

Using RAMPAGE to identify and annotate promoters in insect genomes

R. Taylor Raborn^{*1,2} and Volker P. Brendel^{1,2}

¹Department of Biology, Indiana University

²School of Informatics and Computing, Indiana University

Department of Biology and School of Informatics and Computing,
Indiana University

212 S. Hawthorne Drive 205 Simon Hall, Bloomington, IN 47401, USA
<http://www.brendelgroup.org>

Abstract. Application of Transcription Start Site (TSS) profiling technologies, coupled with large-scale next-generation sequencing (NGS) has yielded valuable insights into the location, structure and activity of promoters across diverse metazoan model systems. In insects, TSS profiling has been used to characterize the promoter architecture of *D. melanogaster*, and, shortly thereafter, to reveal widespread transposon-driven alternative promoter usage.

In this chapter we highlight the utility of one TSS profiling method, RAMPAGE (RNA annotation and mapping of promoters for analysis of gene expression), for the precise, quantitative identification of promoters in insect genomes. We demonstrate this using our tools GoRAMPAGE and TSSrchitect, providing details instructions with the aim of taking the user from raw reads to processed results.

Keywords: *cis*-regulatory regions, promoter architecture, transcription initiation, transcription start sites (TSSs)

1 Introduction

1.1 TSS Profiling Identifies Promoters at Genome-Scale

The promoter, defined in eukaryotes as the genomic region bound by RNA Polymerase II immediately prior to transcription initiation [1], is the site where regulatory signals unite to direct gene expression. The identification of promoter regions is a valuable step for understanding the *cis*-regulatory signals that are present in an organism, and is also important for genome annotation. However, despite the rapid accumulation of genome sequences across metazoan and arthropod diversity, accurate annotation of promoter regions remains sparse. This is because—empirical mapping of TSSs—precisely identifying sequence motifs that demarcate the promoter is unreliable. In contrast with current *in*

* Correspondence: rtraborn@indiana.edu

12 *silico* approaches, direct mapping of TSSs identifies the location of the core pro-
 13 moter. Cap Analysis of Gene Expression (CAGE) [2], one of the first methods
 14 devised to identify 5'-ends of mRNAs at large-scale, involves selective capture
 15 of 5'-capped transcripts, first-strand reverse-transcription and ligation of a short
 16 oligonucleotide (CAGE tag).
 17 CAGE was initially utilized by the FANTOM (Functional Annotation of the
 18 Mammalian Genome) consortium to identify promoter architecture in human
 19 and mouse [3], providing the first glimpse of the global landscape of transcrip-
 20 tion initiation. At the onset of the NGS era, CAGE was coupled with massively-
 21 parallel sequencing to generate 5'-ends of mRNAs at substantially higher scale.
 22 This advance provided more extensive coverage of the expressed transcriptome,
 23 and provided increased sensitivity for quantitative measurements *i.e.* measure-
 24 ment of promoter activity.

25 1.2 Promoter Architecture of *Drosophila melanogaster*

26 Hoskins and colleagues [4] performed CAGE in *D. melanogaster* as part of the
 27 modENCODE consortium, identifying promoters at large-scale and character-
 28 izing the promoter architecture of an insect genome for the first time. Hoskins
 29 [4] indicated that TSS distributions at *Drosophila* promoters exhibit a range
 30 of shapes that can be generally grouped into two major classifications: *peaked*
 31 and *broad*. Peaked promoters have a single, major TSS position occupying a
 32 narrow genomic region, whereas broad promoters lack a single, major TSS and
 33 contain TSSs across a wider region [5][6]. The authors also showed a strong asso-
 34 ciation between promoter class and motif composition (consistent with previous
 35 findings [5, 7]). Peaked promoters were associated with positionally-enriched *cis*-
 36 regulatory motifs including TATA, Initiator (Inr) and DPE, while broad promot-
 37 ers contained an enrichment of less-well characterized motifs, including *Ohler6*
 38 and *Ohler7* [8]. The existence of two promoter classes appears to be conserved
 39 among metazoans, and has been reported (using TSS profiling methodologies) in
 40 insects, cladocerans [9], fish [10] and mammals [11, 6].

41 1.3 Promoter Structure of Insects

42 Beyond *D. melanogaster*, few investigations have utilized TSS profiling in insect
 43 genomes. As a consequence, what is known about promoter architecture in in-
 44 sects is largely restricted to the *Drosophila* genus. As part of the modENCODE
 45 effort, CAGE was performed in multiple tissues and developmental stages of the
 46 *Drosophila pseudoobscura*. TSSs were found to be highly similar between species:
 47 more than 80% of TSSs (81%) of aligned, CAGE-identified TSSs from *D. pseu-*
 48 *doobscura* were positioned within 20nt of their counterparts in *D. melanogaster*.
 49 An enrichment of the CA dinucleotide was detected at the TSS ($[-1, +1]$), and
 50 the motifs corresponding to TATA, Inr and DPE were positioned at the same
 51 locations relative to the TSS in both species. The one other insect species for
 52 which TSS profiling has been applied is the Tsetse fly (*Glossina morsitans morsi-*
 53 *tans*) [12]. Using TSS-seq (specifically Oligo-capping; for details on this method

see [13]), the authors identified 3134 mapping to 1424 genes. The authors found a preference for CA and AA dinucleotides at the TSS, and observe the major core promoter elements observed in *Drosophila*: TATA, Inr, DPE, in addition to MTE (Motif Ten Element). As in *D. melanogaster*, peaked promoters were more likely to contain TATA and Inr than broad promoters. While the taxonomic sampling of species for TSS profiling has been limited, the existing studies are sufficient to provide a general picture of insect promoter architecture. A major demarcation between the promoter architecture of insects and mammals appears to be the large fraction of mammalian promoters found in CpG islands [12]. CpG island promoters (CPIs) form the largest class of promoter in mammals [14]; by contrast, CPIs are not known to exist as a class in invertebrates.

1.4 Paired-end TSS Profiling with RAMPAGE

The most recent major methodological advance in TSS Profiling is RAMPAGE (RNA Annotation and Mapping of Promoters for the Analysis of Gene Expression) . RAMPAGE is a protocol for 5'-cDNA sequencing that combines cap trapping and template-switching with paired-end sequence information. A key advantage of generating paired-end sequence is transcript connectivity, which provides a direct link between a given 5'-end and its associated mRNA molecule. Because short or spurious RNAs are found within the transcriptome, transcript connectivity allows the TSSs (and thus promoters) of full-length mRNAs to be unambiguously identified, which benefits genome annotation. Batut and colleagues generated libraries from total RNA isolated from 36 stages across the life cycle of *D. melanogaster* providing a comprehensive gene expression and promoter atlas for fruit fly and in the process demonstrating the utility of RAMPAGE. RAMPAGE is currently being applied as part of the latest iteration of ENCODE to identify promoters in human, but as of this writing it has not been applied to any non-*Drosophila* insect species. In anticipation of the future application of TSS profiling into other insect model systems here we provide a documented protocol for the computational processing RAMPAGE data, using selected libraries from Batut *et al.*. This method will consist of two parts: first, we will process, filter and align the sequenced RAMPAGE libraries to the *D. melanogaster* genome. Second, we will identify TSSs and promoters from the aligned sequences and associate them with coding regions. In closing, we will consider further applications of this data and discuss the utility of reproducible workflows in bioinformatic analysis.

2 Materials

The analyses described herein require a workstation capable for modern bioinformatics. An intermediate understanding of the Linux/Unix command line will be extremely useful, although we make efforts to explain the procedures with clarity. In addition, it will likely be necessary for the participant to have superuser privileges on the machine. If you do not have a machine (or access to one) that meets

these requirements, it is recommended that you consider cloud-based cyberinfrastructure, including Amazon Web Services (AWS; <https://aws.amazon.com/>) or CyVerse (<http://www.cyverse.org/>). The former is a well-known pay-per-use solution, while the latter is an NSF-funded resource that is made freely available to the public.

2.1 Hardware Requirements

1. x86-64 compatible processors
2. At least 8GB RAM
3. 30GB+ hard disk space

2.2 Software Requirements

1. Operating system: 64 bit Linux (preferred) or Mac OS X (with Command Line Tools from XCode)
2. R (version 3.4)
3. Bioconductor (version 3.5)
4. FASTX-Toolkit (version 0.0.13)
5. Samtools (version 1.3 or above)
6. SRA Toolkit (version 2.3.4-2 or above)
7. STAR aligner (version 2.4 or above)
8. TagDust (version 2.33)

2.3 Online Appendix

We created an online appendix to serve as a companion to this chapter, which contains both scripts and select files to assist you in completing this tutorial.. Please find the repository at https://github.com/rtraborn/MMB_appendix (see **Note 1**).

2.4 Installation of R packages

For installation of the software listed above, please follow the instructions provided by each respective package. Part of our analysis will require the use of R packages found in the Bioconductor suite. To install Bioconductor, please type the following from an R console:

```
source("https://bioconductor.org/biocLite.R")
biocLite()
```

We will use the R package *TSRchitect* to identify promoters from aligned RAMAPGE libraries. First, we will need to install a series of prerequisite packages to *TSRchitect* from Bioconductor. Please install these packages as follows (as before, from an R console):

```

130 source("https://bioconductor.org/biocLite.R")
131 biocLite(c("AnnotationHub", "BiocGenerics", "BiocParallel",
132 "ENCODEExplorer", "GenomicAlignments", "GenomeInfoDb",
133 "GenomicRanges", "IRanges", "methods",
134 "Rsamtools", "rtracklayer", "S4Vectors",
135 "SummarizedExperiment"))

```

136 To install *TSRchitect*, please type the following from an R console:

```

137 source("https://bioconductor.org/biocLite.R")
138 biocLite("TSRchitect")

```

139 Finally, please confirm that *TSRchitect* has been installed correctly by load-
 140 ing it from your R console as follows:

```

141 library(TSRchitect)

```

142 3 Methods

143 3.1 Retrieving the RAMPAGE sequence data from NCBI's Gene 144 Expression Omnibus (GEO)

145 To begin our analysis, we must download the RAMPAGE data to our worksta-
 146 tion. We will utilize tools provided by the SRA Toolkit, which should already
 147 be installed on your machine (see **Materials**). The command *fastq-dump* allows
 148 one to directly retrieve data from the GEO database using the appropriate iden-
 149 tifier(s). While there are 36 RAMPAGE libraries in the Batut *et al.* dataset,
 150 we will select a subset of these to analyze here. We will compare samples from
 151 selected embryonic (E01h-E03h) and larval (L1-L3) tissues, representing the be-
 152 ginning and end of embryonic development. For more information about the
 153 experiment and the available RAMPAGE libraries, please see the following link:
 154 <https://www.ncbi.nlm.nih.gov/Traces/study/?acc=SRP011193>.

155
 156 First, let's proceed with the libraries from early embryonic tissues (see **See**
 157 **Note 2**).

```

158 mkdir fastq_files #creating a new folder to house the downloaded files
159 cd fastq_files #moving into this directory
160 fastq-dump --split-files SRR424683
161 fastq-dump --split-files SRR424684
162 fastq-dump --split-files SRR424685

```

163 We continue by downloading the RAMPAGE libraries from late embryonic
 164 tissues:

```

165 fastq-dump --split-files SRR424707
166 fastq-dump --split-files SRR424708
167 fastq-dump --split-files SRR424709

```

168 Once the download of the aforementioned files are complete, you should see
 169 a total of 12 (6x2) separate fastq files in your current working directory:

```
170 ls -l *.fastq | wc -l
```

171 3.2 Creating symlinks to the files

172 Our workflow expects fastq files that have the format “*.R1/R2.clipped.fq”.
 173 Rather than rename them, we can simply create brand new symbolic links (sym-
 174 links) to the files, as follows:

```
175 mkdir symlinks
176
177 #embryonic libraries
178 ln -s SRR424683_1.fastq symlinks/E01h.R1.clipped.fq
179 ln -s SRR424683_2.fastq symlinks/E01h.R2.clipped.fq
180 ln -s SRR424684_1.fastq symlinks/E02h.R1.clipped.fq
181 ln -s SRR424684_2.fastq symlinks/E02h.R2.clipped.fq
182 ln -s SRR424685_1.fastq symlinks/E03h.R1.clipped.fq
183 ln -s SRR424685_2.fastq symlinks/E03h.R2.clipped.fq
184
185 #larval libraries
186 ln -s SRR424707_1.fastq symlinks/L1.R1.clipped.fq
187 ln -s SRR424707_2.fastq symlinks/L1.R2.clipped.fq
188 ln -s SRR424708_1.fastq symlinks/L2.R1.clipped.fq
189 ln -s SRR424708_2.fastq symlinks/L2.R2.clipped.fq
190 ln -s SRR424709_1.fastq symlinks/L3.R1.clipped.fq
191 ln -s SRR424709_2.fastq symlinks/L3.R2.clipped.fq
```

192 3.3 Downloading genomic data from *D. melanogaster*

193 Now that we have the fastq files from the RAMPAGE libraries downloaded and
 194 named appropriately, we now must retrieve the genome assembly and rRNA
 195 sequences from *D. melanogaster*. The genome assembly is required for aligning
 196 the RAMPAGE reads, and the rRNA sequences are required to filter out match-
 197 ing reads in the sequenced RAMPAGE libraries, since our sample is intended
 198 to contain only capped RNA transcripts. Please download the rRNA sequences
 199 from the link we provide below. These sequences were retrieved separately from
 200 Genbank at the NCBI database.

201 Please download the assembly from the ENSEMBL database as follows:

```
202 wget ftp://ftp.ensembl.org/pub/release-78/fasta/drosophila_melanogaster/dna/Drosophila_m
203 #uncompressing the file
204 gzip -d Drosophila_melanogaster.BDGP5.dna.toplevel.fa.gz
```

205 Please navigate to the rRNA file texttt"Dmel_rRNA.fasta" found in the
 206 Appendix.

```

207 head -n 3
208 >ref|NR_133562.1| Drosophila melanogaster 28S ribosomal RNA (28SrRNA:CR45844), rRNA
209 TTATATACAACCTCAACTCATATGGGACTACCCCTGAATTTAAGCATATTAATTAGGGGAGGAAAAGAA
210 ACTAACAAGGATTTTCTTAGTAGCGGCGAGCGAAAAGAAAACAGTTCAGGACTAAGTCACTTTGTCTATA

```

211 3.4 Filtering and alignment of RAMPAGE reads using 212 GoRAMPAGE

213 At this stage we are ready to commence with the rRNA filtering and alignment
214 of the RAMPAGE libraries. We will use GoRAMPAGE, a tool we developed,
215 to perform these tasks in a concerted workflow. GoRAMPAGE runs TagDust
216 [15] to remove rRNA and low-complexity reads, and uses STAR [16] to align
217 RAMPAGE (or other paired-end) reads to a given genome assembly.
218

219 **Preparing the output directory** It will also be necessary to create an output
220 directory under "outputDir" for the results. GoRAMPAGE expects the results
221 of a given step to be in place prior to initiating a run, so we'll need to create the
222 appropriate folders before proceeding. Please do this as follows:

```

223 mkdir output #omit if you already have an output directory selected
224 mkdir output/reads
225 mkdir output/reads/clipped

```

226 **Setting up the GoRAMPAGE job** Now, once this is complete, please
227 copy the contents of the "symlinks" directory that you created earlier (*i.e.*
228 all of the *.fq files) into the "clipped/" directory. Please refer to the script
229 "GoRAMPAGE_script_MMB.sh" and (using a text editor) provide the appropriate
230 paths to the genome assembly, output directory (see above) and rRNA sequences
231 (*see Note 3*). The script can be executed as follows:

```

232 ./GoRAMPAGE_script_MMB.sh
233 #alternatively 'sh GoRAMPAGE_script_MMB.sh'

```

234 If everything is working correctly you should start to see the results of the
235 job being written to the file "errScript". You can inspect the progress during the
236 run using the *less* command.

```

237 less -S errScript

```

238 Should the run fail before completion, any associated error messages will be
239 printed to the errScript file. Once the job is complete, you should see the message
240 "GoRAMPAGE job is complete!" appear on the command-line terminal.

Inspecting the rRNA filtering results To evaluate the results from Step 3 (rRNA filtering), please navigate to the top level of the "output" directory and open the file "LOGFILES". You'll see the recorded progress of the program Tagdust and a record of the results. We notice that (for the L3h library) 1046448 of reads (78.1%) were "extracted", meaning that slightly more than 20% of reads were removed because of matches with ribosomal sequences. The removed reads from all libraries are found in the "dusted_discard" directory, and the extracted reads are found in the current directory. Due to their sheer abundance within cells, ribosomal RNA sequences are an inevitable contaminant within TSS profiling libraries. For analysis purposes, it is important that these sequences be removed, which is what has been completed here.

Since this step was conducted appropriately, we can proceed to the next step.

Evaluating the alignments The folder "alignments/" in your GoRAMAPGE output folder will now contain 6 .bam files, each representing the distinct RAMAPGE libraries selected for our analysis. Typing "ls -l" from the command line will show that these files are symlinks to the original alignment files found in the "STARoutput/" directory. "STARoutput/", as its name suggests, contains the output from the STAR alignment, and this includes the alignment files "*.sortedByCoord.out.bam", and four additional log files. The files with the suffix "*.STAR.Log.final.out" each contain a summary of the alignment, such as the number of input reads, the percentage of uniquely-mapped reads and the percentage of unmapped reads. An inspection of these log files indicates that the alignments have similar mapping rates (70-80%), a reasonable outcome for our purposes.

Now that our RAMPAGE libraries are filtered and aligned, we can commence with the second half of our analysis.

3.5 Promoter identification from aligned RAMPAGE libraries

We can now use the prepared alignment files to identify TSSs and promoters from the selected RAMPAGE libraries. There are currently several tools available for this purpose. *CAGEr*, developed by Haberle [17], was utilized to perform TSS identification as part of the FANTOM5 efforts. We will use *TSRchitect* in this demonstration, since it was specifically designed to analyze paired-end TSS profiling datasets, and also because it is more flexible with respect to model system (*i.e.* it does not require a corresponding *BSTGenome* package). The latter feature will be helpful when analyzing the non-*D. melanogaster* TSS profiling datasets that we expect to be generated in the near future.

Setting up the Analysis *TSRchitect*, the package we'll use for this analysis, is an R package available in the Bioconductor suite of genomics tools [18]. It makes use of existing packages and data structures within this environment, where available, to identify promoters from sequence alignments. Since you have

282 already installed *TSRchitect* and its dependencies (see section 2.3), we are set
 283 to proceed.

284 There are two general ways one can choose to run *TSRchitect*. The first is in-
 285 teractively *i.e.* typing the instructions directly into an R console. While this
 286 is a perfectly acceptable way to run analyses using package, for larger jobs
 287 it will likely be more efficient (and likely more reproducible) to run a dedi-
 288 cated R script. We have provided a sample script "MMB_chapter_TSRchitect.R"
 289 to make it easier for you to set up an R script. In the section to follow, we
 290 will go through the output of the analysis. For further details on how to use
 291 *TSRchitect*, please see its documentation at its Bioconductor page found here
 292 <https://www.bioconductor.org/packages/release/bioc/html/TSRchitect.html>.
 293

294 **Running the Analysis** To run *TSRchitect* using the batch script provided,
 295 first provide full paths for the variables "BAMDIR" and "DmAnnot" in "MMB_chapter_TSRchitect.R"
 296 using a text editor. *BAMDIR* should be a path to the subdirectory "alignments/"
 297 in RAMPAGE output directory you specified earlier, and *DmAnnot* should be
 298 a full path to the *D. melanogaster* gene annotation listed above. Once this is
 299 complete, we can run the batch script from the Linux command-line as follows:

```
300 R CMD BATCH MMB_chapter_TSRchitect.R
301 #assumes variables BAMDIR and DmAnnot have already been set
302 bg #puts this job in the background
```

303 Once the job is underway, you can monitor its progress by looking at the
 304 contents of the .Rout file (in this case, "MMB_chapter_TSRchitect.Rout"). The
 305 job should complete within an hour on most systems.
 306

307 Before we evaluate the results (which will have been written to your working
 308 directory after running the batch script), there are some important parameters
 309 to review. First, we must initialize the *tssObject* (which stores the information
 310 about the experiment) appropriately (see **Note 4**).
 311

312 The input in this case are BAM files (*inputType*="bam"); *TSRchitect* also
 313 accepts input in BED format.

```
314 DmRAMPAGE <- loadTSSobj(experimentTitle = "RAMPAGE Tutorial", \
315   inputDir=BAMDIR, inputType="bam", isPairedEnd=TRUE, \
316   sampleNames=c("E1h", "E2h", "E3h", "L1", "L2", "L3"), \
317   replicateIDs=c(1,1,1,2,2,2))
```

318 A critical step in our analysis is identifying TSRs from the aligned TSS
 319 data; to do this we use the function *determineTSR*. We have selected the job
 320 to run on 4 cores in this example (*n.cores*=4). Please enter the number of cores
 321 appropriate for your system. Because we want to identify TSRs from every one
 322 of the selected RAMPAGE libraries, we specify *tssSet*="all". The parameter
 323 *tagCountThreshold* was set to 25, meaning that only TSSs supported by 25 or

324 more 5' RAMPAGE reads will be included within a TSR. Setting *writeTable* to
 325 "TRUE" means that the identified TSRs from each set will be written to the
 326 working directory.

```
327 DmRAMPAGE <- determineTSR(experimentName=DmRAMPAGE, n.cores=4, tsrSetType="replicates", \
328 tssSet="all", tagCountThreshold=25, clustDist=20, writeTable=TRUE)
```

329 *TSRchitect* can incorporate the tag abundances from each of the samples
 330 and append them to the list of identified TSRs. This is useful for downstream
 331 analysis of differential expression.

```
332 DmRAMPAGE <- addTagCountsToTSR(experimentName=DmRAMPAGE,
333 tsrSetType="replicates", tsrSet=1, tagCountThreshold=10, \
334 writeTable=TRUE)
```

335 We can use *TSRchitect* to import an annotation file (or, alternatively, use an
 336 existing one from *AnnotationHub*) and use it to associate our set of identified
 337 TSRs with coding genes. We can specify the maximum distances (both up-
 338 and downstream) between the TSR and the annotation using the arguments
 339 *upstreamDist* and *downstreamDist*.

```
340 DmRAMPAGE <- importAnnotationExternal(experimentName=DmRAMPAGE, \
341 fileType="gff3", annotFile=DmAnnot)
342
343 DmRAMPAGE <- addAnnotationToTSR(experimentName=DmRAMPAGE,
344 tsrSetType="replicates", tsrSet=1, \
345 upstreamDist=1000, downstreamDist=200, feature="gene", \
346 featureColumnID="ID", writeTable=TRUE)
```

347 Now we have generated a set of identified TSSs, TSRs from all 6 RAMPAGE
 348 libraries, and have associated the identified TSRs with annotated genes. Next, we
 349 will merge the libraries into two samples according to condition: early embryonic
 350 (E1h, E2h, E3h) and late larval (L1, L2, L3) using the information we provided
 351 when we initialized the *tssObject* at the start of this section. After merging, we
 352 identify promoters i) within the merged samples and ii) within the entire dataset
 353 combined, and associate with the *D. melanogaster* gene annotation as described
 354 previously (not shown).

```
355 #merging the sample data into two groups
356 DmRAMPAGE <- mergeSampleData(DmRAMPAGE)
357
358 # ... identifying TSRs from the merged samples:
359 DmRAMPAGE <- determineTSR(experimentName=DmRAMPAGE, \
360 n.cores=4, tsrSetType="merged", \
361 tssSet="all", tagCountThreshold=40, \
362 clustDist=20, writeTable=TRUE)
```

Evaluating the results Our analysis using *TSRchitect* is now complete. For comparison, the example batch script we provide took just under 44 minutes to run.

Your working directory should now contain the following:

- TSSs from each sample *e.g.* TSSset-1.txt: (6)
- TSRs from each sample (in both .txt and .tab formats): (12)
- TSRs from each merged group (in both .txt and .tab formats): *e.g.* TSRsetMerged-1.txt: (4)
- TSRs from the combined set of TSSs: TSRsetCombined.tab: (1)

Let's briefly review the files. We can quickly obtain the counts on the command line, as follows:

```

wc -l *.tab
8377 TSRset-1.tab
6159 TSRset-2.tab
4814 TSRset-3.tab
17924 TSRset-4.tab
11851 TSRset-5.tab
3242 TSRset-6.tab
13986 TSRsetCombined.tab
7344 TSRsetMerged-1.tab
12126 TSRsetMerged-2.tab
85823 total

```

We will see that we have identified between roughly 3,200 and 18,000 TSRs within the individual RAMPAGE samples, which is attributable to the differences in library sizes. We detect 7,344 TSRs within the early embryonic samples ("TSRsetMerged-1.tab") and 12,126 TSRs in the late larval samples ("TSRsetMerged-2.tab"). Within the combined samples ("TSRsetCombined.tab") we find 13,986 TSRs, which is similar to the number reported by Hoskins *et. al.* [4].

In addition to identifying the position of a given TSRs, *TSRchitect* records other useful information about its properties. The *width* of a TSR refers the span of the genomic region it occupies (in bp), and the *Shape Index* (SI) is measure of the relative peakedness of the TSR. We can see an example of this in the file "TSRsetMerged-1.txt".

seq	start	end	strand	nTSSs	tsrWidth	shapeIndex	featureID
2L.67043.67044.+			2L	67043	67044 +	270 2	1 NA
2L.74089.74115.+			2L	74089	74115 +	341 27	0.13 NA
2L.94739.94752.+			2L	94739	94752 +	1650 14	0.55 FBgn0031
2L.102386.102386.+			2L	102386	102386 +	284 1	2 FBgn0031

403 3.6 Summary

404 The workflow provided here is intended to serve as a useful entry point for the
 405 analysis of TSS profiling data in insects. On the computational side, we have
 406 provided an open source set of tools so that the uninitiated genome scientist
 407 can begin to analyze RAMPAGE (or other forms of TSS profiling data) quickly.
 408 While the analysis centered on *D. melanogaster* via the use of public datasets,
 409 it is anticipated that this will assist groups who may be interested in performing
 410 TSS profiling in their preferred insect model system.
 411 The application of TSS profiling technology across a more representative sample
 412 of insect diversity will improve our understanding of the positions and general
 413 structure *cis*-regulatory regions in this phylum.

414 4 Notes

- 415 1. You can clone this appendix to your workspace on the command line using
 416 git, as follows:

```
417 git clone https://github.com/rtraborn/MMB_appendix.git
```

- 418 The "scripts/" folder in the Appendix contains code for you to run the two
 419 major workflows described in this chapter. The "additional_files/" folder
 420 contains the following files which are necessary for the analysis: i) a fasta file
 421 containing ribosomal RNA sequences for *D. melanogaster* (*Dmel_rRNA.fasta*)
 422 and ii) a gene annotation for *D. melanogaster* (*Drosophila_melanogaster.BDGP5.78.gff*).
 423 2. Since these fastq files are paired-end, we use the argument *-split-files* to
 424 generate separate files for each read pair.
 425 3. If you are running this on a cluster with a job scheduler you'll need to add
 426 the necessary headers to the top of the script and submit the job in the
 427 appropriate manner.
 428 4. Because the samples provided derive from related developmental stages, we
 429 will merge them for annotation purposes using the argument *replicateIDs*,
 430 (though it must be emphasized that they are not replicates).

431 Acknowledgments

432 The authors would like to thank Philippe Batut for generous technical as-
 433 sistance with the RAMPAGE protocol, and to Nathan Keith for his help
 434 establishing the protocol in our laboratory.

435 Disclosure Declaration

436 The authors declare that they have no competing interests.

5 References

References

1. J. T. Kadonaga, "Perspectives on the RNA polymerase II core promoter." *Wiley Interdisciplinary Reviews: Developmental Biology*, vol. 1, no. 1, pp. 40–51, Jan. 2012.
2. R. Kodzius, M. Kojima, H. Nishiyori, M. Nakamura, S. Fukuda, M. Tagami, D. Sasaki, K. Imamura, C. Kai, M. Harbers, Y. Hayashizaki, and P. Carninci, "CAGE: cap analysis of gene expression." *Nature Methods*, vol. 3, no. 3, pp. 211–222, Mar. 2006.
3. P. Carninci, T. Kasukawa, S. Katayama, J. Gough, M. C. Frith, N. Maeda, R. Oyama, T. Ravasi, B. Lenhard, C. Wells, R. Kodzius, K. Shimokawa, V. B. Bajic, S. E. Brenner, S. Batalov, A. R. R. Forrest, M. Zavolan, M. J. Davis, L. G. Wilming, V. Aidinis, J. E. Allen, A. Ambesi-Impimbato, R. Apweiler, R. N. Aturaliya, T. L. Bailey, M. Bansal, L. Baxter, K. W. Beisel, T. Bersano, H. Bono, A. M. Chalk, K. P. Chiu, V. Choudhary, A. Christoffels, D. R. Clutterbuck, M. L. Crowe, E. Dalla, B. P. Dalrymple, B. de Bono, G. Della Gatta, D. di Bernardo, T. Down, P. Engstrom, M. Fagiolini, G. Faulkner, C. F. Fletcher, T. Fukushima, M. Furuno, S. Futaki, M. Gariboldi, P. Georgii-Hemming, T. R. Gingeras, T. Gojobori, R. E. Green, S. Gustincich, M. Harbers, Y. Hayashi, T. K. Hensch, N. Hirokawa, D. Hill, L. Huminiecki, M. Iacono, K. Ikeo, A. Iwama, T. Ishikawa, M. Jakt, A. Kanapin, M. Katoh, Y. Kawasawa, J. Kelso, H. Kitamura, H. Kitano, G. Kollias, S. P. T. Krishnan, A. Kruger, S. K. Kummerfeld, I. V. Kurochkin, L. F. Lareau, D. Lazarevic, L. Lipovich, J. Liu, S. Liuni, S. McWilliam, M. Madan Babu, M. Madera, L. Marchionni, H. Matsuda, S. Matsuzawa, H. Miki, F. Mignone, S. Miyake, K. Morris, S. Mottagui-Tabar, N. Mulder, N. Nakano, H. Nakauchi, P. Ng, R. Nilsson, S. Nishiguchi, S. Nishikawa, F. Nori, O. Ohara, Y. Okazaki, V. Orlando, K. C. Pang, W. J. Pavan, G. Pavesi, G. Pesole, N. Petrovsky, S. Piazza, J. Reed, J. F. Reid, B. Z. Ring, M. Ringwald, B. Rost, Y. Ruan, S. L. Salzberg, A. Sandelin, C. Schneider, C. Schönbach, K. Sekiguchi, C. A. M. Semple, S. Seno, L. Sessa, Y. Sheng, Y. Shibata, H. Shimada, K. Shimada, D. Silva, B. Sinclair, S. Sperling, E. Stupka, K. Sugiura, R. Sultana, Y. Takenaka, K. Taki, K. Tammoja, S. L. Tan, S. Tang, M. S. Taylor, J. Tegner, S. A. Teichmann, H. R. Ueda, E. van Nimwegen, R. Verardo, C. L. Wei, K. Yagi, H. Yamanishi, E. Zabarovsky, S. Zhu, A. Zimmer, W. Hide, C. Bult, S. M. Grimmond, R. D. Teasdale, E. T. Liu, V. Brusic, J. Quackenbush, C. Wahlestedt, J. S. Mattick, D. A. Hume, C. Kai, D. Sasaki, Y. Tomaru, S. Fukuda, M. Kanamori-Katayama, M. Suzuki, J. Aoki, T. Arakawa, J. Iida, K. Imamura, M. Itoh, T. Kato, H. Kawaji, N. Kawagashira, T. Kawashima, M. Kojima, S. Kondo, H. Konno, K. Nakano, N. Ninomiya, T. Nishio, M. Okada, C. Plessy, K. Shibata, T. Shiraki, S. Suzuki, M. Tagami, K. Waki, A. Watahiki, Y. Okamura-Oho, H. Suzuki, J. Kawai, Y. Hayashizaki, F. Consortium, R. G. E. R. Group, and G. S. G. G. N. P. C. Group, "The transcriptional landscape of the mammalian genome," *Science (New York, NY)*, vol. 309, no. 5740, pp. 1559–1563, Sep. 2005.
4. R. A. Hoskins, R. A. Hoskins, J. M. Landolin, J. M. Landolin, J. B. Brown, J. B. Brown, J. E. Sandler, J. E. Sandler, H. Takahashi, H. Takahashi, T. Lassmann, T. Lassmann, C. Yu, C. Yu, B. W. Booth, B. W. Booth, D. Zhang, D. Zhang, K. H. Wan, K. H. Wan, L. Yang, L. Yang, N. Boley, N. Boley, J. Andrews, J. Andrews, T. C. Kaufman, T. C. Kaufman, B. R. Graveley, B. R. Graveley, P. J.

- 485 Bickel, P. J. Bickel, P. Carninci, J. W. Carlson, J. W. Carlson, S. E. Celniker,
486 and S. E. Celniker, "Genome-wide analysis of promoter architecture in *Drosophila*
487 *melanogaster*." *Genome Research*, vol. 21, no. 2, pp. 182–192, Feb. 2011.
- 488 5. E. A. Rach, H.-Y. Yuan, W. H. Majoros, P. Tomancak, and U. Ohler, "Motif
489 composition, conservation and condition-specificity of single and alternative tran-
490 scription start sites in the *Drosophila* genome." *Genome Biology*, vol. 10, no. 7, p.
491 R73, 2009.
- 492 6. B. Lenhard, A. Sandelin, and P. Carninci, "Metazoan promoters: emerging char-
493 acteristics and insights into transcriptional regulation." *Nature Reviews Genetics*,
494 vol. 13, no. 4, pp. 233–245, Apr. 2012.
- 495 7. T. Ni, D. L. Corcoran, E. A. Rach, S. Song, E. P. Spana, Y. Gao, U. Ohler,
496 and J. Zhu, "A paired-end sequencing strategy to map the complex landscape of
497 transcription initiation." *Nature Methods*, vol. 7, no. 7, pp. 521–527, Jul. 2010.
- 498 8. U. Ohler, G.-c. Liao, H. Niemann, and G. M. Rubin, "Computational analysis of
499 core promoters in the *Drosophila* genome." *Genome Biology*, vol. 3, no. 12, pp.
500 research0087.1–0087.12, 2002.
- 501 9. R. T. Raborn, K. Spitze, V. P. Brendel, and M. Lynch, "Promoter Architecture
502 and Sex-Specific Gene Expression in *Daphnia pulex*." *Genetics*, vol. 204, no. 2, pp.
503 593–612, Aug. 2016.
- 504 10. C. Nepal, Y. Hadzhiev, C. Previti, V. Haberle, N. Li, H. Takahashi, A. M. M.
505 Suzuki, Y. Sheng, R. F. Abdelhamid, S. Anand, J. Gehrig, A. Akalin, C. E. M.
506 Kockx, A. A. J. van der Sloot, W. F. J. van IJcken, O. Armant, S. Rastegar,
507 C. Watson, U. Strahle, E. Stupka, P. Carninci, B. Lenhard, and F. Muller, "Dy-
508 namic regulation of the transcription initiation landscape at single nucleotide res-
509 olution during vertebrate embryogenesis," *Genome Research*, vol. 23, no. 11, pp.
510 1938–1950, Nov. 2013.
- 511 11. P. Carninci, A. Sandelin, B. Lenhard, S. Katayama, K. Shimokawa, J. Ponjavic,
512 C. A. M. Semple, M. S. Taylor, P. G. Engström, M. C. Frith, A. R. R. For-
513 rest, W. B. Alkema, S. L. Tan, C. Plessy, R. Kodzius, T. Ravasi, T. Kasukawa,
514 S. Fukuda, M. Kanamori-Katayama, Y. Kitazume, H. Kawaji, C. Kai, M. Naka-
515 mura, H. Konno, K. Nakano, S. Mottagui-Tabar, P. Arner, A. Chesi, S. Gustincich,
516 F. Persichetti, H. Suzuki, S. M. Grimmond, C. A. Wells, V. Orlando, C. Wahlest-
517 edt, E. T. Liu, M. Harbers, J. Kawai, V. B. Bajic, D. A. Hume, and Y. Hayashizaki,
518 "Genome-wide analysis of mammalian promoter architecture and evolution," *Na-
519 ture Genetics*, vol. 38, no. 6, pp. 626–635, Apr. 2006.
- 520 12. S. Mwangi, G. Attardo, Y. Suzuki, S. Aksoy, and A. Christoffels, "TSS seq based
521 core promoter architecture in blood feeding Tsetse fly (*Glossina morsitans mor-
522 sitans*) vector of Trypanosomiasis," *BMC Genomics*, vol. 16, no. 1, p. 722, Sep.
523 2015.
- 524 13. K. Tsuchihara, Y. Suzuki, H. Wakaguri, T. Irie, K. Tanimoto, S.-i. Hashimoto,
525 K. Matsushima, J. Mizushima-Sugano, R. Yamashita, K. Nakai, D. Bentley, H. Es-
526 umi, and S. Sugano, "Massive transcriptional start site analysis of human genes in
527 hypoxia cells," *Nucleic Acids Research*, vol. 37, no. 7, pp. 2249–2263, Apr. 2009.
- 528 14. N. Cvetesic and B. Lenhard, "Core promoters across the genome," *Nature Biotech-
529 nology*, vol. 35, no. 2, pp. 123–124, Feb. 2017.
- 530 15. T. Lassmann, "TagDust2: a generic method to extract reads from sequencing data,"
531 *BMC Bioinformatics*, vol. 16, no. 1, p. 1, Jan. 2015.
- 532 16. A. Dobin and T. R. Gingeras, "Optimizing RNA-Seq Mapping with STAR," in
533 *Transcription Factor Regulatory Networks*. New York, NY: Springer New York,
534 Apr. 2016, pp. 245–262.

- 535 17. V. Haberle, “CAGEr: an R package for CAGE (Cap Analysis of Gene Expression)
536 data analysis and promoterome mining,” 2013.
- 537 18. M. Lawrence and M. Morgan, “Scalable Genomics with R and Bioconductor,”
538 *Statistical Science*, vol. 29, no. 2, pp. 214–226, May 2014.

539 6 Checklist of Items to be Sent to Volume Editors

540 Here is a checklist of everything the volume editor requires from you:

- 541 ☐ The final L^AT_EX source files
- 542 ☐ A final PDF file
- 543 ☐ A copyright form, signed by one author on behalf of all of the authors of the
544 paper.
- 545 ☐ A readme giving the name and email address of the corresponding author.