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Improving small RNA-seq: less bias and better detection of 2'-O-methyl RNAs
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Corresponding Author:	Erwin van Dijk I2BC Gif sur Yvette, AB FRANCE
Corresponding Author's Institution:	I2BC
Corresponding Author E-Mail:	Erwin.VANDIJK@i2bc.paris-saclay.fr
Order of Authors:	Erwin van Dijk
	Evangelia Eleftheriou
	Claude Thermes
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1   **TITLE:**
2   Improving Small RNA-seq: Less Bias and Better Detection of 2'-O-Methyl RNAs
3
4   **AUTHORS AND AFFILIATIONS:**
5   Erwin L. van Dijk, Evangelia Eleftheriou and Claude Thermes
6   Institute for Integrative Biology of the Cell, UMR9198, CNRS CEA Univ Paris-Sud, Université Paris-
7   Saclay, Gif sur Yvette Cedex, France
8
9   Corresponding Author:
10 Erwin L. van Dijk
11 erwin.vandijk@i2bc.paris-saclay.fr
12
13 Email Addresses of Co-authors**:**
14 Evangelia Eleftheriou (evangelia.eleftheriou@i2bc.paris-saclay.fr)
15 Claude Thermes (claude.thermes@i2bc.paris-saclay.fr)
16
17 **KEYWORDS:**
18 small RNA, small RNA-seq, bias, library preparation, next-generation sequencing, NGS, 2'-O-
19 methyl (2'-OMe) RNA, plant microRNA, plant miRNA
20
21 **SUMMARY:**
22 We present a detailed small RNA library reparation protocol with less bias than standard methods
23 and an increased sensitivity for 2'-O-methyl RNAs. This protocol can be followed using
24 homemade reagents to save cost or using kits for convenience.
25
26 **ABSTRACT:**
27 The study of small RNAs (sRNAs) by next-generation sequencing (NGS) is challenged by bias issues
28 during library preparation. Several types of sRNA such as plant microRNAs (miRNAs) carry a 2'-O-
29 methyl (2'-OMe) modification at their 3' terminal nucleotide. This modification adds another
30 difficulty as it inhibits 3' adapter ligation. We previously demonstrated that modified versions of
31 the 'TruSeq (TS)' protocol have less bias and an improved detection of 2'-OMe RNAs. Here we
32 describe in detail protocol 'TS5', which showed the best overall performance. TS5 can be followed
33 either using homemade reagents or reagents from the TS kit, with equal performance.
34
35 **INTRODUCTION:**
36 Small RNAs (sRNAs) are involved in the control of a diversity of biological processes[1]. Eukaryotic
37 regulatory sRNAs are typically between 20 and 30 nt in size; the three major types are microRNAs
38 (miRNA), piwi-interacting RNAs (piRNA) and small interfering RNAs (siRNA). Aberrant miRNA
39 expression levels have been implicated in a variety of diseases[2]. This underscores the importance
40 of miRNAs in health and disease and the requirement for accurate, quantitative research tools to
41 detect sRNAs in general.
42
43 Next-generation sequencing (NGS) is a widely used method to study sRNAs. Main advantages of
44 NGS as compared with other approaches, such as quantitative PCR or microarray techniques

45 (qPCR), are that it does not need a priori knowledge of the sRNA sequences and can therefore be
46 used to discover novel RNAs, and in addition it suffers less of background signal and saturation
47 effects. Further, it can detect single nucleotide differences and has a higher throughput than
48 microarrays. However, NGS also has some drawbacks; the cost of a sequencing run remains
49 relatively high and the multistep process required to convert a sample into a library for
50 sequencing may introduce biases. In a typical sRNA library preparation process, a 3' adapter is
51 first ligated to the sRNA (often gel-purified from total RNA) using a truncated version of RNA
52 ligase 2 (RNL2) and a preadenylated 3' adapter (**Figure 1**) in the absence of ATP. This increases
53 the efficiency of sRNA-adapter ligation and reduces the formation of side reactions such as sRNA
54 circularization or concatemerization. Subsequently, a 5' adapter is ligated by RNA ligase 1 (RNL1),
55 followed by reverse transcription (RT) and PCR amplification. All these steps may introduce
56 bias[3,4]. Consequently, read numbers may not reflect actual sRNA expression levels leading to
57 artificial, method-dependent expression patterns. Specific sRNAs may be either over- or
58 underrepresented in a library, and strongly underrepresented sRNAs may escape detection. The
59 situation is particularly complicated with plant miRNAs, siRNAs in insects and plants, and piRNAs
60 in insects, nematodes and mammals, in which the 3' terminal nucleotide has a 2'-O-methyl (2'-
61 OMe) modification[1]. This modification strongly inhibits 3' adapter ligation[5], making library
62 preparation for these types of RNA a difficult task.
63
64 Previous work demonstrated that adapter ligation introduces serious bias, due to RNA
65 sequence/structure effects[6-11]. Steps downstream of adapter ligation such as reverse
66 transcription and PCR do not significantly contribute to bias[6,11,12]. Ligation bias is likely due to the
67 fact that adapter molecules with a given sequence will interact with sRNA molecules in the
68 reaction mixture to form co-folds, that may either lead to favorable or unfavorable configurations
69 for ligation (**Figure 2**). Data from Sorefan et al[7] suggest that RNL1 prefers a single stranded
70 context, while RNL2 prefers a double strand for ligation. The fact that the adapter/sRNA co-fold
71 structures are determined by the specific adapter and sRNA sequences explains why specific
72 sRNA are over- or underrepresented with a given adapter set. It is also important to note that
73 within a series of sRNA libraries to be compared, the same adapter sequences should be used.
74 Indeed, it has previously been observed that changing adapters by the introduction of different
75 barcode sequences alters miRNA profiles in sequencing libraries[9,13].
76
77 Randomization of adapter sequences near the ligation junction likely reduces these biases.
78 Sorefan and colleagues[7] used adapters with 4 random nucleotides at their extremities,
79 designated "High Definition" (HD) adapters, and showed that the use of these adapters lead to
80 libraries that better reflect true sRNA expression levels. More recent work confirmed these
81 observations and revealed that the randomized region does not need to be adjacent to the
82 ligation junction[11]. This novel type of adapters was named "MidRand" adapters. Together, these
83 results demonstrate that improved adapter design can reduce bias.
84
85 Instead of modifying the adapters, bias can be suppressed through the optimisation of reaction
86 conditions. Polyethylene glycol (PEG), a macromolecular crowding agent known to increase
87 ligation efficiency[14], has been shown to significantly reduce bias[15,16]. Based on these results,
88 several "low bias" kits appeared on the market. These include kits that use PEG in the ligation

89 reactions, either in combination with classical adapters or HD adapters. Other kits avoid ligation
90 altogether, and use 3' polyadenylation and template switching for 3' and 5' adapter addition,
91 respectively[12]. In yet another strategy, 3' adapter ligation is followed by a circularization step,
92 thus omitting 5' adapter ligation[17].
93
94 In a previous study, we searched for a sRNA library preparation protocol with the lowest possible
95 levels of bias and the best detection of 2'-OMe RNAs[12]. We tested some of the above-mentioned
96 'low bias' kits, which had a better detection of 2'-OMe RNAs than the standard protocol (TS).
97 Surprisingly however, upon modification (the use of randomized adapters, PEG in the ligation
98 reactions and removal of excess 3' adapter by purification) the latter outperformed the other
99 protocols for the detection of 2'-OMe RNAs. Here, we describe in detail a protocol based on the
100 TS protocol, 'TS5', which had the best overall detection of 2'-OMe RNAs. The protocol can be
101 followed using reagents from the TS kit and one reagent from the 'Nf' kit or, to save money, using
102 homemade reagents, with equal performance. We also provide a detailed protocol for the
103 purification of sRNA from total RNA and the preparation of preadenylated 3' adapter.
104
105 **PROTOCOL:**
106
107 **1.   Isolation of small RNAs**
108
109 1.1.   Extract total RNA using phenol-based reagents or any other method. Verify if the RNA is
110 of good quality.
111
112 1.2.   Pre-run a 15% TBE-urea gel (see **Table of Materials**) for 15 min at 200 V.
113
114 1.3.   While the gel is pre-running, mix 5-20 µg of total RNA in a 5-15 µL volume with an equal
115 volume of formamide loading dye (see **Table of Materials**; 95% deionized formamide, 0.025%
116 bromophenol blue, 0.025% xylene cyanol, 5 mM EDTA pH 8) in a 200 µL PCR tube. Likewise, mix
117 10 µL (200 ng) of small-RNA ladder (see **Table of Materials**) with an equal volume of formamide
118 loading dye. Incubate for 5 min at 65 °C in a thermocycler with heated lid, then place the tubes
119 immediately on ice.
120
121 1.4.   Load the ladder and the sample on the same gel with at least one lane between them and
122 run at 200 V until the bromophenol has migrated about two-third of the gel length
123 (approximately 40 min).
124
125 1.5.   Prepare a system to elute the RNA from gel as follows: puncture the bottom of a nuclease-
126 free 0.5 mL micro centrifuge tube with a 21-gauge needle. Place the punctured 0.5 mL tube in a
127 nuclease-free round-bottom 2 mL micro centrifuge tube.
128
129 1.6.   Remove the gel, and incubate at room temperature with nucleic acid gel stain (see **Table
130 of Materials**) in water for 10-15 min.
131
132 1.7.   View the gel on a 'Dark Reader' trans illuminator (it is strongly recommended to avoid UV

as this might damage the RNA) and cut out the sample RNA between the 17 nt and the 29 nt ladder bands. Transfer the gel piece to the 0.5 mL tube from step 1.5.

1.8.   Centrifuge the 0.5 mL tube in the 2 mL tube in a micro centrifuge at maximum speed for 2 min. Remove the 0.5 mL tube, which should be empty now.

1.9.   Add 300 µL of nuclease-free 0.3 M NaCl to the 2 mL tube containing the crushed gel and rotate for at least 2 h at room temperature or at 4 °C overnight (16 h).

1.10.   Transfer the suspension of crushed gel pieces to a spin column (see **Table of Materials**) and centrifuge for 2 min at maximum speed in a micro centrifuge.

1.11.   Add 1 µL (20 µg/µL) of glycogen (see **Table of Materials**) and 950 µL of room temperature 100% ethanol. Incubate for at least 30 min at -80 °C.

1.12.   Centrifuge for 20 min at maximum speed in a micro centrifuge at 4 °C. Remove the supernatant, wash the pellet with 800 µL of cold 80 % ethanol. Centrifuge again for 5 min, and carefully remove all supernatant. Resuspend RNA pellet in 15 µL of nuclease free water. Typically, ~5-20 ng of small RNA should be recovered, depending on the amount of input total RNA (~1 ng of small RNA per 1 µg of input total RNA).

1.13.   **Optional step:** Check the quantity and quality of the recovered sRNA (e.g., by capillary gel electrophoresis using a small RNA kit; see **Table of Materials**).

2.   Preparation of preadenylated 3' HD adapter

NOTE: Preadenylation of 3' HD adapter was done in a manner similar to the protocol described by Chen et al[18]. Note that preadenylated adapter can be ordered directly (/5rApp/ modification), but this is quite expensive.

2.1.   Order 5' phosphorylated, 3' blocked 3' HD adapter oligonucleotide. See **Table 1** for sequence and modifications. Note that '3AmMO' is a 3' amino modifier group, most suppliers can produce oligonucleotide with this modification. Dilute in nuclease free water to 100 µM.

2.2.   Set up a 100 µL reaction containing the following reagents: 10 µL of oligo (100 µM), 10 µL of T4 RNA ligase buffer (10x), 10 µL of ATP (10 mM), 40 µL of 50% PEG8000, 5 µL of T4 RNA ligase 1 (50 units), 25 µL of nuclease-free water. Incubate overnight at 20 °C.

2.3.   Perform a classical phenol-chloroform extraction of the preadenylated oligonucleotide followed by ethanol precipitation. Add 100 µL of acid (pH 4.5) phenol:chloroform and vortex. Spin for 5 min at maximum speed. Carefully transfer 90 µL of the upper phase to a new tube; add 10 µL of 3 M sodium acetate pH 5.2, 1 µL of ultra-pure glycogen and 250 µL of cold 100% ethanol.

2.4.   Keep at -20 °C for at least 30 min. Centrifuge for 30 min at 4 °C maximum speed. Remove

177 the supernatant, wash the pellet once with cold 80% ethanol. As an alternative to phenol-
178 chloroform extraction and ethanol precipitation, a nucleotide removal kit can be used (see **Table**
179 **of Materials**). Resuspend in 10 µL of water or 10 mM Tris pH 8.
180
181 2.5. Verify the efficacy of preadenylation by migrating a small amount on a 15% TBE-urea gel
182 (**Table of Materials**) along with untreated oligonucleotide. The preadenylated adapter should
183 migrate slightly slower than untreated oligonucleotide. It is recommended to gel purify the
184 preadenylated adapter; proceed as described for the isolation of sRNA (steps 1.2-1.12).
185
186 **3. Library preparation – Protocol TS5**
187
188 NOTE: We present here the modified TS protocol 'TS5' that we described previously[12] and that
189 can be performed either with reagents from the kit or with self-provided reagents. It should be
190 noted that we obtained similar or even slightly better results with a different protocol, 'TS7'.
191 However, with TS7 it is more difficult to eliminate adapter dimers. We have therefore preferred
192 to describe TS5 in detail, but TS7 can be followed by simply replacing the adapters. For TS7 use
193 the 'MidRand-Like (MRL)' adapter sequences (**Table 1**). Note that here the randomized regions
194 are in the middle of the adapters. Primers for reverse transcription and PCR will hybridize to the
195 sequences downstream of the randomized region in the 3' adapter and upstream of the
196 randomized region in the 5' adapter. Sequencing will start from the first randomized nucleotide
197 in the 5' adapter.
198
199 3.1. **3' adapter ligation.**
200
201 3.1.1. Combine 1 µL of preadenylated 3' HD adapter (10 µM) with 1 µL of purified small RNA
202 (~0.1-1 µM) in a 0.2 mL micro centrifuge tube. Incubate for 2 min at 72 °C in a thermo cycler, then
203 put directly on ice.
204
205 3.1.2. Add 4 µL of 50 % PEG 8000 (viscous solution; pipet slowly), 1 µL of RNA ligase buffer (10x),
206 1 µL of H_2O, 1 µL of T4 RNA ligase2 truncated, and 1 µL of RNase inhibitor. Incubate overnight at
207 16 °C.
208
209 3.2. **Elimination of unligated 3' adapter**
210
211 3.2.1. Add 10 µL of nuclease-free water and mix well. Add 6 µL of 3 M NaOAc pH 5.2 or 'Adapter
212 Depletion Solution' from the Nf kit (**Table of Materials**) and mix well. Add 40 µL of magnetic
213 purification beads (**Table of Materials**) and 60 µL of isopropanol and mix well. Incubate for 5 min
214 at room temperature.
215
216 3.2.2. Put the sample in a magnetic rack until the solution appears clear. Remove and discard
217 the supernatant.
218
219 3.2.3. Add 180 µL of freshly prepared 80 % ethanol. Incubate for ~30 s, then remove. Take care
220 to use freshly prepared 80% ethanol and do not incubate with 80% ethanol for extended periods.

221

<mark>3.2.4. Briefly spin the tube and remove residual liquid that may have collected at the bottom of the well. Let the beads dry for 2 min, then resuspend in 22 µL of 10 mM Tris pH 8 or resuspension buffer from the Nf kit. Incubate for 2 min, then magnetize the sample until the solution appears clear.</mark>

<mark>3.2.5. Add 6 µL of 3 M NaOAc pH 5.2 to a new tube. Transfer 20 µL of the supernatant from the previous step to this new tube and mix by pipetting. Add 40 µL of magnetic beads and 60 µL of 100% isopropanol and mix well by pipetting. Incubate for 5 min.</mark>

<mark>3.2.6. Magnetize the sample until the solution appears clear, then remove and discard the supernatant.</mark>

<mark>3.2.7. Add 180 µL of freshly prepared 80 % ethanol. Incubate for ~30 s, then remove. Take care to use freshly prepared 80% ethanol and do not incubate for with 80% ethanol for extended periods.</mark>

<mark>3.2.8. Briefly spin the tube and remove residual liquid that may have collected at the bottom of the well. Let the beads dry for 2 min, and resuspend in 10 µL of nuclease-free water. Incubate for 2 min, then magnetize the sample until the solution appears clear.</mark>

<mark>3.2.9. Transfer 9 µL of supernatant to a new tube. Add 1 µL of T4 RNA ligase buffer (10x) and 1 µL of water or 2 µL of ligase buffer from the TS kit.</mark>

3.3. Ligation of 5' adapter.

3.3.1. Add 1 µL of 5' HD adapter (10 µM; **Table 1**) to a 200 µL PCR tube in a thermo cycler with heated lid. Incubate for 2 min at 70 °C, then put the tube directly on ice.

3.3.2. Add 1 µL of 10 mM ATP and 1 µL of T4 RNA ligase 1. Mix well by gently pipetting. Add 3 µL of this mix to the 3' ligated RNA from step 3.2.9 and mix by pipetting. Incubate for 1 h at 28 °C.

3.4. Reverse transcription (RT)

3.4.1. Transfer 6 µL of 3' and 5' adapter ligated RNA to a new 200 µL PCR tube (keep the remaining ~8 µL at -80 °C for later use if necessary). Add 1 µL of RT primer (10 µM) and mix by pipetting. Incubate for 2 min at 70 °C, then put the tube directly on ice.

3.4.2. Add the following reagents for RT: 2 µL of 5x first strand buffer, 0.5 µL of 12.5 mM dNTP mix, 1 µL of 100 mM DTT, 1 µL of RNase inhibitor, and 1 µL of reverse transcriptase (**Table of Materials**). Incubate for 1 h at 50 °C.

3.5. PCR amplification

265

266 3.5.1. Add the following reagents to the 12.5 µL of RT reaction mixture: 10 µL of PCR polymerase
267 buffer, 2 µL of universal P5 primer (10 µM), 2 µL of P7-index primer (10 µM), 1 µL of 12.5 mM
268 dNTPs, 0.5 µL of DNA polymerase, and 22 µL of water. Keep the reaction on ice until use.

269

270 3.5.2. Run the following PCR program: 98 °C for 30 s, 11 cycles (98 °C for 10 s, 60 °C for 30 s and
271 72 °C for 15 s) and 72 °C for 10 min. Keep the reaction at 4 °C when finished.

272

273 NOTE: Concerning the number of PCR cycles: we typically perform 11 cycles, but this should be
274 optimized by the user. Try to perform the smallest possible number of PCR cycles.

275

276 3.6. **Gel purification**

277

278 3.6.1. Run 5, 10 and 20 µL of PCR product on a native 6% TBE gel (see **Table of Materials**) along
279 with a suitable ladder (see **Table of Materials**). Run the gel for about 1 h (until the bromophenol
280 blue reaches the bottom; this dye migrates at the 65 bp position).

281

282 3.6.2. Remove the gel, and incubate with nucleic acid gel stain (see **Table of Materials**) in water
283 for 10-15 min.

284

285 3.6.3. View the gel on a "Dark Reader" trans illuminator (it is strongly recommended to avoid
286 UV as this might damage the RNA) and cut out the library band at 150 bp. Prepare a system to
287 elute the RNA from gel as described in step 1.5 and transfer the gel piece to the 0.5 mL tube.

288

289 3.6.4. Centrifuge in a micro centrifuge at maximum speed for 2 min. Remove the 0.5 mL tube,
290 which should be empty now.

291

292 3.6.5. Add 300 µL of nuclease-free water to the 2 mL tube containing the crushed gel and rotate
293 for at least 2 h at room temperature or at 4 °C overnight.

294

295 3.6.6. Transfer the suspension of crushed gel pieces in water to a spin column and centrifuge
296 for 2 min at maximum speed.

297

298 3.6.7. Add 1 µL of (20 µg/µL) glycogen, 30 µL of 3 M NaOAc and 975 µL of ice cold 100% ethanol.
299 Centrifuge for 20 min at max speed at 4 °C.

300

301 3.6.8. Resuspend the pellet in 20 µL of 10 mM Tris pH8. Use 1 µL for concentration measurement
302 and 1 µL for quality control.

303

304 **4. Data analysis**

305

306 NOTE: The data analysis procedure described below is based on the Linux operating system
307 Ubuntu 16.04 LTS.

308

309 **4.1. Treatment of raw sequence files**
310
311 4.1.1. Download the FASTQ sequence file(s) generated during the sequencing run. If required,
312 perform demultiplexing with bcl2fastq2 (version V2.2.18.12; a manual can be downloaded from
313 the   following   link:
314 http://emea.support.illumina.com/sequencing/sequencing_software/bcl2fastq-conversion-
315 software/documentation.html).
316
317 Use the following command:
318 *nohup **Pathway_of_bcl2fastq**/bcl2fastq --runfolder-dir **Pathway_of_Run** --ignore-missing-bcl --*
319 *output-dir **Pathway_of_Output_Directory** --barcode-mismatches 1 --aggregated-tiles AUTO -r 16*
320 *-d 16 -p 16 -w 16*
321
322 4.1.2. Remove adapter sequences using Cutadapt[19] version 1.15. A manual can be downloaded
323 here: https://cutadapt.readthedocs.io/en/stable/guide.html.
324 Use the following command:
325 ***Pathway_to_cutadapt/***cutadapt -a TGGAATTCTCGGGTGCCAAGG -n 5 -O 4 -m 10 -j 0 --nextseq-*
326 *trim 10 -o **Output_File_Read1_cutadapt**.fastq.gz **Input_File_Read1**.fastq.gz*
327
328 Note that the sequence in the command corresponds to the 3' HD adapter **without** the 4 random
329 nucleotides; these will therefore not be removed during this step.
330
331 4.1.3. Use seqtk (https://github.com/lh3/seqtk) for the removal of the terminal random
332 nucleotides in the sequencing reads. Use the following command (variable names in bold):
333 *seqtk trimfq -b 4 -e 4 **Output_File_Read1_cutadapt**.fastq > **Output_File_Read1_trimmed**.fastq*
334
335 4.1.4. Use the following awk command in order to discard the sequences shorter than 10 nt:
336 *awk 'BEGIN {FS = "\t" ; OFS = "\n"} {header = $0 ; getline seq ; getline qheader ; getline qseq ; if*
337 *(length(seq) >= 10) {print header, seq, qheader, qseq}}' **Output_File_Read1_trimmed**.fastq >*
338 ***Length_Filtered**.fastq*
339
340 **4.2. Mapping of the trimmed sequences**
341
342 4.2.1. Download the database corresponding to the organism of study from miRBase as follows.
343 Go to http://www.mirbase.org/ftp.shtml and download the 'mature.fa' file. Note that the
344 sequences are indicated in RNA notation. Replace the U residues by T with the following
345 command:
346 *sed -i '/^>/! s/U/T/g' mature.fa*
347
348 NOTE: This will yield a complete list of all miRNAs in miRBase, originating from a variety of
349 organisms.
350
351 4.2.2. Select the miRNA sequences of your organism of interest with the following command:
352

353 *awk   '/**name_of_the_organism**/{print;   nr[NR+1];   next};   NR   in   nr'   mature.fa   >*
354 *mature_**name_of_the_organism_**mirs.fa*

359 *bowtie2-build mature_**name_of_the_organism_**mirs.fa mature_**name_of_the_organism_**mirs*

363 *bowtie2 -N 0 -L 10 --score-min C,0,0 --end-to-end --time -x*
364 *mature_**name_of_the_organism_**mirs -U **Length_Filtered**.fastq -S*
365 ***Length_Filtered_ALIGNMENT**.sam*

367 NOTE: The option: *--score-min C,0,0* ensures that alignment is without any mismatches. For an
368 explanation of the various parameters in the tool, please visit the following website:
369 http://bowtie-bio.sourceforge.net/bowtie2/manual.shtml

372 *samtools view -F 4 **Length_Filtered_ALIGNMENT**.sam > **Reads_aligned_to_Mirs**.sam*

377 **REPRESENTATIVE RESULTS:**
378 Critical steps are the isolation of the small RNA fraction of the starting total RNA material (**Figure
379 3**) and the desired final library product (**Figure 4**). Both steps involve polyacrylamide gel
380 purification; small RNA is isolated from 15% TBE urea gels, while the final libraries are isolated
381 from 6% native TBE gels. Small RNA isolated from gel can be analyzed on a small RNA capillary
382 electrophoresis chip (**Table of Materials**; **Figure 3B**). This will allow users to estimate the amount
383 of small RNA recovered and the proportion of miRNA in the preparation.

385 Gel purification of the final library product is a delicate step as a number of additional products
386 are formed that migrate close to the desired library. It is important to not overload the gel as this
387 will increase the risk to contaminate the library with other species such as adapter dimers. As An
388 example (**Figure 4**), increasing amounts of PCR-amplified library (from *B. napus* RNA) were loaded
389 on the gel and the product corresponding to the expected size (150 bp) was cut out (**Figure 4A**).
390 After elution, the purified library was checked on a capillary gel electrophoresis chip; in addition
391 to the expected 150 bp product, an increasing proportion of a 130 bp species, corresponding to
392 adapter dimers was observed as increasing amounts of PCR product were loaded (**Figure 4B,C**).

394 We have tested if protocol TS5 performs similarly with homemade reagents as with reagent from
395 the kits. To this end, we prepared libraries from a mix of synthetic small RNAs 1-6, each present
396 without or with a 2'-OMe modification, as done in our previous study[12]. **Figure 5** shows the

proportion of reads corresponding to each of these RNAs obtained previously and with the new libraries made using reagents from the TS and Nf kits or from other suppliers. As can be seen, very similar results were obtained.

Figure 6 shows a comparison of the performance of protocol TS5 with the standard TS protocol for the detection of plant (*A. thaliana and B. napus*) miRNAs, which are 2'-OMe modified, and of unmodified human miRNAs. We also tested the detection of piRNAs, 2'-OMe modified in human samples. As can be seen, TS5 performs significantly better than TS for the detection of 2'-OMe RNAs but not for unmodified RNAs. However, also for unmodified sRNAs, even though not a larger number of RNAs are detected, the obtained read numbers probably better reflect true expression levels with TS5 than with TS due to lower levels of bias.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic representation of the sRNA library preparation workflow for Illumina sequencing. First, sRNAs are isolated by a gel purification step. Here, the size range of miRNAs is indicated but any other size range could be selected, depending on the RNAs of interest. A quality control (QC) step is then performed to check the quality and quantity of isolated sRNA. During library preparation, a preadenylated (App) 3' adapter is first ligated to the sRNA. Then, a 5' adapter is ligated. Subsequently reverse transcription is performed using a primer complementary to the 3' adapter, followed by PCR amplification, during which the Illumina P5, P7, and index ('In') sequences are added. The resulting library is gel purified, followed by a quality control step. Then the library is sequenced, and data are analyzed.

Figure 2. Bias due to sequence-dependent adapter-sRNA co-folding. In the adapter ligation mixture, adapters will cofold with sRNAs in a manner that depends on the sequence of the adapter and the sRNA. Thus, different sRNAs (illustrated by examples a, b, and c; indicated by different shades of green) will cofold differently with a given adapter (the 3' adapter is indicated in red, the 5' adapter is indicated in blue). The black arrows indicate ligation junctions. This may lead to a favorable or unfavorable context for ligation. As RNL2 appears to prefer a double stranded environment, 3' ligation is expected to be more efficient for RNAs a and b than for RNA c. With RNL1 having a preference for a single stranded region around the ligation junction, 5' adapter ligation may be most efficient for RNA a, followed by b and least efficient for c. Together this may result in an overrepresentation of sRNA a, an intermediate representation of RNA b and an underrepresentation of RNA c in the final library, even if the three RNAs are present at equal amounts in the original RNA sample. Note that in a similar fashion, a given sRNA will be represented differently when changing the adapter sequence (e.g., by adding different barcodes).

Figure 3. Isolation of small RNA and quality control. (A). Electrophoretic separation of *Brassica napus* total RNA (10 µg) on a 15% TBE urea denaturing polyacrylamide gel. A small RNA ladder (see **Table of Materials)** was migrated along as a molecular size marker. After migration the gel was stained and the RNA was visualized on a trans illuminator. The region from 17 to 29 nucleotides was cut out (indicated by a red rectangle) and RNA was eluted. **(B).** The quality of the purified RNA was checked by capillary gel electrophoresis. Note that this analysis provides

441 information on the proportion of miRNA in the sample (93% in this case).
442
443 **Figure 4. Gel purification of a *B. napus* small RNA library prepared following protocol TS5 and**
444 **quality control. (A).** Increasing amounts of a PCR amplified library from *B. napus* small RNA were
445 loaded on a 6% native TBE gel; 2.5 µL (a), 5 µL (b), 10 µL (c) or 20 µL (d) PCR product. A 50 bp
446 ladder was migrated alongside. PCR products migrating at the expected 150 pb position were
447 isolated (red rectangle), DNA was eluted and purified. **(B).** Quality control of the purified library;
448 gel representation. **(C).** Electropherogram representation of the same analysis. As can be seen,
449 the 150 pb product is increasingly contaminated with adapter dimers (~130 bp) as larger amounts
450 of PCR product are migrated on gel.
451
452 **Figure 5. Protocol TS5 performs similarly with reagents from the TS and Nf kits or with reagents**
453 **from other suppliers.** Histograms representing the percentage of the total numbers of raw reads
454 (before trimming) corresponding to RNA(OMe)1-6 with protocol TS5 followed with reagents from
455 the TS and Nf kits (blue bars), or reagents from other suppliers (orange bars). For comparison,
456 results from our previous study are shown by grey bars. The total numbers of reads
457 corresponding to RNA1-6 (*total RNA*) or RNA-OMe1-6 (*total RNA-OMe*) are shown as well. Shown
458 are the mean values of at least two independent experiments. Error bars represent standard
459 deviations. Part of this figure has been modified from Dard-Dascot et al, 2018[12]
460
461 **Figure 6. Comparison of miRNA detection of protocol TS5 with the classical TS method. (A).** The
462 proportion of reads mapping to *A. thaliana, B. napus,* or *H. sapiens* miRNAs in miRBase were
463 determined. We also mapped the reads of the human libraries to piRBase for piRNA detection.
464 Note that *A.thaliana* and *B.napus* miRNAs, as well as human piRNAs are 2'-OMe modified, in
465 contrast to human miRNAs. Shown are the mean values of at least two independent experiments
466 and the error bars represent standard deviations. **(B).** Numbers of miRNAs (or piRNAs) identified.
467 We determined the numbers of known miRNAs from the different species identified with
468 protocols TS or TS5. Note that for *A. thaliana, B. napus, or H. sapiens*, 427, 92, and 2588 miRNAs
469 have been registered in miRBase, respectively. 0.5 million reads from the TS or TS5 libraries were
470 mapped to *B. napus* miRNAs in miRbase, 1 million reads were mapped to the *A. thaliana* or
471 human databases. Shown are the mean values of at least two independent experiments with
472 standard deviations represented by error bars. This figure has been modified from Dard-Dascot
473 et al, 2018[12].
474
475 **Table 1. Oligonucleotides used with this protocol.**
476
477 **DISCUSSION:**
478 Small RNA library preparation remains challenging due to bias, mainly introduced during adapter
479 ligation steps. RNAs with a 2'-OMe modification at their 3' end such as plant miRNAs, piRNA in
480 insects, nematodes and mammals, and small interfering RNAs (siRNA) in insects and plants are
481 particularly difficult to study because the 2'-OMe modification inhibits 3' adapter ligation. A
482 number of solutions have been proposed in the literature to improve sRNA library preparation
483 protocols, but most commercially available kits are still based on the classical TS protocol, which
484 has severe bias. A few 'low bias' kits exist, however, including the Nf kit with randomized adapters

485　and PEG in the ligation reactions, and a few kits appeared recently that avoid adapter ligation
486　altogether[12]. We reported previously that Nf detects more different miRNAs than the standard
487　TS protocol, but protocol 'S' (without adapter ligation) performed relatively poorly due to a
488　significant formation of side-products[12]. Surprisingly, upon modification the TS protocols had a
489　more sensitive detection of 2'-OMe sRNAs than the other protocols, but not of normal sRNAs.
490　We chose here to describe in detail the TS5 protocol, in which the adapters are randomized at
491　their extremities, PEG is used in the ligation reactions and excess 3' adapter is eliminated by
492　purification on beads. It should be noted here that a different protocol (TS7), using MRL adapters
493　may perform slightly better than the TS5 protocol. However, as there are only minor differences
494　between the two and because with TS7, it is more difficult to separate the desired library product
495　from adapter dimers, we preferred here to describe in detail the TS5 protocol. However, users
496　can, if desired, replace the TS5 adapters by TS7 adapters. Note that these are slightly longer
497　leading to a final library product of ~170 bp rather than ~150 bp.

498

499　The possibility to perform protocol TS5 or TS7 with 'home-made' materials allows to substantially
500　reduce cost. However, there may be a larger variability in terms of quality with home-made
501　materials; especially home-made pre-adenylated 3' adapter may be subject to variable quality
502　due to varying efficacies of pre-adenylation. It is therefore recommended to prepare a large stock
503　and if a new stock is prepared, compare its performance with the previous one. A control RNA
504　sample can be used for this purpose.

505

506　A disadvantage of the protocols describe herein is the relatively strong formation of side-
507　products and the difficulty to separate the desired library from these products. Care must be
508　taken to not overload the acrylamide gel for purification. Recently, modified adapters were
509　developed that have a strongly reduced tendency to form dimers[21]. A 2'-OMe modification at the
510　3' end of the 5' adapter combined with a methylphosphonate modification at the 5' extremity of
511　the 3' adapter efficiently suppressed adapter dimers. It will be interesting to test such modified
512　adapters in the herein described protocol.

513

514　The gel purification steps in protocol TS5 are relatively labor-intensive. If the use of modified
515　adapters efficiently reduces the formation of adapter dimers, gel purification of the final library
516　may not be necessary anymore. In addition, as an alternative to the gel purification step to isolate
517　small RNA (step 1), a strategy using magnetic beads to enrich for small RNAs exists
518　(https://ls.beckmancoulter.co.jp/files/appli_note/Supplemental_Protocol_for_miRNA_.pdf).
519　We have not used this method ourselves, but it is worth testing and if it works well it could
520　significantly simplify the protocol.

521

522　In conclusion, while protocol TS5 could be further improved, it performs better than
523　commercially available kits, at least those tested in our previous comparative analysis, for the
524　detection of 2'OMe sRNA. It can be followed using home-made materials, allowing significant
525　cost reduction. For convenience, and perhaps more constant performance, reagents from the TS
526　and Nf kits can be used.

527

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DISCLOSURES:
The authors have nothing to disclose.

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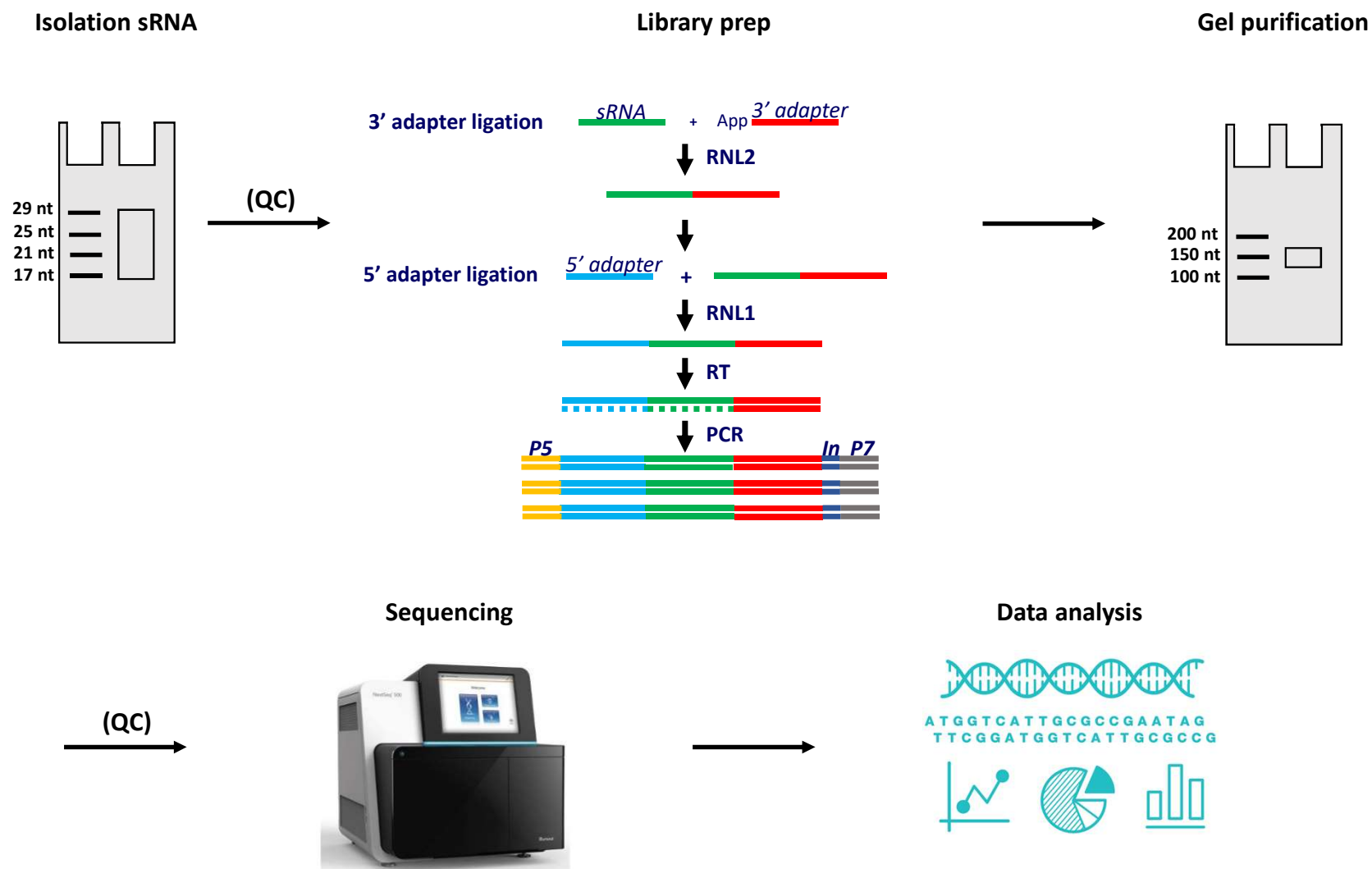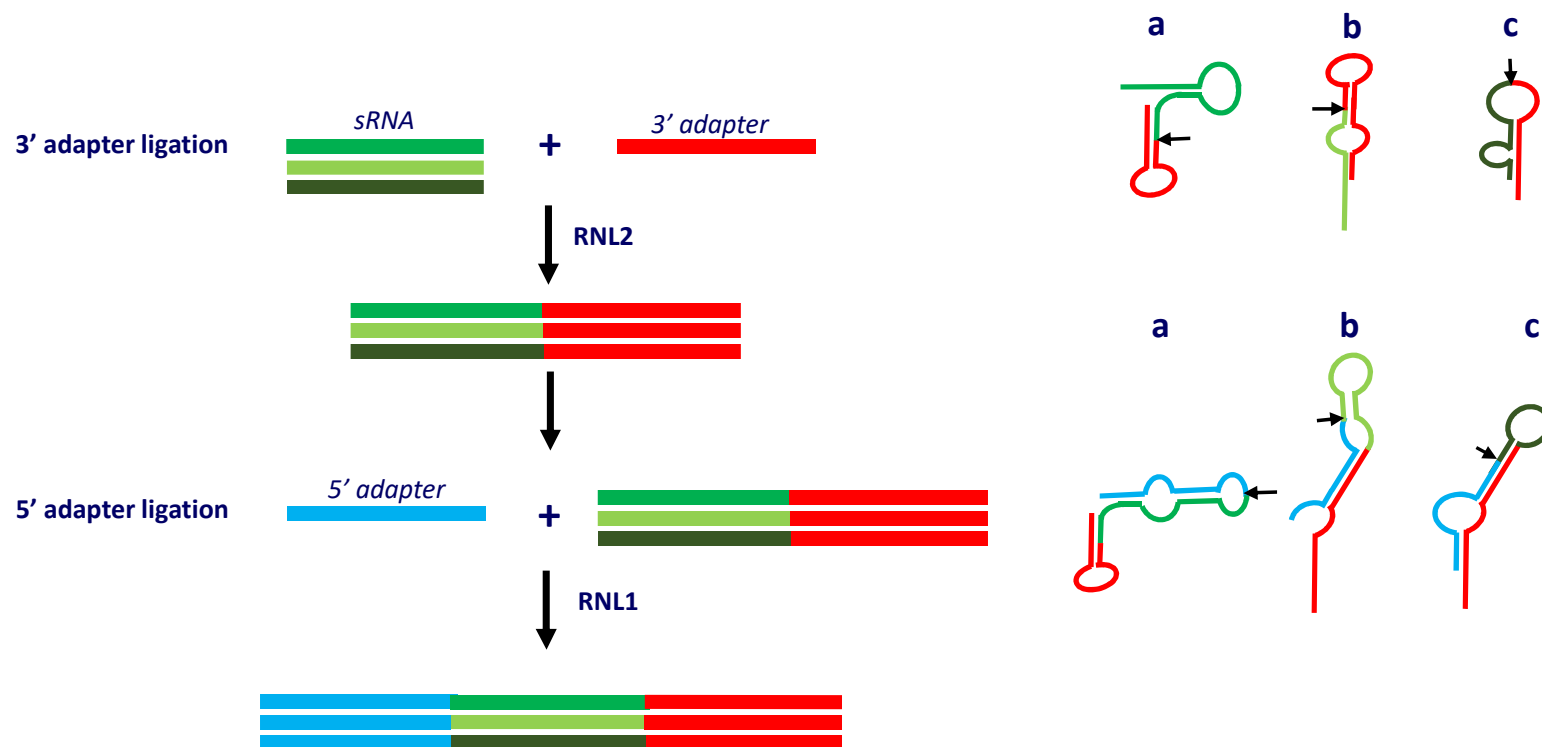596 doi:10.1371/journal.pone.0167009 [doi];PONE-D-16-33596 [pii] (2016).
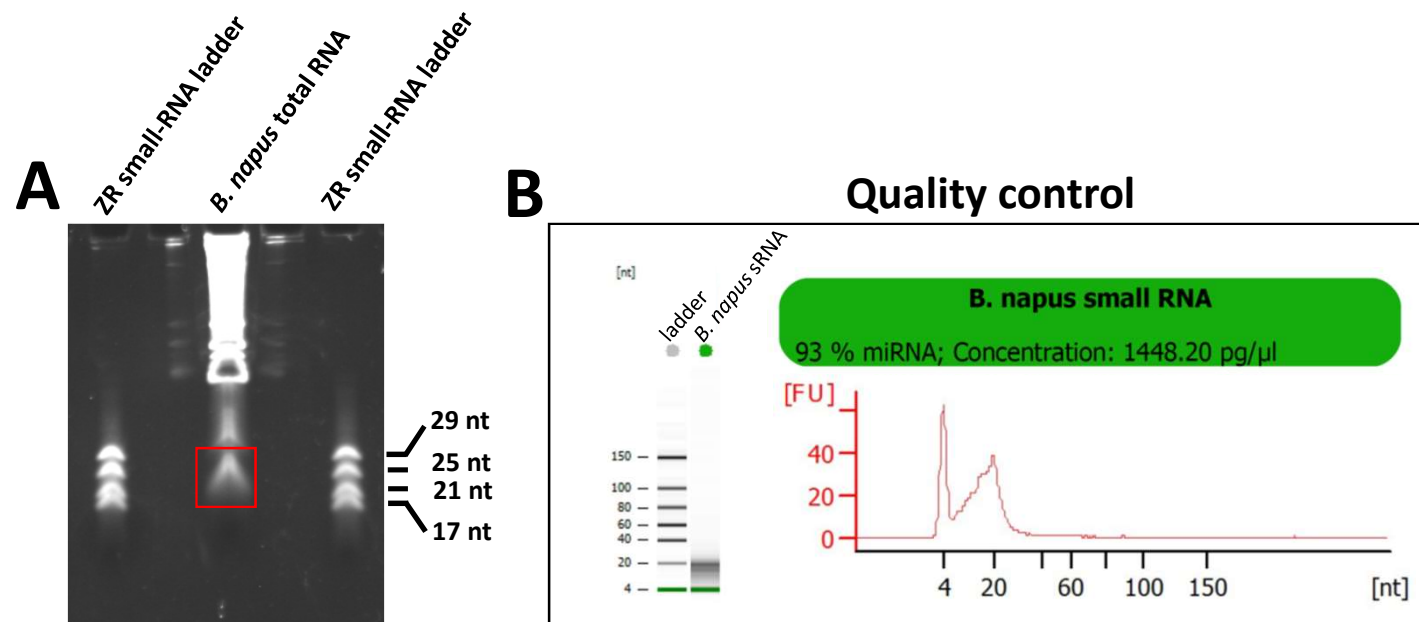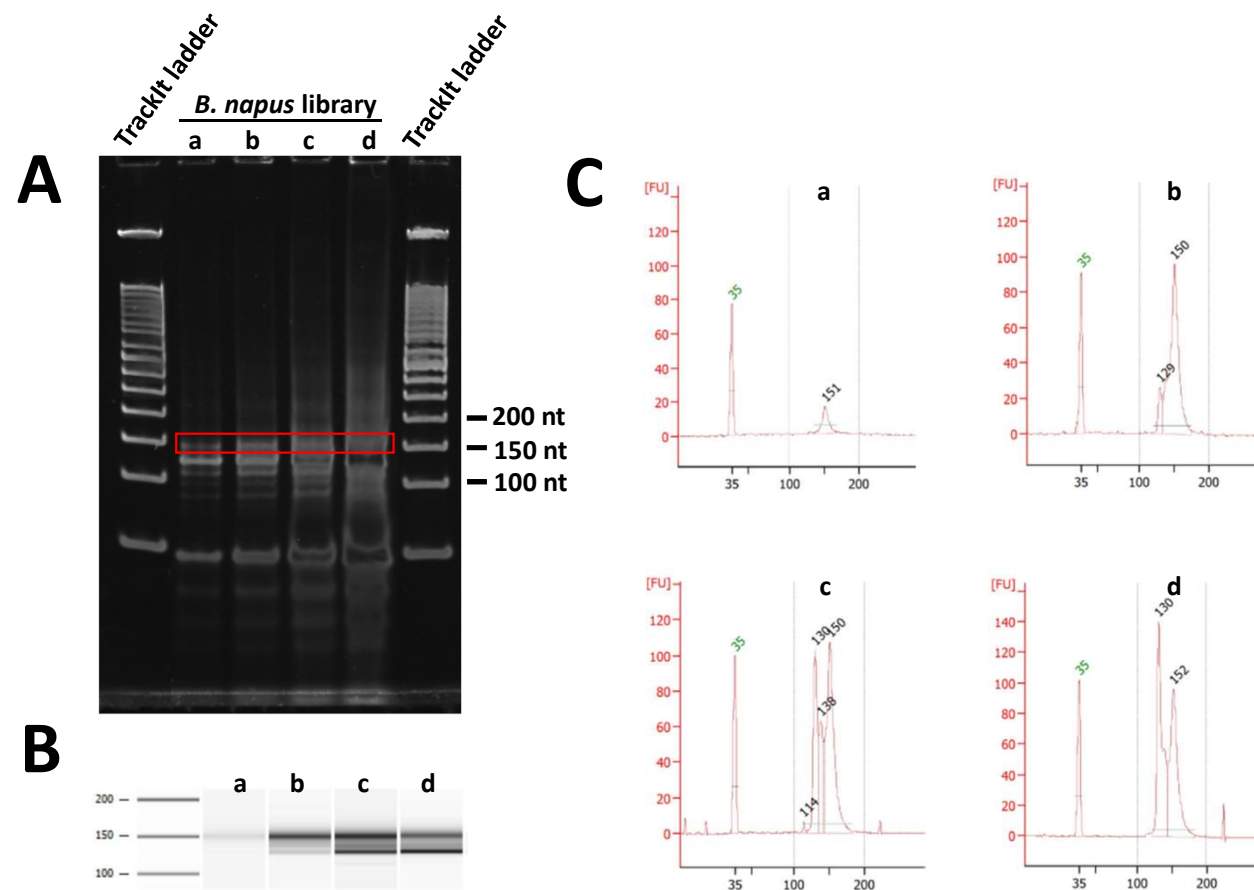597

Figure 1

Figure 1.

Figure 2

Figure 2

Figure 3

Figure 3.

Figure 4

Figure 4.
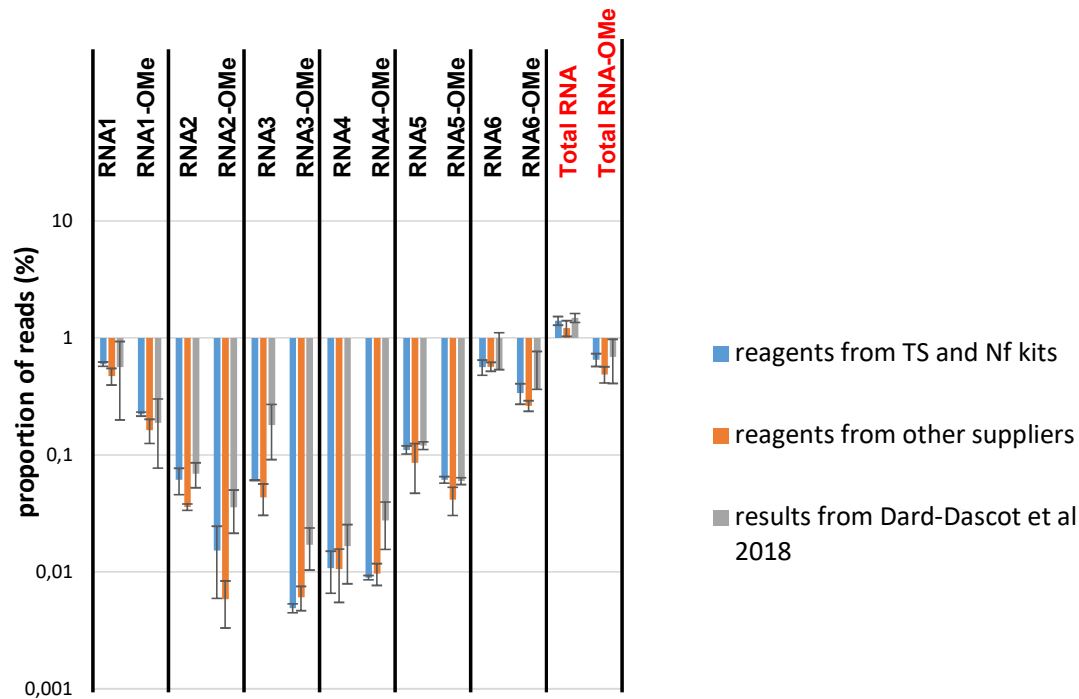
Figure 5

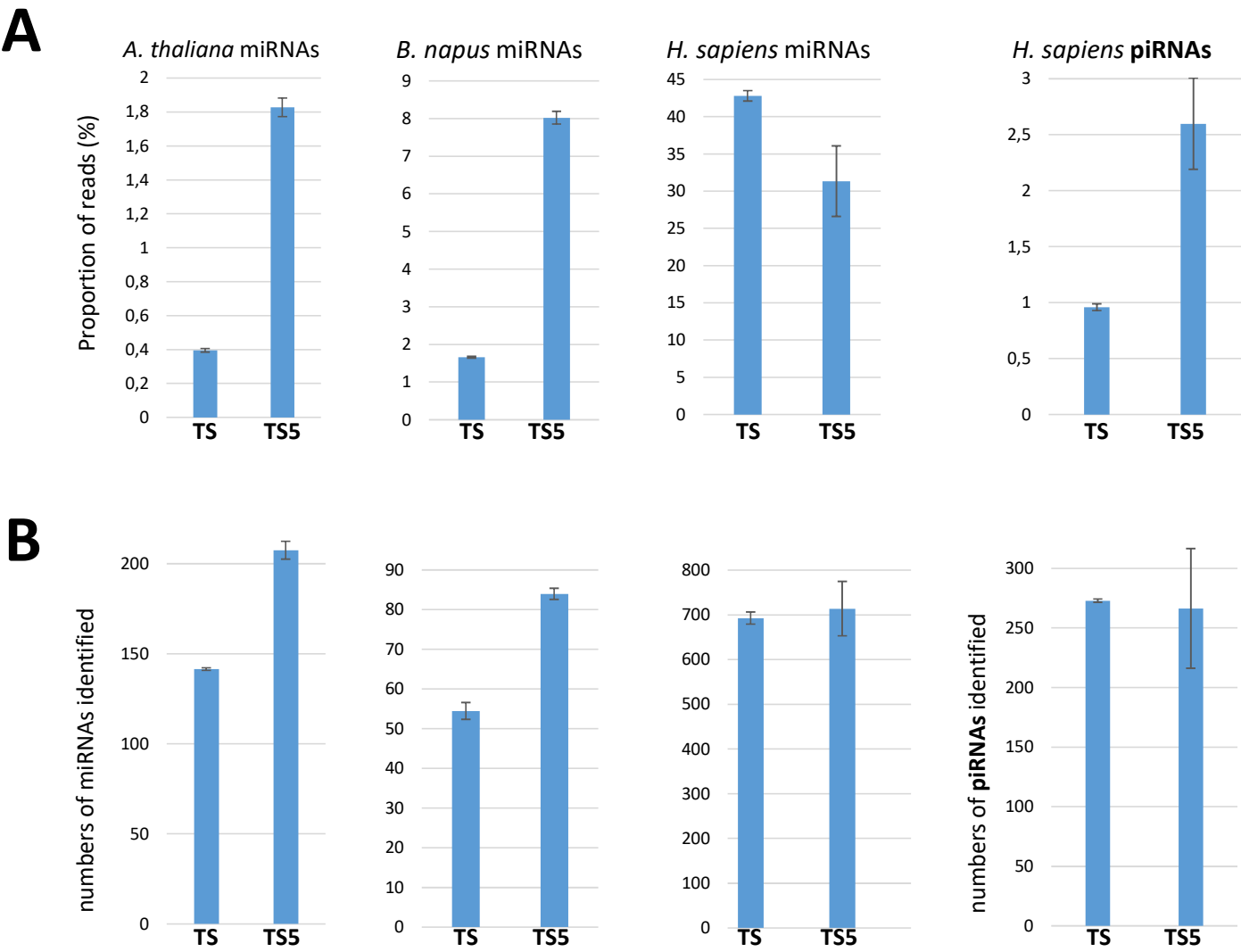**Figure 5.**

Figure 6

Figure 6.

Table

Table 1. Oligonucleotides required for this protocol

Name oligonucleotide	5' modification	3' modification	sequence 5' to 3'	purification
5' HD (TS5) adapter	5AmMC6		[5AmMC6]GTTCAGAGTTCTACAGTCCGACGATCNrNrNrN (note that this oligo is a DNA-RNA chimeric; the three 3' terminal nts are RNA)	HPLC
3' HD (TS5) adapter	phosphate	3AmMO	[Phos]rNrNrNrNTGGAATTCTCGGGTGCCAAGG[3AaMO] (note that this oligo is a DNA-RNA chimeric; the four 5' terminal nts are RNA)	HPLC
5' MRL (TS7) adapter	5AmMC6		[5AmMC6]GTTCAGAGTTCTACAGTCCGACGATCNNNNrArCrGrArUrArC (note that this oligo is a DNA-RNA chimeric; the seven 3' terminal nts are RNA)	HPLC
3' MRL (TS7) adapter	phospate	3AmMO	[Phos]GTATCGTNNNNNNTGGAATTCTCGG[3AmMO]	HPLC
RT primer			GCCTTGGCACCCGAGAATTCCA	HPLC
Universal P5 primer			AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA	HPLC
P7-index primer			CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA (NNNNNN = index)	HPLC

index 1: CGTGAT
index 2: ACATCG
index 3: GCCTAA
index 4: TGGTCA
index 5: CACTGT
index 6: ATTGGC
index 7: GATCTG
index 8: TCAAGT
index 9: CTGATC
index 10: AAGCTA
index 11: GTAGCC
index 12: TACAAG

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2100 Bioanalyzer Instrument	Agilent	G2939BA	
Acid-Phenol:Chloroform, pH 4.5 (with IAA, 125:24:1)	ThermoFisher	AM9720	
Adenosine 5'-Triphosphate (ATP)	Nex England Biolabs	P0756S	
Agencourt AMPure XP beads	Beckman Coulter	A63880	
Bioanalyzer High Sensitivity DNA Kit	Agilent	5067-4626	
Bioanalyzer Small RNA Kit	Agilent	5067-1548	
Corning Costar Spin-X centrifuge tube filters	Sigma Aldrich	CLS8162-96EA	
Dark Reader transilluminator	various suppiers		
HotStart PCR Kit, with dNTPs	Kapa Biosystems	KK2501	
NEXTflex small RNA-seq V3 kit	BIOO Scientific	NOVA-5132-05	optional
Novex TBE gels 6%	ThermoFisher	EC6265BOX	
Novex TBE Urea gels 15%	ThermoFisher	EC6885BOX	
QIAquick Nucleotide Removal Kit	Qiagen	28304	
Qubit 4 Quantitation Starter Kit	ThermoFisher	Q33227	
RNA Gel Loading Dye (2X)	ThermoFisher	R0641	
RNA Gel Loading Dye (2X)	ThermoFisher	R0641	
RNase Inhibitor, Murine	Nex England Biolabs	M0314S	
SuperScript IV Reverse Transcriptase	ThermoFisher	18090200	
SYBR Gold Nucleic Acid Gel Stain	ThermoFisher	S11494	
T4 RNA Ligase 1 (ssRNA Ligase)	Nex England Biolabs	M0204S	
T4 RNA Ligase 2, truncated	Nex England Biolabs	M0242S	
TrackIt 50 bp DNA ladder	ThermoFisher	10488043	
TruSeq Small RNA Library Prep Kit	Illumina	RS-200-0012/24/36/48	optional
UltraPure Glycogen	ThermoFisher	10814010	
XCell SureLock Mini-Cell	ThermoFisher	EI0001	
XCell SureLock Mini-Cell	ThermoFisher	EI0001	
ZR small RNA ladder	Zymo Research	R1090	
			the last two numbers correspond to the set of indexes

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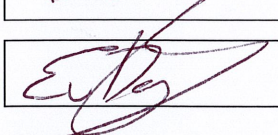
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CORRESPONDING AUTHOR

Name: _Erwin van Dyk_

Department: _I2BC_

Institution: _CNRS_

Title: _Dr._

Signature: _[signature]_   Date: _28/03/2019_

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612542.6   For questions, please contact us at submissions@jove.com or +1.617.945.9051.

Rebuttal letter 2.

We are grateful for the editorial comments and we have taken advantage of these to improve our manucript. Please find below our detailed responses to the comments:

1. We have carefully proofread the manuscript and we have corrected some remaining errors.
2. We acknowledge that the paper still contained a number of company names. We have now carefully removed all of these and we have checked if all materials required to perform the protocol are referenced in the Table of Materials.
3. Paragraph 3, lines 188-197: 'We present here…the 5' adapter.' The text here is not really a note but rather an introduction. We have therefore removed the word 'NOTE' at the beginning.
4. Step 1.6: incubation should be done at room temperature. This has been clarified in the description of the step.
5. We agree that step 2.3 was a bit long. We have therefore subdivided this step in three separate steps 2.3-2.5.
6. We have rewritten the requested sections to make sure original language is used.