end overlaps from `U3.OV00`:

```
> U3.acompOV00 <- U3.OV00[U3.OV00_is_acomp]
```

8.2.2 Tabulate the "almost splice compatible" paired-end overlaps

Number of "almost splice compatible" transcripts for each alignment pair in `U3.GALP`:

```
> U3.GALP_nacomptx <- countQueryHits(U3.acompOV00)
> mcols(U3.GALP)$nacomptx <- U3.GALP_nacomptx
> head(U3.GALP)

GAlignmentPairs object with 6 pairs, strandMode=1, and 3 metadata columns:
                     seqnames strand :    ranges --    ranges |       ntx   ncomptx  nacomptx
                        <Rle>  <Rle> : <IRanges> -- <IRanges> | <integer> <integer> <integer>
  SRR031715.1138209     chr4      + :   169-205 --   326-362 |         0         0         0
   SRR031714.756385     chr4      + :   943-979 -- 1086-1122 |         0         0         0
  SRR031714.5054563     chr4      + :   946-982 --  986-1022 |         0         0         0
  SRR031715.1722593     chr4      + :  966-1002 -- 1108-1144 |         0         0         0
  SRR031715.2202469     chr4      + :  966-1002 -- 1114-1150 |         0         0         0
  SRR031714.3544437     chr4      - : 1087-1123 --   963-999 |         0         0         0
  -------
  seqinfo: 8 sequences from an unspecified genome

> table(U3.GALP_nacomptx)

U3.GALP_nacomptx
    0     1     2     3     4     5    11
45734    74     4    13     1     1     1

> mean(U3.GALP_nacomptx >= 1)

[1] 0.002051148
```

Only 0.2% of the alignment pairs in `U3.GALP` are "almost splice compatible" with at least 1 transcript in `exbytx`.

Number of "almost splice compatible" alignment pairs for each transcript:

```
> U3.exbytx_nacompOV00 <- countSubjectHits(U3.acompOV00)
> names(U3.exbytx_nacompOV00) <- names(exbytx)
> table(U3.exbytx_nacompOV00)

U3.exbytx_nacompOV00
    0     1     5     8    12    13    66
29143    22     4     1     1     1     1

> mean(U3.exbytx_nacompOV00 >= 50)

[1] 3.427827e-05
```

Only 0.0034% of the transcripts in `exbytx` are "almost splice compatible" with at least 50 alignment pairs in `U3.GALP`.

Finally note that the "query start in transcript" values returned by `extractQueryStartInTranscript` are also defined for "almost splice compatible" paired-end overlaps:

```
> head(subset(U3.OV00_Lqstart, U3.OV00_is_acomp))

      startInTranscript firstSpannedExonRank startInFirstSpannedExon
27617              1549                   12                      45
27629              1562                   12                      58
27641              1562                   12                      58
27690              1567                   12                      63
27812              1549                   12                      45
42870               659                    4                     101

> head(subset(U3.OV00_Rqstart, U3.OV00_is_acomp))

      startInTranscript firstSpannedExonRank startInFirstSpannedExon
27617              2135                   14                     115
27629              2135                   14                     115
27641              2141                   14                     121
27690              2048                   14                      28
27812              2136                   14                     116
42870               866                    6                      19
```

9 Detect novel splice junctions

9.1 By looking at single-end overlaps

An alignment in `U1.GAL` with "almost splice compatible" overlaps but no "splice compatible" overlaps suggests the presence of one or more transcripts that are not in our annotations.

First we extract the index of those alignments (*nsj* here stands for "**n**ovel **s**plice **j**unction"):

```
> U1.GAL_is_nsj <- U1.GAL_nacomptx != 0L & U1.GAL_ncomptx == 0L
> head(which(U1.GAL_is_nsj))

[1] 57972 57974 58321 67251 67266 67267
```

We make this an index into `U1.OV00`:

```
> U1.OV00_is_nsj <- queryHits(U1.OV00) %in% which(U1.GAL_is_nsj)
```

We intersect with `U1.OV00_is_acomp` and then subset `U1.OV00` to keep only the overlaps that suggest novel splicing:

```
> U1.OV00_is_nsj <- U1.OV00_is_nsj & U1.OV00_is_acomp
> U1.nsjOV00 <- U1.OV00[U1.OV00_is_nsj]
```

For each overlap in `U1.nsjOV00`, we extract the ranks of the skipped exons (we use a list for this as there might be more than 1 skipped exon per overlap):

```
> U1.nsjOV00_skippedex <- extractSkippedExonRanks(U1.ovenc)[U1.OV00_is_nsj]
> names(U1.nsjOV00_skippedex) <- queryHits(U1.nsjOV00)
> table(elementNROWS(U1.nsjOV00_skippedex))

  1   2   3   4   5
234 116   7   1   1
```

Finally, we split `U1.nsjOV00_skippedex` by transcript names:

```
> f <- factor(names(exbytx)[subjectHits(U1.nsjOV00)], levels=names(exbytx))
> U1.exbytx_skippedex <- split(U1.nsjOV00_skippedex, f)
```

`U1.exbytx_skippedex` is a named list of named lists of integer vectors. The first level of names (outer names) are transcript names and the second level of names (inner names) are alignment indices into `U1.GAL`:

```
> head(names(U1.exbytx_skippedex))  # transcript names

[1] "FBtr0300689" "FBtr0300690" "FBtr0330654" "FBtr0309810" "FBtr0306539" "FBtr0306536"
```

Transcript FBtr0089124 receives 7 hits. All of them skip exons 9 and 10:

```
> U1.exbytx_skippedex$FBtr0089124

$`104549`
[1]  9 10

$`104550`
[1]  9 10

$`104553`
[1]  9 10

$`104557`
[1]  9 10

$`104560`
[1]  9 10

$`104572`
[1]  9 10

$`104577`
```

```
[1]  9 10
```

Transcript FBtr0089147 receives 4 hits. Two of them skip exon 2, one of them skips exons 2 to 6, and one of them skips exon 10:

```
> U1.exbytx_skippedex$FBtr0089147

$`72828`
[1] 10

$`74018`
[1] 2 3 4 5 6

$`74664`
[1] 2

$`74670`
[1] 2
```

A few words about the interpretation of `U1.exbytx_skippedex`: Because of how we've conducted this analysis, the aligments reported in `U1.exbytx_skippedex` are guaranteed to not have any "splice compatible" overlaps with other known transcripts. All we can say, for example in the case of transcript FBtr0089124, is that the 7 reported hits that skip exons 9 and 10 show evidence of one or more unknown transcripts with a splice junction that corresponds to the gap between exons 8 and 11. But without further analysis, we can't make any assumption about the exons structure of those unknown transcripts. In particular, we cannot assume the existence of an unknown transcript made of the same exons as transcript FBtr0089124 minus exons 9 and 10!

9.2 By looking at paired-end overlaps

[COMING SOON...]

10 `sessionInfo()`

```
> sessionInfo()

R Under development (unstable) (2025-10-20 r88955)
Platform: x86_64-pc-linux-gnu
Running under: Ubuntu 24.04.3 LTS

Matrix products: default
BLAS:   /home/biocbuild/bbs-3.23-bioc/R/lib/libRblas.so
LAPACK: /usr/lib/x86_64-linux-gnu/lapack/liblapack.so.3.12.0  LAPACK version 3.12.0

locale:
 [1] LC_CTYPE=en_US.UTF-8       LC_NUMERIC=C               LC_TIME=en_GB
 [4] LC_COLLATE=C               LC_MONETARY=en_US.UTF-8    LC_MESSAGES=en_US.UTF-8
 [7] LC_PAPER=en_US.UTF-8       LC_NAME=C                  LC_ADDRESS=C
[10] LC_TELEPHONE=C             LC_MEASUREMENT=en_US.UTF-8 LC_IDENTIFICATION=C
```

Overlap encodings

```
time zone: America/New_York
tzcode source: system (glibc)

attached base packages:
[1] stats4    stats     graphics  grDevices utils     datasets  methods   base

other attached packages:
 [1] BSgenome.Dmelanogaster.UCSC.dm3_1.4.0     BSgenome_1.79.0
 [3] rtracklayer_1.71.0                        BiocIO_1.21.0
 [5] TxDb.Dmelanogaster.UCSC.dm3.ensGene_3.2.2 GenomicFeatures_1.63.1
 [7] AnnotationDbi_1.73.0                      pasillaBamSubset_0.49.0
 [9] GenomicAlignments_1.47.0                  SummarizedExperiment_1.41.0
[11] Biobase_2.71.0                            MatrixGenerics_1.23.0
[13] matrixStats_1.5.0                         Rsamtools_2.27.0
[15] Biostrings_2.79.1                         XVector_0.51.0
[17] GenomicRanges_1.63.0                      IRanges_2.45.0
[19] S4Vectors_0.49.0                          Seqinfo_1.1.0
[21] BiocGenerics_0.57.0                       generics_0.1.4
[23] RNAseqData.HNRNPC.bam.chr14_0.49.0        BiocStyle_2.39.0

loaded via a namespace (and not attached):
 [1] KEGGREST_1.51.0     rjson_0.2.23        xfun_0.54           bslib_0.9.0
 [5] lattice_0.22-7      vctrs_0.6.5         tools_4.6.0         bitops_1.0-9
 [9] curl_7.0.0          parallel_4.6.0      RSQLite_2.4.3       blob_1.2.4
[13] pkgconfig_2.0.3     Matrix_1.7-4        cigarillo_1.1.0     lifecycle_1.0.4
[17] compiler_4.6.0      codetools_0.2-20    htmltools_0.5.8.1   sass_0.4.10
[21] RCurl_1.98-1.17     yaml_2.3.10         crayon_1.5.3        jquerylib_0.1.4
[25] BiocParallel_1.45.0 DelayedArray_0.37.0 cachem_1.1.0        abind_1.4-8
[29] digest_0.6.37       restfulr_0.0.16     bookdown_0.45       fastmap_1.2.0
[33] grid_4.6.0          cli_3.6.5           SparseArray_1.11.1  S4Arrays_1.11.0
[37] XML_3.99-0.19       bit64_4.6.0-1       rmarkdown_2.30      httr_1.4.7
[41] bit_4.6.0           png_0.1-8           memoise_2.0.1       evaluate_1.0.5
[45] knitr_1.50          rlang_1.1.6         DBI_1.2.3           BiocManager_1.30.26
[49] jsonlite_2.0.0      R6_2.6.1
```