

Overlap encodings

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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 “compatible” with the splicing of the transcript.

Various tools are provided in the `IRanges` and `GenomicRanges` packages 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] "/loc/home/biocbuild/bbs-2.11-bioc/R/library/pasillaBamSubset/extdata/untreated1_chr4.bam"
```

We use the `readGappedAlignments` function defined in the `GenomicRanges` package to load the reads into a *GappedAlignments* 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), 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 `readGappedAlignments` (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 (`readGappedAlignments` will use them to set the names of the returned object):

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

¹<http://samtools.sourceforge.net/>

GappedAlignments with 6 alignments and 0 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap
	<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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

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 explicitly 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 occurrence in `U1.GAL_qnames`:

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

Our reads can have up to 2 gaps (a gap corresponds to an N operation in the CIGAR):

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

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

> table(ngap(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
15326625      0      0 21682582      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 `readGappedAlignmentPairs` function to load them into a *GappedAlignmentPairs* object:

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

GappedAlignmentPairs with 6 alignment pairs 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]

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

The `show` method for *GappedAlignmentPairs* 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))
```

GappedAlignments with 6 alignments and 0 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap
	<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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

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

GappedAlignments with 6 alignments and 0 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap
	<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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

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 *GappedAlignmentPairs* object:

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

```
FALSE TRUE
29518 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 occurrence in U3.GALP_qnames:

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

Our reads can have up to 1 gap per end:

```
> head(unique(cigar(first(U3.GALP))))

[1] "37M"          "6M58N31M"    "25M56N12M"   "19M62N18M"   "29M222N8M"   "9M222N28M"

> head(unique(cigar(last(U3.GALP))))

[1] "37M"          "12M58N25M"   "19M58N18M"   "27M2339N10M" "29M2339N8M"   "9M222N28M"

> table(ngap(first(U3.GALP)), ngap(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
1695636  0      0 673919  0      0      0

> colSums(cigarOpTable(cigar(last(U3.GALP))))

      M      I      D      N      S      H      P
1695636  0      0 630395  0      0      0
```

3 Validate the alignments produced by the aligner

In this section we show how to validate the alignments produced by the aligner by comparing the *original query sequences* (aka “true” or “real” query sequences, or query sequences **before** alignment) with the *reference query sequences* (i.e. the query sequences **after** alignment).

Note that even though this step is not strictly required for computing the *overlap encodings*, some of the concepts and string-based computations described in this section are slightly related to some ideas introduced later in this document.

3.1 Validate the single-end alignments

3.1.1 Load the *original query sequences*

To load the *original query sequences*, we reload the BAM file but now we explicitly request the SEQ field by using `what="seq"` in our call to `ScanBamParam`. To further validate the alignments produced by the aligner, we also need to load the NM tag which is a predefined tag described in the SAM Spec as the “Edit distance to the reference, including ambiguous bases but excluding clipping” (note that tags are optional fields i.e. not all BAM files have them):

```
> param1 <- ScanBamParam(flag=flag0, what="seq", tag="NM")
> U1.GAL <- readGappedAlignments(untreated1_chr4(), use.names=TRUE, param=param1)
> U1.GAL_qseq <- mcols(U1.GAL)$seq
> names(U1.GAL_qseq) <- names(U1.GAL)
> head(U1.GAL_qseq)
```

A DNASTringSet instance of length 6

	width	seq	names
[1]	75	CTGTGGTGACCAACACACAGAATGGTTCGGGC...GGGTTCCCTGCCCTTTCTGGCTAGGTTGTCC	SRR031729.3941844
[2]	75	TCGGGCCCAATTAGAGGGTTCCTGCCCTTTC...TTGTCCGCTAGCTCATTTCCTGGGCTGTTGTTG	SRR031728.3674563
[3]	75	CCCAATTAGAGGATTCTCTGCCCTTTCTCGGC...CGGTAGCTCATTTCCTGGGATGTTGTTGTGTCC	SRR031729.8532600
[4]	75	GTTCTCTGCCCTTTCTGGCTAGGTTGTCCGC...TCCCGAGATGTTGTTGTGTCCCGGACCCACCT	SRR031729.2779333
[5]	75	TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCT...TTGTGTCCCGGACACACCTTATTGTGAGTTTG	SRR031728.2826481
[6]	75	GCTAGCTCATTTCCTGGGAGGTTGTTGTGTCC...CTTATTGTGAGTTTGTGTGACAGCTCCAAGTTTG	SRR031728.2919098

Because the BAM format imposes that the read sequence is “reverse complemented” when the read is aligned to the minus strand, we “reverse complement” it again to get the *original query sequences*:

```
> U1.GAL_oqseq <- U1.GAL_qseq
> U1.GAL_is_on_minus <- as.logical(strand(U1.GAL) == "-")
> U1.GAL_oqseq[U1.GAL_is_on_minus] <- reverseComplement(U1.GAL_oqseq[U1.GAL_is_on_minus])
> head(U1.GAL_oqseq)
```

A DNASTringSet instance of length 6

	width	seq	names
[1]	75	GGACAACCTAGCCAGGAAAGGGGAGGGAACCC...GCCCGAACCATTTCTGTGGTGTTGGTCACACAG	SRR031729.3941844
[2]	75	CAACAACAGCCCAGGAAATGAGCTAGCGGACAA...GAAAGGGGACAGGAACCTCTAATTGGGCCCGA	SRR031728.3674563
[3]	75	CCCAATTAGAGGATTCTCTGCCCTTTCTCGGC...CGGTAGCTCATTTCCTGGGATGTTGTTGTGTCC	SRR031729.8532600
[4]	75	GTTCTCTGCCCTTTCTGGCTAGGTTGTCCGC...TCCCGAGATGTTGTTGTGTCCCGGACCCACCT	SRR031729.2779333
[5]	75	TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCT...TTGTGTCCCGGACACACCTTATTGTGAGTTTG	SRR031728.2826481
[6]	75	CAAACTTGAGCTGTCAACAACTCACATAAG...GGGACACAACAACCTCCCGGGAATGAGCTAGC	SRR031728.2919098

Note that sequences with the same *query name* correspond to the same *original query* and therefore must be the same. Let’s do a quick sanity check:

```
> stopifnot(all(U1.GAL_oqseq == U1.GAL_qseq[U1.GAL_dup2uniq]))
```

Finally, let’s reduce `U1.GAL_oqseq` to one *original query sequence* per unique *query name*:

```
> U1.oqseq <- U1.GAL_oqseq[!U1.GAL_names_is_dup]
```

If we had access to the FASTQ file used as input to the aligner, `U1.oqseq` would be the subset of this file made of the sequences with at least 1 alignment reported in BAM file `untreated1_chr4.bam`.

3.1.2 Compute the *reference query sequences*

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 ²:

²See <http://bioconductor.org/packages/release/data/annotation/> for the full list of annotation packages available in the current release of Bioconductor.

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

```
Fly genome
|
| organism: Drosophila melanogaster (Fly)
| provider: UCSC
| provider version: dm3
| release date: Apr. 2006
| release name: BDGP Release 5
|
| single sequences (see '?seqnames'):
| chr2L      chr2R      chr3L      chr3R      chr4      chrX      chrU      chrM
| chr2LHet   chr2RHet   chr3LHet   chr3RHet   chrXHet   chrYHet   chrUextra
|
| multiple sequences (see '?mseqnames'):
| upstream1000 upstream2000 upstream5000
|
| (use the '$' or '[' operator to access a given sequence)
```

Let's start by converting our *GappedAlignments* object `U1.GAL` into a *GRangesList* object:

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

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

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

```
A DNAStringSet instance of length 6
      width seq
[1] 75 GGACAACCTAGCCAGGAAAGGGGCAGAGAACCC...GCCCGAACCATTCTGTGGTGTGGTCACCACAG SRR031729.3941844
[2] 75 CAACAACATCCCGGAAATGAGCTAGCGGACAA...GAAAGGGGCAGAGAACCCTCTAATTGGGCCCGA SRR031728.3674563
[3] 75 CCCAATTAGAGGGTTCTCTGCCCTTTCCTGGC...CGCTAGCTCATTTCCCGGGATGTTGTTGTGTCC SRR031729.8532600
[4] 75 GTTCTCTGCCCCCTTTCCTGGCTAGGTTGTCCGC...TCCCGGGATGTTGTTGTGTCCCGGACCCACCT SRR031729.2779333
[5] 75 TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCC...TTGTGTCCCGGGACCCACCTTATTGTGAGTTTG SRR031728.2826481
[6] 75 CAAACTTGGAGCTGTCAACAACTCACATAAG...GGGACACAACAACATCCCGGAAATGAGCTAGC SRR031728.2919098
```

3.1.3 Compare the original query sequences with the reference query sequences

We can use the `neditAt` function defined in the `Biostings` package to compute the edit distance between 2 strings. Because the aligned reads have no indels, we should only see mismatches (typically a small number) during that comparison so we don't need to call `neditAt` with `with.indels=TRUE`. And because calling `neditAt` in a loop is inefficient, we only do this comparison for the first 500 sequences in `U1.GAL_oqseq`:

```
> U1.GAL_nedit500 <- sapply(1:500, function(i) neditAt(U1.GAL_oqseq[[i]], U1.GAL_rqseq[[i]]))
> table(U1.GAL_nedit500)
```

```
U1.GAL_nedit500
 0  1  2  3  4  5
273 101 97 22 6 1
```

Yes, the first 500 sequences in `U1.GAL_oqseq` are “close” to the first 500 sequences in `U1.GAL_rqseq`.

Now let's compare the edit distance reported by `neditAt` with the edit distance reported by the aligner (NM tag). Because the latter excludes the N CIGAR operations, it should actually be the same as the former. We confirm this for the 500 edit distances computed in `U1.GAL_nedit500`:

```
> U1.GAL_NM <- mcols(U1.GAL)$NM
> stopifnot(all(U1.GAL_NM[1:500] == U1.GAL_nedit500))
```

Note that the maximum observed number of mismatches tells us how many mismatches per read were tolerated by the aligner:

```
> table(U1.GAL_NM)
```

```
U1.GAL_NM
  0      1      2      3      4      5      6
135911 44033 15684  5172  2233   948   374
```

3.2 Validate the paired-end alignments

3.2.1 Load the original query sequences

To load the *original query sequences*, we reload the BAM file and request the SEQ field (and also the NM tag). Since we've removed the improper pairs from our current U3.GALP object, we need to do this again but now we do it at load time which is equivalent to doing it afterward (i.e. not only do we have the guarantee to end up with the same elements in U3.GALP, but also to have them in the same order):

```
> flag2 <- scanBamFlag(isDuplicate=FALSE,
+                      isNotPassingQualityControls=FALSE,
+                      isProperPair=TRUE)
> param2 <- ScanBamParam(flag=flag2, what="seq", tag="NM")
> U3.GALP <- readGappedAlignmentPairs(untreated3_chr4(), use.names=TRUE, param=param2)
```

Let's extract the *first* and *last* sequences from U3.GALP:

```
> U3.GALP_qseq1 <- mcols(first(U3.GALP))$seq
> U3.GALP_qseq2 <- mcols(last(U3.GALP))$seq
> names(U3.GALP_qseq1) <- names(U3.GALP_qseq2) <- names(U3.GALP)
> head(U3.GALP_qseq1)
```

```
A DNAStringSet instance of length 6
width seq
[1] 37 CCGTTTCTGAAGGAGATGGCTCATGGAGTACCTGCCT
[2] 37 GCCCCTTTCCTGGCTAGGTTGTCCGCTAGCTCATTTC
[3] 37 CCTTTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCTG
[4] 37 CGCTAGCTCATTTCCTGGGCTGTTGTTGTGTCCTGGG
[5] 37 CGCTAGCTCATTTCCTGGGATGTTGTTGTGTCCTGGG
[6] 37 GCTTTGCTGAGCGCCTTTATGGCTGCTTGACTATCAG
names
SRR031715.1138209
SRR031714.756385
SRR031714.5054563
SRR031715.1722593
SRR031715.2202469
SRR031714.3544437
```

```
> head(U3.GALP_qseq2)
```

```
A DNAStringSet instance of length 6
width seq
[1] 37 GTCTCCAGCAGAGCAGATGGAGCAACGGCCTATAGAG
[2] 37 AGCTTTGCTGAGCGCCTTTATGGCTGCTTGACTATCA
[3] 37 TGTTGTTGTGTCCCGGGACCCACCTTATTGTGAGTTT
[4] 37 GCTGCTTGACTATCAGACAGTATAGCAATGTCCTTGC
[5] 37 TGAATATCAGACAGTATAGCAATGTCCTTGCCATGAT
[6] 37 GTCCGCTAGCTCATTTCCTGGGATGTTTTGTGTCC
names
SRR031715.1138209
SRR031714.756385
SRR031714.5054563
SRR031715.1722593
SRR031715.2202469
SRR031714.3544437
```

To obtain the *original query sequences* we “reverse complement” the sequences that are aligned to the minus strand:

```
> U3.GALP_oqseq1 <- U3.GALP_qseq1
> U3.GALP_first_is_on_minus <- as.logical(strand(first(U3.GALP)) == "-")
> U3.GALP_oqseq1[U3.GALP_first_is_on_minus] <- reverseComplement(U3.GALP_oqseq1[U3.GALP_first_is_on_minus])
> U3.GALP_oqseq2 <- U3.GALP_qseq2
> U3.GALP_last_is_on_minus <- as.logical(strand(last(U3.GALP)) == "-")
> U3.GALP_oqseq2[U3.GALP_last_is_on_minus] <- reverseComplement(U3.GALP_oqseq2[U3.GALP_last_is_on_minus])
```

Note that sequence pairs with the same *query template name* correspond to the same *original query pairs* and therefore should be the same. Let's do a quick sanity check:


```
> stopifnot(all(U3.GALP_oqseq1 == U3.GALP_oqseq1[U3.GALP_dup2uniq]))
> stopifnot(all(U3.GALP_oqseq2 == U3.GALP_oqseq2[U3.GALP_dup2uniq]))
```

Finally, let's reduce U3.GALP_oqseq1 and U3.GALP_oqseq2 to one *original query sequence* per unique *query template name*:

```
> U3.oqseq1 <- U3.GALP_oqseq1[!U3.GALP_names_is_dup]
> U3.oqseq2 <- U3.GALP_oqseq2[!U3.GALP_names_is_dup]
```

If we had access to the 2 FASTQ files used as input to the aligner, U3.oqseq1 and U3.oqseq2 would be the subsets of those files made of the sequence pairs with at least 1 alignment pair reported in BAM file `untreated3_chr4.bam`.

3.2.2 Compute the reference query sequences

Because our reads are paired-end, we 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):

```
> U3.grl_first <- grglist(first(U3.GALP), order.as.in.query=TRUE)
> U3.grl_last <- grglist(last(U3.GALP, invert.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 <- extractTranscriptsFromGenome(Dmelanogaster, U3.grl_first)
> U3.GALP_rqseq2 <- extractTranscriptsFromGenome(Dmelanogaster, U3.grl_last)
```

3.2.3 Compare the original query sequences with the reference query sequences

Because the aligned reads have no indels, we should only see mismatches (typically a small number) during that comparison so we don't need to call `neditAt` with `with.indels=TRUE`. Let's do this comparison for the first 500 sequences in U3.GALP_oqseq1 and in `reverseComplement(U3.GALP_oqseq2)`:

```
> U3.GALP_first_nedit500 <- sapply(1:500, function(i)
+   neditAt(U3.GALP_oqseq1[[i]], U3.GALP_rqseq1[[i]])
+ )
> table(U3.GALP_first_nedit500)
```

```
U3.GALP_first_nedit500
```

```
 0  1  2
337 135 28
```

```
> U3.GALP_last_nedit500 <- sapply(1:500, function(i)
+   neditAt(reverseComplement(U3.GALP_oqseq2[[i]]), U3.GALP_rqseq2[[i]])
+ )
> table(U3.GALP_last_nedit500)
```

```
U3.GALP_last_nedit500
```

```
 0  1  2
341 121 38
```

Yes, the first 500 sequences in U3.GALP_oqseq1 and in `reverseComplement(U3.GALP_oqseq2)` are “close” to the first 500 sequences in U3.GALP_rqseq1 and in U3.GALP_rqseq2, respectively.

Now let's compare the edit distance reported by `neditAt` with the edit distance reported by the aligner (NM tag). Because the latter excludes the N CIGAR operations, it should actually be the same as the former. We confirm this for the 500 edit distances computed in U3.GALP_first_nedit500 and U3.GALP_last_nedit500:

```
> U3.GALP_first_NM <- mcols(first(U3.GALP))$NM
> stopifnot(all(U3.GALP_first_NM[1:500] == U3.GALP_first_nedit500))
> U3.GALP_last_NM <- mcols(last(U3.GALP))$NM
> stopifnot(all(U3.GALP_last_NM[1:500] == U3.GALP_last_nedit500))
```

Note that the following table tells us how many mismatches per read were tolerated by the aligner:

```
> table(U3.GALP_first_NM, U3.GALP_last_NM)
```

```

      U3.GALP_last_NM
U3.GALP_first_NM  0    1    2
      0 30140 4648 1292
      1  5023 1522  760
      2  1240  700  503

```

Up to 2 mismatches per end.

3.3 Conclusion

In addition to validate the alignments produced by the aligner, the validation described in this section is also an efficient and accurate way to make sure that the reference genome we've picked up is the same as the reference genome used by the aligner, at least for the regions covered by the reads.

In other words, if it's known that the 2 reference genomes are different, then this validation could still be performed, and, if successful, would indicate that the 2 genomes are probably substitutable for most analysis happening downstream of the BAM file.

4 Find all the overlaps between the reads and transcripts

4.1 Load the transcripts from a *TranscriptDb* 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 `makeTranscriptDbFromUCSC` function defined in the `GenomicFeatures` package to make a *TranscriptDb* object containing the dm3 transcripts and their exons retrieved from the UCSC Genome Browser³. The Bioconductor project however provides a few annotation packages containing *TranscriptDb* 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 *TranscriptDb* object that was made by pointing the `makeTranscriptDbFromUCSC` function to the dm3 genome and *Ensembl Genes* track⁴. We can use it here:

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

TranscriptDb object:

```

| Db type: TranscriptDb
| Supporting package: GenomicFeatures
| Data source: UCSC
| Genome: dm3
| Genus and Species: Drosophila melanogaster
| 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: 23017
| exon_nrow: 69155
| cds_nrow: 59573
| Db created by: GenomicFeatures package from Bioconductor
| Creation time: 2012-09-10 13:00:23 -0700 (Mon, 10 Sep 2012)
| GenomicFeatures version at creation time: 1.9.39
| RSQLite version at creation time: 0.11.1
| DBSCHEMAVERSION: 1.0

```

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

³<http://genome.ucsc.edu/cgi-bin/hgGateway>

⁴See <http://genome.ucsc.edu/cgi-bin/hgTrackUi?hgsid=276880911&g=ensGene> for a description of this track.

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] 23017
```

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 analysis⁵. 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(elementLengths(runLength(seqnames(exbytx))))
```

```
1
23017
```

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

```
1
23017
```

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 *TranscriptDb* object **txdb**, and then we construct a named factor that represents the mapping:

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

GRanges with 6 ranges and 2 metadata columns:

	seqnames	ranges	strand	tx_name	gene_id
	<Rle>	<IRanges>	<Rle>	<character>	<CompressedCharacterList>
[1]	chr2L	[7529, 9484]	+	FBtr0300689	FBgn0031208
[2]	chr2L	[7529, 9484]	+	FBtr0300690	FBgn0031208
[3]	chr2L	[67044, 71390]	+	FBtr0078100	FBgn0067779
[4]	chr2L	[72388, 76211]	+	FBtr0078101	FBgn0031213
[5]	chr2L	[73485, 76211]	+	FBtr0302164	FBgn0031213
[6]	chr2L	[74485, 76211]	+	FBtr0301733	FBgn0031213

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4 ...	chr3Rhet	chrXhet	chrYhet	chrUextra
23011544	21146708	24543557	27905053	1351857 ...	2517507	204112	347038	29004656

```
> 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 FBtr0078100 FBtr0078101 FBtr0302164 FBtr0301733
FBgn0031208 FBgn0031208 FBgn0067779 FBgn0031213 FBgn0031213 FBgn0031213
14869 Levels: FBgn0031208 FBgn0067779 FBgn0031213 FBgn0031214 FBgn0031216 FBgn0031217 ... FBgn0085792
```

```
> nlevels(exbytx2gene) # nb of genes
```

```
[1] 14869
```

⁵Dealing with trans-splicing events is not covered in this document.

4.2 Single-end overlaps

4.2.1 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] 496485
```

4.2.2 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:

```
> nhitPerQuery <- function(x) tabulate(queryHits(x), nbins=queryLength(x))
> nhitPerSubject <- function(x) tabulate(subjectHits(x), nbins=subjectLength(x))
```

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

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

GappedAlignments with 6 alignments and 3 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap	
	<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	

	seq
	<DNASTringSet>
SRR031729.3941844	CTGTGGTGACCAACACCACAGAATGGTTCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCC
SRR031728.3674563	TCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTCTGGGCTGTTGTTG
SRR031729.8532600	CCCAATTAGAGGATTCTCTGCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTCCCGGATGTTGTTGTGTCC
SRR031729.2779333	GTTCTCTGCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTCCCGAGATGTTGTTGTGTCCCGGGACCCACCT
SRR031728.2826481	TTCCTGGCTAGGTTGTCCGCTAGCTCATTCCCGGGCTGTTGTTGTGTGTCCCGGGACACACCTTATTGTGAGTTTG
SRR031728.2919098	GCTAGCTCATTTCCTGGGAGGTTGTTGTGTCCCGGGACCCACCTTATTGTGAGTTTGTGACAGCTCCAAGTTTG

	NM	ntx
	<integer>	<integer>
SRR031729.3941844	1	0
SRR031728.3674563	3	0
SRR031729.8532600	2	0
SRR031729.2779333	1	0
SRR031728.2826481	2	0
SRR031728.2919098	1	0

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U1.GAL_ntx)
```

```
U1.GAL_ntx
  0     1     2     3     4     5     6     7     8     9    10
49079 37954 16036 58831 11042  5683 13877  2897  6914  1890  152
```

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

```
[1] 0.7598346
```

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, query.map=U1.GAL_qnames)
> U1.uqnames_ntx <- nhitPerQuery(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
41205 30281 16063 59904 11909  5683 13876  2893  6914  1890  152
```

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

```
[1] 0.7840069
```

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

Number of reads for each transcript:

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

```
[1] 0.00868923
```

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)
```

```
FBtr0089175 FBtr0089176 FBtr0089177 FBtr0112904 FBtr0289951 FBtr0089243 FBtr0089186 FBtr0089173
  40582      40582      40582      11735      11661      11656      10084      6750
FBtr0089172 FBtr0300498
  6749      6748
```

4.3 Paired-end overlaps

4.3.1 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] 102883
```

4.3.2 Tabulate the paired-end overlaps

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

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

GappedAlignmentPairs with 6 alignment pairs 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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U3.GALP_ntx)
```

U3.GALP_ntx											
0	1	2	3	4	5	6	7	8	9	10	14
13219	8661	3581	11449	2384	1197	2708	423	1905	271	29	1

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

```
[1] 0.7115519
```

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, query.map=U3.GALP_qnames)
> U3.uqnames_ntx <- nhitPerQuery(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	14
12094	7108	3701	11692	2530	1197	2708	423	1905	271	29	1

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

```
[1] 0.7229895
```

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

Number of templates for each transcript:

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

```
[1] 0.00755963
```

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)

FBtr0089175 FBtr0089176 FBtr0089177 FBtr0112904 FBtr0089243 FBtr0289951 FBtr0089186 FBtr0089100
      7573      7572      7556      2750      2732      2732      2260      1691
FBtr0089099 FBtr0089098
      1691      1691
```

5 Encode the overlaps between the reads and transcripts

5.1 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.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 of length 496485

	Offset	Roffset	encoding	flippedQuery
[1]	0	3	1:i:	TRUE
[2]	6	0	1:i:	TRUE
[3]	6	0	1:i:	TRUE
[4]	6	0	1:i:	TRUE
[5]	6	0	1:i:	TRUE
[6]	6	0	1:i:	FALSE
[7]	6	0	1:i:	TRUE
[8]	6	0	1:i:	TRUE
[9]	6	0	1:i:	TRUE
...
[496477]	23	0	1:i:	TRUE
[496478]	24	0	1:i:	TRUE
[496479]	22	0	1:i:	TRUE
[496480]	23	0	1:i:	FALSE
[496481]	24	0	1:i:	FALSE
[496482]	22	0	1:i:	FALSE
[496483]	23	0	1:i:	TRUE
[496484]	24	0	1:i:	TRUE
[496485]	22	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] 116
```

```
> 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:j: 1:k:c: 1:k: 1:c: 2:jm:af: 1:i:
1384 1919 10103 12659 65169 395278
```

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.

5.2 Paired-end encodings

Let's encode the overlaps in U3.OV00:

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

OverlapEncodings of length 102883

	Loffset	Roffset	encoding	flippedQuery
[1]	6	0	1--1:i--m:	FALSE
[2]	5	1	1--1:i--i:	FALSE
[3]	4	2	1--1:i--i:	TRUE
[4]	4	2	1--1:i--i:	TRUE
[5]	4	2	1--1:i--i:	TRUE
[6]	4	2	1--1:i--i:	TRUE
[7]	4	2	1--1:i--i:	FALSE
[8]	4	2	1--1:i--i:	FALSE
[9]	4	2	1--1:i--i:	FALSE
...
[102875]	22	0	2--1:jm--m:af--i:	FALSE
[102876]	23	0	2--1:jm--m:af--i:	FALSE
[102877]	21	0	2--1:jm--m:af--i:	FALSE
[102878]	23	0	1--1:i--i:	FALSE
[102879]	24	0	1--1:i--i:	FALSE
[102880]	22	0	1--1:i--i:	FALSE
[102881]	23	0	1--1:i--i:	TRUE
[102882]	24	0	1--1:i--i:	TRUE
[102883]	22	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--e:" "1--1:a--f:" "1--1:a--i:" "1--1:a--j:" "1--1:a--k:"

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

      1--1:i--k:      1--1:i--m:      1--1:c--i: 1--2:i--jm:a--af: 2--1:jm--m:af--i:
      1531          1534          1778          2023          2562
      1--1:i--i:
      87951
```

6 “Compatible” overlaps

We are interested in a particular type of overlap where the read overlaps the transcript in a “compatible” way, that is, in a way 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.

6.1 “Compatible” single-end overlaps

6.1.1 “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:f:      1:j:      2:jm:af:
      25          54          342          1336          1384          65169
      1:i:
      395278
```

Encodings "1:i:" (395278 occurrences in `U1.ovenc`), "2:jm:af:" (65169 occurrences in `U1.ovenc`), and "3:jmm:agm:aaf:" (342 occurrences in `U1.ovenc`), correspond to the following overlaps:

- "1:i:"
 - read (no gap): oooooooooo
 - transcript: ... >>>>>>>>>>>>>> ...
- "2:jm:af:"
 - read (1 gap): oooooo---ooo
 - transcript: ... >>>>>>>> >>>>>>>> ...
- "3:jmm:agm:aaf:"
 - read (2 gaps): 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 in the 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 gap): oooooooooo
 - transcript: ... >>>>>>>>>>>>>> ...

- "1:j:"

```
- read (no gap):          oooooooooo
- transcript:      ...  >>>>>>>>>>>>  ...
```

```
> U1.OV00_is_comp <- isCompatibleWithSplicing(U1.ovenc)
> table(U1.OV00_is_comp) # 476124 "compatible" overlaps
```

```
U1.OV00_is_comp
FALSE  TRUE
32897 463588
```

Finally, let's extract the "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 "compatible" overlaps directly between a *GappedAlignments* object (containing reads) and a *GRangesList* object (containing transcripts):

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

6.1.2 Tabulate the "compatible" single-end overlaps

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

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

GappedAlignments with 6 alignments and 4 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap
	<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

seq

<DNASTringSet>

```
SRR031729.3941844 CTGTGGTGACCAACACCACAGAATGGTTCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCC
SRR031728.3674563 TCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTTCCTGGGCTGTTGTTG
SRR031729.8532600 CCCAATTAGAGGATTCTCTGCCCCCTTTCTGGCTAGGTTGTCCGGTAGCTCATTTCCTGGGATGTTGTTGTGTC
SRR031729.2779333 GTTCTCTGCCCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTTCCTGGAGATGTTGTTGTGTCCTCCGGGACCCACCT
SRR031728.2826481 TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCTGGGCTGTTGTTGTGTCCTCCGGGACACACCTTATTGTGAGTTTG
SRR031728.2919098 GCTAGTTCATTTCCTGGGAGGTTGTTGTGTCCTCCGGGACCCACCTTATTGTGAGTTTGTGACAGCTCCAAGTTTG
```

	NM	ntx	ncomptx
	<integer>	<integer>	<integer>

SRR031729.3941844	1	0	0
SRR031728.3674563	3	0	0
SRR031729.8532600	2	0	0
SRR031729.2779333	1	0	0
SRR031728.2826481	2	0	0
SRR031728.2919098	1	0	0

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U1.GAL_ncomptx)
```

```
U1.GAL_ncomptx
  0    1    2    3    4    5    6    7    8    9   10
53514 43731 16616 50092 10949 5404 13088 2502 6688 1723 48
```

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

```
[1] 0.7381322
```

73.8% of the alignments in U1.GAL are “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 “compatible” transcripts for each read:

```
> U1.compOV10 <- remapHits(U1.compOV00, query.map=U1.GAL_qnames)
> U1.uqnames_ncomptx <- nhitPerQuery(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
44832 36563 17029 51158 11735 5404 13088 2502 6688 1723 48
```

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

```
[1] 0.7649945
```

76.5% of the reads are “compatible” with at least 1 transcript in exbytx.

Number of “compatible” reads for each transcript:

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

```
[1] 0.008602337
```

Only 0.860% of the transcripts in exbytx are “compatible” with at least 50 reads.

Top 10 transcripts:

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

```
FBtr0089175 FBtr0089177 FBtr0089176 FBtr0089243 FBtr0289951 FBtr0112904 FBtr0089186 FBtr0089173
  40289      33978      33621      11365      11332      11284      10025      6606
FBtr0300497 FBtr0089172
  6601      6599
```

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

6.2 “Compatible” paired-end overlaps

6.2.1 “Compatible” paired-end encodings

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	9	12
2--1:jm--m:af--f:	1--1:i--m:a--f:	1--1:j--m:a--i:
24	48	56
2--2:jm--mm:af--jm:aa--af:	1--1:i--m:a--i:	1--1:i--j:
122	353	361
1--1:f--i:	1--2:i--jm:a--af:	2--1:jm--m:af--i:
544	2023	2562
1--1:i--i:		
87951		

Paired-end encodings "1--1:i--i:" (87951 occurrences in U3.ovenc), "2--1:jm--m:af--i:" (2562 occurrences in U3.ovenc), "1--2:i--jm:a--af:" (2023 occurrences in U3.ovenc), "1--1:i--m:a--i:" (353 occurrences in U3.ovenc), and "2--2:jm--mm:af--jm:aa--af:" (122 occurrences in U3.ovenc), correspond to the following paired-end overlaps:

- "1--1:i--i:"
 - paired-end read (no gap on the first end, no gap on the last end):


```

          oooo  oooo
          - transcript:    ...  >>>>>>>>>>>>>>>>  ...
          
```
- "2--1:jm--m:af--i:"
 - paired-end read (1 gap on the first end, no gap on the last end):


```

          ooo---o  oooo
          - transcript:    ...  >>>>>>>  >>>>>>>>>>>  ...
          
```
- "1--2:i--jm:a--af:"
 - paired-end read (no gap on the first end, 1 gap on the last end):


```

          oooo  oo---oo
          - transcript:    ...  >>>>>>>>>>>>>  >>>>>>>>  ...
          
```
- "1--1:i--m:a--i:"
 - paired-end read (no gap on the first end, no gap on the last end):


```

          oooo  oooo
          - transcript:    ...  >>>>>>>>  >>>>>>>>  ...
          
```
- "2--2:jm--mm:af--jm:aa--af:"
 - paired-end read (1 gap on the first end, 1 gap 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) # 94068 "compatible" paired-end overlaps

U3.OV00_is_comp
FALSE TRUE
8815 94068

```

Finally, let's extract the “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 “compatible” paired-end overlaps directly between a *GappedAlignmentPairs* 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))

```

6.2.2 Tabulate the “compatible” paired-end overlaps

Number of “compatible” transcripts for each alignment pair in U3.GALP:

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

GappedAlignmentPairs with 6 alignment pairs 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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U3.GALP_ncomptx)
```

U3.GALP_ncomptx										
0	1	2	3	4	5	6	7	8	9	10
14242	10842	3569	8652	2345	1135	2589	356	1833	263	2

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

```
[1] 0.6892293
```

68.9% of the alignment pairs in U3.GALP are “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 “compatible” transcripts for each template:

```
> U3.compOV10 <- remapHits(U3.compOV00, query.map=U3.GALP_qnames)
> U3.uqnames_ncomptx <- nhitPerQuery(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
13044	9376	3695	8914	2452	1135	2589	356	1833	263	2

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

```
[1] 0.70123
```

70.1% of the templates are “compatible” with at least 1 transcript in exbytx.

Number of “compatible” templates for each transcript:

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

```
[1] 0.007516184
```

Only 0.752% of the transcripts in `exbytx` are “compatible” with at least 50 templates.

Top 10 transcripts:

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

FBtr0089175 FBtr0089176 FBtr0089177 FBtr0289951 FBtr0089243 FBtr0112904 FBtr0089186 FBtr0089096
      7490      5252      5234      2686      2684      2640      2246      1638
FBtr0089100 FBtr0089099
      1632      1632
```

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

7 Project the alignments on the transcriptome

7.1 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
2             3584              7              26
3             3579              7              21
4             3579              7              21
5             3576              7              18
6             3574              7              16
```

`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 “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 the `extractTranscriptsFromGenome` to extract the transcript sequences (aka transcriptome) from the dm3 reference genome:

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

For each “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 “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 “compatible” overlap.

7.2 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	3406	6	31
3	3158	5	22
4	3156	5	20
5	3140	5	4
6	3148	5	12
7	3174	5	38

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	3414	6	39
3	3315	5	179
4	3297	5	161
5	3297	5	161
6	3291	5	155
7	3289	5	153

Like with single-end reads, having this information allows us for example to compare the read and transcript nucleotide sequences for each “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 “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])
+ ))
```

8 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 “compatible” overlaps per *original query*. Supporting junction reads (i.e. reads that align with at least 1 gap) 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 gap 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 “compatible” overlaps that we will call “encoding-based compatible” from now on, unless the context is clear).

8.1 Align the single-end reads to the transcriptome

8.1.1 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) “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)), elementLengths(x))
+     new("Hits", queryHits=query_hits, subjectHits=subject_hits,
+         queryLength=pattern_length, subjectLength=length(x))
+   }
+   .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@queryLength <- length(qseq)
+   hits0@queryHits <- which(is_clean)[hits0@queryHits]
+
+   ## 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@queryLength <- length(qseq)
+   hits1@queryHits <- which(!is_clean)[hits1@queryHits]
+
+   ## 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@subjectLength <- length(txseq)
+   }
+
+   ## Order the hits.
+   oo <- IRanges::orderIntegerPairs(query_hits, subject_hits)
+   hits0@queryHits <- query_hits[oo]
+   hits0@subjectHits <- 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)
```

8.1.2 Tabulate the “hits”

Number of “string-based compatible” transcripts for each read:

```
> U1.uqnames_nsbcomptx <- nhitPerQuery(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
42494 36484 16641 52677 12214 5542 13446 2642 6777 1787 66
```

```
> mean(U1.uqnames_nsbcomptx >= 1)
[1] 0.7772501
```

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

```
[1] 0.008645784
```

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)
FBtr0301886 FBtr0078118 FBtr0301887 FBtr0077816 FBtr0077815 FBtr0077817 FBtr0077837 FBtr0078047
      40520      34703      34406      11605      11579      11548      10066      6710
FBtr0078128 FBtr0078131
      6704      6701
```

8.1.3 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”:

```
> ## FIXME: 11 September, 2012 requires U1.sbcompHITS object update
> ## stopifnot(length(setdiff(U1.compOV10, U1.sbcompHITS)) == 0)
```

but the reverse is not true:

```
> length(setdiff(U1.sbcompHITS, U1.compOV10))
[1] 474492
```

8.2 Align the paired-end reads to the transcriptome

[COMING SOON...]

9 “Almost compatible” overlaps

In many aspects, “compatible” overlaps can be seen as perfect. We are now interested in a less perfect type of overlap where the read overlaps the transcript in a way that *would* be “compatible” if 1 or more exons were removed from the transcript. In that case we say that the overlap is “almost 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.

9.1 “Almost compatible” single-end overlaps

9.1.1 “Almost compatible” single-end encodings

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

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

2:jm:am:am:am:am:am:af:      2:jm:am:am:am:am:af:      2:gm:am:af:  3:jmm:agm:aam:aam:aaf:
                        1                        2                        3                        3
      3:jmm:agm:aam:aaf:      2:jm:am:am:am:af:      2:jm:am:af:
                        18                        112                        721
```

Encodings "2:jm:am:am:af:" (721 occurrences in `U1.ovenc`), "2:jm:am:am:am:af:" (112 occurrences in `U1.ovenc`), and "3:jmm:agm:aam:aaf:" (18 occurrences in `U1.ovenc`), correspond to the following overlaps:

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

```
- read (1 gap):      ooooo-----ooo
- transcript:      ... >>>>>> >>>> >>>>>>> ...
```
- "2:jm:am:am:am:af:"

```
- read (1 gap):      ooooo-----ooo
- transcript:      ... >>>>>> >>>> >>>>> >>>>>>> ...
```
- "3:jmm:agm:aam:aaf:"

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

```
> U1.OV00_is_acomp <- isCompatibleWithSkippedExons(U1.ovenc)
> table(U1.OV00_is_acomp) # 860 "almost compatible" overlaps
```

```
U1.OV00_is_acomp
FALSE  TRUE
495625  860
```

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

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

9.1.2 Tabulate the “almost compatible” single-end overlaps

Number of “almost compatible” transcripts for each alignment in U1.GAL:

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

GappedAlignments with 6 alignments and 5 metadata columns:

	seqnames	strand	cigar	qwidth	start	end	width	ngap
	<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

seq

<DNASet>

```
SRR031729.3941844 CTGTGGTGACCAACACCACAGAATGGTTCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCC
SRR031728.3674563 TCGGGCCCAATTAGAGGGTTCCTGCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTTCTGGGCTGTTGTTG
SRR031729.8532600 CCCAATTAGAGGATTCTCTGCCCCCTTTCTGGCTAGGTTGTCCGGTAGCTCATTTCCCGGGATGTTGTTGTGCC
SRR031729.2779333 GTTCTCTGCCCCCTTTCTGGCTAGGTTGTCCGCTAGCTCATTTCCCGAGATGTTGTTGTGTCCCGGGACCCACCT
SRR031728.2826481 TTCCTGGCTAGGTTGTCCGCTAGCTCATTTCCCGGGCTGTTGTTGTGTCCCGGGACACACCTTATTGTGAGTTTG
SRR031728.2919098 GCTAGCTCATTTCCCGGGAGGTTGTTGTGTCCCGGGACCCACCTTATTGTGAGTTTGTGACAGCTCCAAGTTTG
```

	NM	ntx	ncomptx	nacomptx
	<integer>	<integer>	<integer>	<integer>

SRR031729.3941844	1	0	0	0
SRR031728.3674563	3	0	0	0
SRR031729.8532600	2	0	0	0
SRR031729.2779333	1	0	0	0
SRR031728.2826481	2	0	0	0
SRR031728.2919098	1	0	0	0

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U1.GAL_nacomptx)
```

U1.GAL_nacomptx

0	1	2	3	4	5	6	7	8	9
203902	299	28	79	20	1	12	6	3	5

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

[1] 0.002216731

Only 0.22% of the alignments in U1.GAL are “almost compatible” with at least 1 transcript in exbytx.

Number of “almost compatible” alignments for each transcript:

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

U1.exbytx_nacompOV00

0	1	2	3	4	6	7	8	9	10	12	13	14	18	20	21
22911	43	5	14	8	2	4	4	9	2	1	1	1	2	1	2
32	34	44	55	170											
1	1	3	1	1											

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

```
[1] 8.68923e-05
```

Only 0.0087% of the transcripts in `exbytx` are “almost 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 compatible” overlaps:

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

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
132461	66	1	66
132472	84	1	84
134409	757	7	39
134410	689	8	39
134411	631	6	39
134412	877	7	39

9.2 “Almost compatible” paired-end overlaps

9.2.1 “Almost compatible” paired-end encodings

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

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

```
2--1:jm--m:am--m:af--i:      1--2:i--jm:a--am:a--am:a--af:
                                1                                4
2--2:jm--mm:am--mm:af--jm:aa--af:      1--2:i--jm:a--am:a--af:
                                9                                36
2--1:jm--m:am--m:af--i:
                                54
```

Paired-end encodings “2--1:jm--m:am--m:af--i:” (54 occurrences in `U3.ovenc`), “1--2:i--jm:a--am:a--am:a--af:” (36 occurrences in `U3.ovenc`), and “2--2:jm--mm:am--mm:af--jm:aa--af:” (9 occurrences in `U3.ovenc`), correspond to the following paired-end overlaps:

- “2--1:jm--m:am--m:af--i:”
 - paired-end read (1 gap on the first end, no gap on the last end):
ooo-----o oooo
 - transcript: ... >>>> >>> >>>>>>> ...
- “1--2:i--jm:a--am:a--am:a--af:”
 - paired-end read (no gap on the first end, 1 gap on the last end):
oooo oo-----oo
 - transcript: ... >>>>>>>>> >>> >>>>> ...
- “2--2:jm--mm:am--mm:af--jm:aa--af:”
 - paired-end read (1 gap on the first end, 1 gap 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) # 104 "almost compatible" paired-end overlaps
```

```
U3.OV00_is_acomp
FALSE  TRUE
102779  104
```

Finally, let’s extract the “almost compatible” paired-end overlaps from `U3.OV00`:

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

9.2.2 Tabulate the “almost compatible” paired-end overlaps

Number of “almost compatible” transcripts for each alignment pair in U3.GALP:

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

GappedAlignmentPairs with 6 alignment pairs 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

seqlengths:

chr2L	chr2R	chr3L	chr3R	chr4	chrM	chrX	chrYHet
23011544	21146708	24543557	27905053	1351857	19517	22422827	347038

```
> table(U3.GALP_nacomptx)
```

U3.GALP_nacomptx				
0	1	3	4	6
45745	75	5	2	1

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

```
[1] 0.00181112
```

Only 0.18% of the alignment pairs in U3.GALP are “almost compatible” with at least 1 transcript in **exbytx**.

Number of “almost compatible” alignment pairs for each transcript:

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

U3.exbytx_nacompOV00				
0	1	5	8	66
22997	15	3	1	1

```
> mean(U3.exbytx_nacompOV00 >= 50)
```

```
[1] 4.344615e-05
```

Only 0.0043% of the transcripts in **exbytx** are “almost 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 compatible” paired-end overlaps:

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

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
43359	785	4	6
43366	808	4	29
43373	819	4	40
43380	785	4	6
43387	785	4	6
43394	810	4	31

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

	startInTranscript	firstSpannedExonRank	startInFirstSpannedExon
43359	845	4	66
43366	847	4	68
43373	849	4	70
43380	842	4	63
43387	847	4	68
43394	847	4	68

10 Detect novel splice junctions

10.1 By looking at single-end overlaps

An alignment in `U1.GAL` with “almost compatible” overlaps but no “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 != OL & U1.GAL_ncomptx == OL
> 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(elementLengths(U1.nsjOV00_skippedex))

 1  2  3  5
226 91  2  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" "FBtr0078100" "FBtr0078101" "FBtr0302164" "FBtr0301733"
```

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 alignments reported in `U1.exbytx_skippedex` are guaranteed to not have any “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!

10.2 By looking at paired-end overlaps

[COMING SOON...]

11 sessionInfo()

```
> sessionInfo()
```

```
R version 2.15.2 (2012-10-26)
Platform: x86_64-unknown-linux-gnu (64-bit)
```

```
locale:
```

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

```
attached base packages:
```

```
[1] stats      graphics  grDevices  utils      datasets  methods    base
```


other attached packages:

```
[1] TxDb.Dmelanogaster.UCSC.dm3.ensGene_2.8.0 BSgenome.Dmelanogaster.UCSC.dm3_1.3.19
[3] pasillaBamSubset_0.0.6 BSgenome.Scerevisiae.UCSC.sacCer2_1.3.19
[5] org.Sc.sgd.db_2.8.0 RSQLite_0.11.2
[7] DBI_0.2-5 GenomicFeatures_1.10.1
[9] AnnotationDbi_1.20.3 leeBamViews_0.99.21
[11] BSgenome_1.26.1 Biobase_2.18.0
[13] EatonEtAlChIPseq_0.0.8 rtracklayer_1.18.2
[15] ShortRead_1.16.3 latticeExtra_0.6-24
[17] RColorBrewer_1.0-5 lattice_0.20-13
[19] Rsamtools_1.10.2 Biostrings_2.26.2
[21] GenomicRanges_1.10.6 IRanges_1.16.4
[23] BiocGenerics_0.4.0
```

loaded via a namespace (and not attached):

```
[1] RCurl_1.95-3 XML_3.95-0.1 biomaRt_2.14.0 bitops_1.0-5 grid_2.15.2 hwriter_1.3
[7] parallel_2.15.2 stats4_2.15.2 tools_2.15.2 zlibbioc_1.4.0
```