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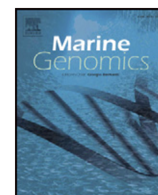

Method paper

High-quality RNA extraction from copepods for Next Generation Sequencing: A comparative study

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ABSTRACT

Despite the ecological importance of copepods, few Next Generation Sequencing studies (NGS) have been performed on small crustaceans, and a standard method for RNA extraction is lacking. In this study, we compared three commonly-used methods: TRIzol®, Aurum Total RNA Mini Kit and Qiagen RNeasy Micro Kit, in combination with preservation reagents TRIzol® or RNA*later*®, to obtain high-quality and quantity of RNA from copepods for NGS. Total RNA was extracted from the copepods *Calanus helgolandicus*, *Centropages typicus* and *Temora stylifera* and its quantity and quality were evaluated using NanoDrop, agarose gel electrophoresis and Agilent Bioanalyzer. Our results demonstrate that preservation of copepods in RNA*later*® and extraction with Qiagen RNeasy Micro Kit were the optimal isolation method for high-quality and quantity of RNA for NGS studies of *C. helgolandicus*. Intriguingly, *C. helgolandicus* 28S rRNA is formed by two subunits that separate after heat-denaturation and migrate along with 18S rRNA. This unique property of protostome RNA has never been reported in copepods. Overall, our comparative study on RNA extraction protocols will help increase gene expression studies on copepods using high-throughput applications, such as RNA-Seq and microarrays.
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1. Introduction

Copepods are the most abundant multicellular organisms on the planet, with extraordinary diversity in their morphologies, physiologies and life-strategies, forming an important link between phytoplankton and fish in the 'classic' pelagic food web (Humes, 1994; Runge, 1988). Through their life processes and vertical migrations, they play an important role in carbon transfer to the deep ocean and thus contribute to biogeochemical cycling (Frangoulis et al., 2005). Ultimately, they are also an emerging model system for ecotoxicological and environmental genomics studies (Raisuddin et al., 2007). Despite their global abundance and ecological importance, however, very few large-scale genomic resources exist for copepods.

With the recent development of Next Generation Sequencing (NGS) platforms (e.g. Illumina, 454, and SOLiD), it is now possible to address specific ecological and evolutionary questions in non-model organisms using transcriptome sequencing (RNA-Seq) (Ekblom and Galindo, 2011; Hudson, 2008). Although, several RNA-Seq studies have been performed on calanoids (Lenz et al., 2014; Ning et al., 2013), no information on the quantity or quality of the extracted RNA was given, making it difficult to ascertain the true efficiency of the method for isolating RNA from copepods.

To date, no systematic comparison of preservation and RNA isolation protocols for copepod RNA-Seq has been reported. Since copepod transcriptomics is a growing field and more studies are expected in the near future, it is critical to define a standard and reliable total RNA extraction protocol. The aim of the present study was to perform a comparative analysis of two preservation reagents, TRIzol® and RNA*later*®, and three RNA extraction methods: a GTPC separation method using TRIzol® reagent, and two widely-used Silica Membrane (SM) based commercial kits, Qiagen RNeasy Micro Kit (Qiagen) and Aurum Total RNA Mini Kit (BioRad), in the ubiquitous large-sized calanoid copepod *Calanus helgolandicus* to obtain high-quality total RNA for NGS. All methods were evaluated with regard to the quantity and quality of the isolated RNA, in terms of purity (measured as A260/280 and A260/230 ratios), and integrity (measured as RNA Integrity Number, RIN). After optimization on *C. helgolandicus*, the optimal protocol was also tested on the small-sized calanoid copepods *Centropages typicus* and *Temora stylifera*. Our results provide a standard protocol for isolating high quality copepod RNA for high-throughput NGS studies and will help increase the number of copepod genomic resources in the near future.

2. Material and methods

2.1. Sample collection

Zooplankton samples were collected weekly from April to May 2012 at a fixed coastal station in the Gulf of Naples (40.80°N, 14.25°E),

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with vertical hauls using a 200-μm mesh size plankton net, and samples were brought to the laboratory within 4 h. Live *C. helgolandicus* males and females (n = 100–200) were sorted from the sample under a stereomicroscope (Leica), kept for several days on a diet of the dinoflagellate *Prorocentrum minimum* and finally preserved for RNA extraction. Males and females of *C. typicus* and *T. stylifera* were collected similarly from the zooplankton sample in May 2012 and January 2014, respectively.

2.2. Sample preservation

Two different preservation techniques were tested on *C. helgolandicus* specimens (N = 5–15 individuals), according to the manufacturer's instructions: (i) immersion in 0.5 mL TRIzol® Reagent (Invitrogen, San Diego, CA, US) and flash freezing in liquid nitrogen, followed by immediate storage at −80 °C, and (ii) soaking in 0.5 mL RNA*later*® (Qiagen, Austin, TX, US) overnight at 4 °C, removal of excess reagent before subsequent storage at −80 °C. *C. typicus* and *T. stylifera* were collected similarly and stored in RNA*later*® at −80 °C.

2.3. RNA extraction

Three different RNA extraction protocols were tested and compared:

2.3.1. TRIzol® RNA extraction method

Copepods frozen in TRIzol® reagent were thawed on ice and homogenized with TissueLyser (Qiagen, Austin, TX, US) using 3 mm sterile aluminium beads at 20.1 Hz for 3 min and 2 min in succession. After centrifuging at 12,000 rpm for 10 min at 4 °C to remove debris, the supernatant was passed 5–6 times through a 0.1 mm syringe-needle. The RNA was then phase separated using chloroform, precipitated with an equal volume of isopropanol and washed with 75% ethanol following the manufacturer's instructions. Total RNA was suspended in 10 μL of 0.1% v/v diethylpyrocarbonate (DEPC)-treated water and stored at −80 °C. The phase separation step was also performed on previously homogenized copepods with TRIzol® before storage at −80 °C, but the results did not change (data not shown).

2.3.2. Qiagen RNeasy Micro Kit

The Qiagen RNeasy Micro Kit (Qiagen, Austin, TX, US) was used to process either samples preserved in TRIzol® or in RNA*later*®. Samples preserved in RNA*later*® were homogenized with a heat-sterilized Teflon micropestle in 350 μL of RLT buffer and 4 μL of β-mercaptoethanol. RNA was extracted following the manufacturer's protocol with on-column DNaseI treatment. To avoid RNA degradation, all centrifugations were carried out at 4 °C and samples were kept on ice during the entire procedure. The RNA was eluted in 14 μL of RNase-free water. Samples frozen in TRIzol® were processed similarly following homogenization by pestle and chloroform phase separation.

2.3.3. Aurum Total RNA Mini Kit

The Aurum Total RNA Mini Kit (BioRad, Hercules, CA, US) was also used to process samples preserved in TRIzol® or in RNA*later*®. Samples preserved in RNA*later*® were homogenized with a pestle and RNA was extracted following the manufacturer's protocol with on-column DNaseI treatment. Finally, RNA was eluted in 15 μL of elution buffer. Samples frozen in TRIzol® were processed similarly following homogenization by pestle and chloroform phase separation.

2.4. RNA quantity and quality determination

Quantity and quality (purity and integrity) of total RNA were assessed by NanoDrop (ND-1000 UV–vis spectrophotometer; NanoDrop Technologies Inc., Wilmington, DE, US), agarose gel electrophoresis, and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US). Purity was evaluated as A260/230 and A260/280 ratios. Integrity was assessed by running 100–200 ng of RNA sample in each lane of a 6000 Nano LabChip, using an Agilent Bioanalyzer 2100 system to obtain the RNA integrity values (RIN). We modified the standard sample preparation procedure as suggested in the Agilent 2100 Bioanalyzer manufacturer' instructions by omitting the RNA denaturation step at 70 °C for 2 min (Krupp, 2005).

2.5. RNA extraction from other copepod species

The optimal protocol was also tested on the smaller-sized copepods *C. typicus* and *T. stylifera*. RNA was extracted from whole *C. typicus* (60 animals) and *T. stylifera* (30 animals) specimens preserved in RNA*later*®. Samples were homogenized using a micropestle and RNA was extracted using the Qiagen RNeasy Micro Kit. The quality and quantity of RNA samples were assessed as described above.

2.6. Statistical analysis

The effect of different RNA extraction procedures on RNA quantity and quality (A260/280 and A260/230 ratios, and RIN values), was tested using the One-way analysis of variance (ANOVA), followed by a Tukey's post hoc pair-wise comparison test. All statistical analyses were performed using GraphPad PRISM v.4 software (San Diego, CA, US).

3. Results and discussion

Overall, we performed 55 extractions of total RNA from *C. helgolandicus*. Results are summarized in Table 1. Total RNA quantity has been indicated separately for males and females, since significantly higher amounts of RNA were obtained from females than males (540.2 ± 403.6 ng/female vs. 224.9 ± 203.9 ng/male, respectively) (unpaired *t*-test, $t = 3.831$ $df = 51$, $p < 0.001$). This difference is probably related to the females' large body size and active metabolism during reproduction, as suggested by Zhang and co-workers, who observed the same tendency in the copepod *Acartia hudsonica* (Zhang et al., 2013).

3.1. RNA quantity

Similar results were obtained for males and females, except for TRIzol method. Significantly higher quantity of RNA was, in fact, extracted using RNA*later* + Qiagen and TRIzol + Qiagen as compared to TRIzol + Aurum and RNA*later* + Aurum methods (One way ANOVA, $F_{4,33} = 29.12$, $p < 0.0001$, for females and $F_{4,20} = 18.83$, $p < 0.001$, for

Table 1
Quantity (ng/individual), purity (A260/230 and A260/280 ratios) and integrity (agarose gel electrophoretic profile and RIN values), of total RNA extracted from *Calanus helgolandicus* males and females using different extraction methods: TRIzol, TRIzol + Aurum, TRIzol + Qiagen, RNA*later* + Aurum and RNA*later* + Qiagen. Values represent mean ± SD. N represents number of samples analyzed. NA indicates that no values were assigned.

Extraction method	N	Quantity (ng/individual)		A260/230	A260/280	Gel profile		RIN
		Males	Females			18S	28S	
TRIzol	9	120.0 ± 60.2	819.3 ± 373.5	1.27 ± 0.48	1.81 ± 0.13	+	−	3.90 ± 1.13
TRIzol + Aurum	16	94.8 ± 60.6	108.8 ± 64.6	1.49 ± 0.66	1.99 ± 0.21	+	−	3.93 ± 1.19
TRIzol + Qiagen	10	486.8 ± 165.1	798.7 ± 215.5	2.47 ± 0.26	2.05 ± 0.04	+	−	NA
RNA*later* + Aurum	8	73.9 ± 20.1	128.5 ± 30.47	1.58 ± 0.61	2.06 ± 0.08	+	+	9.43 ± 0.53
RNA*later* + Qiagen	12	461.7 ± 53.7	865.4 ± 179.7	1.94 ± 0.40	2.02 ± 0.04	+	+	9.90 ± 0.14

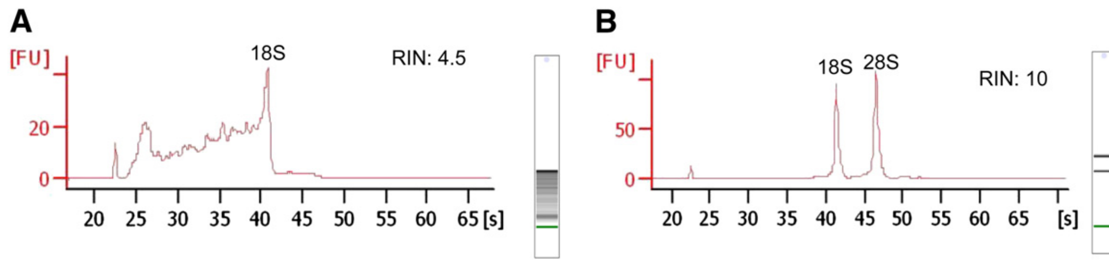

Fig. 1. Representative Agilent Bioanalyzer electropherograms and calculated RIN values of *Calanus helgolandicus* total RNA. Relative Fluorescent Unit (FU) and seconds of migration (s), of *C. helgolandicus* RNA sample isolated according to the (A) TRIzol and TRIzol + Aurum, (B) RNA*later* + Aurum and RNA*later* + Qiagen methods. For the TRIzol + Qiagen sample, RIN value was not calculated by the instrument software due to lack of 28S rRNA (see text).

males. Tukey's Multiple Comparison Test, p > 0.05) (Table 1). Thus, all extraction procedures, except combination with Aurum kit, yielded sufficient amounts of total RNA from as few as six females or ten males to construct cDNA libraries of *C. helgolandicus* for NGS (~5 μg of total RNA generally recommended by the Illumina protocol).

3.2. RNA purity

We did not detect significant differences in RNA purity (A260/230 and A260/280 ratios) between males and females (unpaired *t*-test, t = 1.377 df = 53, p = 0.174 and unpaired *t*-test, t = 0.391 df = 51, p = 0.698, for A260/230 and A260/280 ratios, respectively), which were therefore analyzed together (Table 1). Total RNA extracted with the TRIzol method had lower purity compared to the other treatments (One way ANOVA, $F_{3,46} = 11.76$, p < 0.0001 for A260/230, and One way ANOVA, $F_{4,52} = 5.392$, p < 0.001 for A260/280) (Table 1), thus, suggesting that commercial kits gave better purity than the TRIzol standard method. These results support the observations by Gayral et al. (2011) who suggested a combination of the GTPC and SM methods for the extraction of total RNA from non-model organisms, such as wax moth, nematode, oyster and nemertea for NGS application.

3.3. RNA integrity

Representative Bioanalyzer Agilent profiles showing electropherogram with low or high quality of total RNA extracted from *C. helgolandicus*, together with corresponding RIN values, are shown in Fig. 1. Total RNA from *C. helgolandicus* extracted with the TRIzol and TRIzol + Aurum method showed a single peak corresponding to the 18S rRNA at 42 s, absence of a 28S rRNA peak, a high amount of small size RNA occurring between 25 s and 42 s and RNA degradation with a very low RIN value (RIN: 4.5) (Fig. 1A). RNA extracted with the TRIzol + Qiagen method also lacked a sharp 28S rRNA peak, though no small sizes of RNA were observed before 42 s (data not shown). Comparable results were observed in agarose gel electrophoresis (Table 1). A similar finding was recently reported in insects and molluscs, and was probably related to the denaturing effect of the TRIzol® reagent towards the 28S rRNA (Gayral et al., 2011).

The integrity of *C. helgolandicus* RNA significantly improved with the whole-body copepods preservation in RNA*later*® reagent, with very high RIN value of 9.4 and 10 for Aurum and Qiagen kits, respectively (Fig. 1B). Such high RIN values are considered suitable for NGS analysis (Perez-Portela and Riesgo, 2013). Additionally, agarose gel electrophoresis analysis showed two distinct bright bands corresponding to 18S and 28S rRNA for both procedures (Table 1). Overall, RIN values obtained with the RNA*later* + Aurum (9.4 ± 0.53) and RNA*later* + Qiagen (9.9 ± 0.14) protocols were significantly higher than those obtained with the TRIzol (3.9 ± 1.13) and TRIzol + Aurum (3.9 ± 1.2) methods (Table 1) (One way ANOVA, $F_{3,14} = 40.39$, p < 0.001, Tukey's Multiple Comparison Test, p < 0.001). Total RNA extraction using RNA*later* + Qiagen procedure has been previously used for microarray hybridization studies in the copepods *Calanus finmarchicus* (Lenz et al., 2012), and *Lepeophtheirus salmonis* (Eichner et al., 2008), or for transcriptome sequencing of *C. finmarchicus* (Christie et al., 2013) and *Calanus sinicus* (Ning et al., 2013). The Qiagen RNeasy Kit has been also used for total RNA extraction from other crustaceans such as krill for 454 sequencing (Clark et al., 2011). Moreover, this RNA*later*®-based method also provides an effective alternative for preserving copepods during field experiments, when no flash freezing of animal samples in liquid nitrogen is possible.

Intriguingly, loading these RNA*later* + Aurum and RNA*later* + Qiagen extracted RNA samples onto the Agilent Nanochip after heat denaturation at 70 °C for 2 min, as usually recommended (Krupp, 2005), resulted in the disappearance of the 28S rRNA peak from the electropherograms (Fig. 2). A similar lack of a 28S rRNA peak was recently reported in the insect *Apis mellifera* (Winnebeck et al., 2010), the crustacean *Artemia parthenogenetica* and the planarian *Dugesia japonica* (Sun et al., 2012), and was related to the 'hidden break' present in protostome 28S rRNA (Ishikawa, 1977). Upon heat-denaturation, the two fragments of 28S rRNA separate and migrate with the 18S rRNA (Winnebeck et al., 2010). Thus, we suggest omitting the heat denaturation step of copepod RNA prior to loading onto the Agilent Nanochip should be incorporated into the routine procedure to assess RNA integrity for these non-model organisms.

3.4. RNA extraction from other copepod species

Total RNA extracted from *C. typicus* (n = 60) and *T. stylifera* (n = 30) samples using the RNA*later* + Qiagen protocol had a concentration of 195.53 ± 69.29 ng/individual and 208.91 ± 37.96 ng/individual,

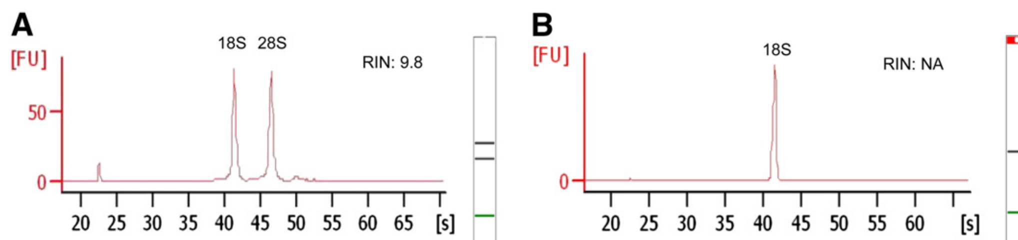

Fig. 2. Agilent Bioanalyzer electropherograms of total RNA extracted from *Calanus helgolandicus* females with RNA*later* + Qiagen method. RNA without heat-denaturation (A) and with heat-denaturation (B), with the latter lacking the 28S rRNA peak.

respectively. The RNA extracts from *C. typicus* and *T. stylifera* had a very high purity, accordingly to the A260/230 (2.51 ± 0.19 and 2.61 ± 0.04, respectively) and A260/280 ratios (2.09 ± 0.04 and 2.02 ± 0.01, respectively), as well as integrity. The gel electrophoresis profile showed two bright bands for 18S and 28S rRNA (data not shown) and the Agilent profile for RNA samples from *C. typicus* and *T. stylifera* showed two distinct peaks corresponding to 18S and 28S rRNA, with RIN values of 9.8 and 10, respectively (Similar to Fig. 1B). These results suggest a wider application of this method to other copepod species as well.

4. Conclusion

To our knowledge, this is the first systematic assessment of different protocols for extraction of total RNA from copepods which is suitable for Next Generation Sequencing technologies. We were able to optimize extraction of high quality and quantity RNA from the copepods *C. helgolandicus, C. typicus* and *T. stylifera* by a combination of whole-body preservation with RNA*later*®, followed by homogenization using a micropestle, and extraction of total RNA using the Qiagen RNeasy Micro Kit.

Author contributions

S. A. and Y. C. designed and performed the experiments and drafted the manuscript. C. L. helped in RNA extraction and copepod sorting. Y. C. and A. I. conceived the study and revised the manuscript along with P.K.L. All authors have read and approved the final manuscript.

Additional information

The authors declare that they have no competing financial interests.

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