Optimizing preservation protocols to extract high-quality RNA from different tissues of echinoderms for next-generation sequencing

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Abstract

Transcriptomic information provides fundamental insights into biological processes. Extraction of quality RNA is a challenging step, and preservation and extraction protocols need to be adjusted in many cases. Our objectives were to optimize preservation protocols for isolation of high-quality RNA from diverse echinoderm tissues and to compare the utility of parameters as absorbance ratios and RIN values to assess RNA quality. Three different tissues (gonad, oesophagus and coelomocytes) were selected from the sea urchin *Arbacia lixula*. Solid tissues were flash-frozen and stored at −80 °C until processed. Four preservation treatments were applied to coelomocytes: flash freezing and storage at −80 °C, RNA later and storage at −20 °C, preservation in TRIzol reagent and storage at −80 °C and direct extraction with TRIzol from fresh cells. Extractions of total RNA were performed with a modified TRIzol protocol for all tissues. Our results showed high values of RNA quantity and quality for all tissues, showing nonsignificant differences among them. However, while flash freezing was effective for solid tissues, it was inadequate for coelomocytes because of the low quality of the RNA extractions. Coelomocytes preserved in RNA later displayed large variability in RNA integrity and insufficient RNA amount for further isolation of mRNA. TRIzol was the most efficient system for stabilizing RNA which resulted on high RNA quality and quantity. We did not detect correlation between absorbance ratios and RNA integrity. The best strategies for assessing RNA integrity was the visualization of 18S rRNA and 28S rRNA bands in agarose gels and estimation of RIN values with Agilent Bioanalyzer chips.

Keywords: coelomocytes, RIN, RNA extraction, sea urchin, transcriptome

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Introduction

During the last years, as a consequence of global warming, ocean acidification and pollution increase, the study of stress response in animal species has become a pivotal subject within the scientific community (Kassahn et al. 2009). In this sense, echinoderms have been ideal models for monitoring marine environmental hazards (Dupont et al. 2010) because they are keystone species, which often act as ecosystem engineers and play an important role within food chains in most oceans around the world (Harrold & Reed 1985; Wangensteen et al. 2011). Among the diversity of tissues in echinoderms, coelomocytes have been selected as biomarkers to study stress response on adults because of their prompt response to stressors (Matranga et al. 2000, 2005; Pinsino et al. 2008). These cells, present in the coelomic fluid within the body cavity of adult echinoderms, are recognized as the immune effectors. Environmental stressors as temperature shift, UV radiation, pollutants and pH decrease can reduce protective capacity of coelomocytes and induce activation of the heat shock protein 70 (hsp70) expression (Matranga et al. 2000, 2012; Pinsino et al. 2008), but the massive sequencing of the coelomocyte transcriptome under the influence of different stressors has not been taken so far, because the extraction of messenger RNA proved challenging, among other reasons.

The rapidly decreasing costs of high-throughput sequencing are currently pushing the boundaries of the applications of short reads (either from genomic or transcriptomic origin) in all fields (Collins et al. 2008; Riesgo et al. 2012a). Transcriptomic information is used in a wide range of studies and provides fundamental insights into biological processes and applications (Surget-Groba & Montoya-Burgos 2010), but its analysis involves complementary DNA (cDNA) library construction from total...
or messenger RNA of usually large numbers of samples. For some cases, the extraction of RNA proves as one of the most challenging steps of the whole library construction processes (Gayral et al. 2011; Hillyard & Clark 2012; Riesgo et al. 2012b). In these cases, the optimization of the extraction protocol is essential for ensuring the required amount of RNA (depending on the protocol) with the adequate RNA integrity, which is the main requirement for subsequent retrotranscription of RNA into cDNA. Most protocols involve some sort of preservation of the RNA, because immediate RNA extraction is not always possible. Since recently, flash freezing of tissue or cell pellets and preservation in RNA later are among the most frequent preservation methods for animals. However, for certain tissues, those preservation strategies have proved to be subideal (Hillyard & Clark 2012; Riesgo et al. 2012b). Therefore, protocol optimization is often crucial to ensure further procedures with critical samples.

The extraction of RNA of coelomocytes, with the further aim of analysing the stress response of adults at the transcriptomic level, was a defiant process, because protocols usually performed for other echinoderm tissues were not adequate. Therefore, we established two objectives for our study: (i) to optimize preservation and storage methods to isolate high-quality RNA from different tissues of echinoderms, an animal group extensively used as a model system in research, and (ii) to test accuracy for two different measures of RNA quality, absorbance ratios (A260/280 and A260/230) and RIN values (see below in Material and Methods). This study looked at two important parameters of the RNA extraction, concentration and quality.

Material and methods

Sample and tissue collection

Thirty-two specimens of Arbacia lixula, a common sea urchin in the Mediterranean Sea and Atlantic Ocean, were collected by snorkelling or SCUBA diving at Punta Santa Anna, Blanes (41°40′22.47″N, 2°48′10.81″E, Northwestern Mediterranean) and maintained in an aquarium for a few hours until processed. Samples from three different tissue types: coelomocytes from the coelomic fluid, gonads and oesophagus (digestive) were collected from the sea urchins.

Coelomic fluid was withdrawn from the body cavity with sterile syringes (21-gauge needle) through an insertion in the peristomial membrane. Syringes were pre-loaded with 5 mL of cold anticoagulant buffer composed of 80% CM-ASW (Ca²⁺/Mg²⁺ free sea water, artificially made in DEPC-treated water) and 20% EDTA stock solution (13.53 g/L) (see Matranga et al. 2012). Approximately 10 mL of the cell suspension containing 15–10 × 10⁶ coelomocytes cells was immediately centrifuged at 12 000 g for 6 min at 4 °C, and a pellet of coelomocytes recovered. The coelomocyte cells were then preserved following four different treatments: (i) flash freezing in liquid nitrogen and immediate stored at −80 °C (LN₂), (ii) immersion in 2 mL of RNA later (Qia-gen, www.qiagen.com) for 12 h at 4 °C and overnight incubation at −20 °C (RNA later), (iii) pellet fixation in 1 mL of TRIzol Reagent (Invitrogen, www.invitrogen.com) and storage at −80 °C (TRIzol −80 °C) for 24 h and (iv) pellet fixation in 1 mL of TRIZol Reagent for immediate extraction of total RNA (TRIzol) (see Table 1 and Table S1, Supporting information).

Tissue samples from the gonads and digestive tract were dissected out of the animals, flash-frozen in liquid nitrogen and stored at −80 °C until processed. Tissue extraction was always performed with sterilized razor blades and forceps rinsed with RNase AWAY (Sigma-Aldrich, www.sigmaaldrich.com) to avoid RNA degradation.

### Table 1 Tissue type, preservation treatment, number of samples analysed (n), RIN value, RNA concentration (ng/µL) and profile features

| Tissue       | Preservation          | N | RIN     | Concentration (ng/µL) | Profile features                                      |
|--------------|-----------------------|---|---------|-----------------------|-------------------------------------------------------|
| Coelomocytes | LN₂                   | 10| 1.0–7.2 | 210–1524              | Good quantity but very low quality. Degraded RNA mRNA for cDNA library construction |
| Coelomocytes | TRIzol −80 °C         | 6 | 9.1–9.7 | 34–1650               | High variability in quantity but good quality         |
| Coelomocytes | TRIzol                | 10| 8.3–10  | 220–1022              | Good quantity and quality                             |
| Gonad        | LN₂                   | 6 | 8.3–10  | 388–1680              | Good quantity and quality                             |
| Digestive    | LN₂                   | 3 | 8.6–9.4 | 424–852               | Good quality and quality                             |

RNA extraction

Besides the coelomocyte pellets, approximately 20 mg of gonad tissue or oesophagus (the whole length) was used for extraction of total RNA. For samples preserved in liquid nitrogen and stored at −80 °C, two different methods of extraction were tested for best suitability in echinoderm samples: (i) for direct extraction of poly(A)
mRNA, we used the Dynabeads® mRNA DIRECT™ Kit (Invitrogen) following the manufacturer’s instructions and (ii) for total RNA extraction, we used an optimized TRIzol protocol. Due to the high viscosity of the tissue and cell samples, the direct mRNA extraction could not be successfully accomplished, and RNA extraction was always performed using TRIzol.

Either fresh or frozen tissues were homogenized with micropestles in 1 mL of TRIzol. One BCP (1-bromo-3-chloropropane) extraction was performed using 0.2 mL, followed by precipitation in 0.5 mL of isopropanol plus 1 μL of RNaseOUT (Invitrogen), and overnight incubation at −20°C. Total RNA was then precipitated and pelleted using a 15 min centrifugation (16 000 g) at 4 °C, then the pellet washed twice in 75% ethanol with previous centrifugations (16 000 g) for 5 min at 4 °C and redissolved in 55 μL RNaše-free water plus 1 μL of RNaseOUT. To avoid RNA degradation, the whole extraction protocol was developed on ice.

Integrity of total RNA was initially evaluated by visualizing the 28S rRNA and 18S rRNA bands into a 1% agarose gel in 1 × TAE Buffer. In addition, RNA has an absorbance maximum at 260 nm, and the ratio of the absorbance at 260 and 280 nm and 260 and 230 nm has been used to assess the RNA purity. An A260/230 ratio has been also used to estimate the presence of contaminants, while A260/280 ratio was used to estimate the purity of RNA (Riesgo et al. 2012b). Absorbance ratios A260/230 and A260/280 and concentration of our extractions were assessed in a Hellma spectrophotometer (Hellma Analytics). An RNA sample is considered ‘pure’ when values for the A260 and A260/230 are between 1.8 and 2.2, and concentration over 200 ng/μL is considered acceptable, according to the manufacturer’s instructions of the kit used for isolation of mRNA (TruSeq RNA sample preparation kit from Illumina Inc.). RNA extractions were finally run in an Agilent 2100 Bioanalyzer (Agilent Technologies) at the Scientific and Technical Services of the University of Barcelona for quality measurements. RNA integrity was measured using the RIN value, which is calculated based on the comparison of the areas of 18S rRNA and 28S rRNA, the height of the 28S rRNA peak and the fast area ratio (Schroeder et al. 2006), with 1 being the most degraded profile and 10 being the most intact. RIN values over 8 were considered nondegraded usable RNA extractions.

Statistical analyses

The total number of samples consisted of 41 extractions from three different tissue types and four different treatments for coelomocytes (Table 1).

We first investigated whether measures of RNA quality, absorbance ratios (A260/230 and A260/280) and RIN value variables were correlated in our data. Because RIN did not follow a normal distribution (Shapiro–Wilk test: W = 0.7593, P < 0.001), even after we applied the logarithmic transformation to the original values (Shapiro–Wilk test: W = 0.6169, P < 0.001), a nonparametric Spearman’s correlation coefficient was applied. To test for differences in the RNA concentration of different tissues and treatments, we initially used a two-way ANOVA, after confirming normality and homoscedasticity of the dependent variable. One-way ANOVA was also applied to evaluate the effect of the treatments on the RNA concentration from coelomocytes. We also investigated if RNA quality, measured as RIN values, depended on either the tissue type or the different treatments (here considering only the coelomocytes) by Kruskal–Wallis nonparametric analyses.

Distribution of the variables RIN and RNA concentration was graphically represented in boxplots for different treatments in coelomocytes. Statistical analyses and boxplots were performed using the software ‘R v. 2.15.2’.

Results

The Spearman’s coefficients did not detect correlation between RNA quality variables based on absorbance ratios (A260/280 and A260/230) and RIN values (ρ = 0.498 and ρ = 0.7496, respectively; P < 0.001). In some samples with A260/280 and A260/230 ratios over 2.0, we observed RIN values lower than 8 (see examples in Fig. 1) (Table S1, Supporting information), showing that the 28S rRNA and 18S rRNA peaks were close to intact, but the fast region (mRNA) and the 5.8S rRNA were completely degraded (Fig. 2).

We did not detect significant differences in RNA quantity and quality for the three tissues analysed. RNA concentration was not significantly different among tissues (ANOVA, F = 7.4, P > 0.05). Both digestive and gonad tissues presented good values of concentration and RIN for further mRNA isolation. RIN values did not either display significant differences between the three tissue types (Kruskal–Wallis, H = 1.6549, P > 0.05). The high variability of RIN values in coelomocytes was mostly due to the different preservation treatments applied (see below).

For coelomocytes, we observed significant differences in both RNA quality, based on RIN values (Kruskal–Wallis, H = 18.45, d.f. = 3, P < 0.001), and quantity (ANOVA, F = 5.548, P = 0.004) depending on the treatment applied (Fig. 3). Flash freezing of coelomocytes provided high RNA concentrations but degraded RNA for most samples. RIN values for flash-frozen samples were between 1 and 7.2, which were significantly lower than those obtained from ‘TRIzol’ and ‘TRIzol −80 °C’ treatments (see Fig. 3 and Table S1, Supporting informa-
Fixation in RNA later resulted in variable values of RIN (from 5.6 to 9.0, with median about 8). The concentration of RNA later samples was significantly lower than that of all the other treatments (from 22 ng/µL to 70 ng/µL) (Fig. 3 and Table S1, Supporting information). We did not observe significant differences in RNA quality and quantity between ‘TRIzol’ and ‘TRIzol/C0 80°C’ treatments (Kruskal–Wallis, H = 16.32, P > 0.05; ANOVA, H = 1.119, P > 0.05 for quality and quantity, respectively), but there was a wider variability in RNA concentration values in the ‘TRIzol –80 ºC’ treatment. In agarose gels, the quality of RNA samples varied greatly among treatments (Fig. 2). While samples flash-frozen in liquid nitrogen presented degraded RNA with no visible 18S rRNA and 28S rRNA bands and a wide smear in the fast region, the samples preserved in TRIzol (whether or not conserved at –80 ºC) showed the sharpest and cleanest bands for 18S rRNA and 28S rRNA. For the RNA later
preserved samples, the quantity was so low (always below 70 ng/μL) that hampered the visualization of the bands using standard agarose electrophoresis (Fig. 2).

Discussion
Assessment of RNA quality can be performed measuring different features: overall degradation through visualization of 18S rRNA and 28S rRNA bands in a standard agarose gel, A260/280 and A260/230 ratios, and estimation of the RIN value (Gayral et al. 2011; Hillyard & Clark 2012; Riesgo et al. 2012b). In our samples, the most efficient strategies for assessing the RNA integrity were the electrophoresis in agarose gels and the estimation of the RIN value using Agilent Bioanalyzer chips. For coelomocytes, there were no consistent correlations between the RNA integrity and the A260/280 and A260/230 ratios. This could be due to the different stability of the RNAs, being the ribosomal RNA more stable than the mRNA (Houseley & Tollervey 2009). Then, even though the mRNA might be degraded, the A260/280 could still render values around 2 due to the intact nature of the ribosomal RNA. If working with coelomocytes, it would be important to assess the RNA integrity using bioanalyzer profiles, because in this case, the bioanalyzer profile would show degradation in the fast and 5.8S rRNA regions.

All tissues extracted during the study contained enough RNA amounts to further construct cDNA libraries for next-generation sequencing technologies. However, the preservation method needed to be adjusted in the case of coelomocytes to obtain good-quality RNA. Undegraded RNA was successfully extracted with TRIzol from flash-frozen digestive and gonad tissues, as occurred in other flash-frozen solid tissues or biological fluids of other nonmodel invertebrates (Santiago-Vázquez et al. 2006; Pinsino et al. 2008; Gayral et al. 2011; Simister et al. 2011; Hillyard & Clark 2012; Riesgo et al. 2012a,b). However, that was not the case for coelomocytes of Arbacia lixula. Flash-frozen coelomocytes rendered considerable RNA amounts with very low quality (estimated using RIN values and observed also in agarose gels). Coelomocytes are cells containing a rich selection of lysosomal enzymes (Stabili et al. 1994; Haug et al. 2002), among which RNases may be present. Therefore, cell lysis should be avoided to prevent RNA degradation by the echinoderm own RNases. During sample freezing, cell lysis can occur when the produced microcrystals break the cellular membranes; hence, although flash freezing is advisable for solid tissues in general, it should be avoided when dealing with coelomic fluids unless an RNase inhibitor is added to the fluid. One solution for preventing cell lysis is the use of imidazole, which is commonly added to the anticoagulant buffer used for withdrawal of coelomic fluid in other echinoderms (Gross et al. 1999). Imidazole inhibits the activity of lysosomal enzymes (such as lysozyme) (Shintzky et al. 1966), and therefore, cell lysis is prevented.

Another solution equally effective in maintaining the RNA integrity is the use of TRIzol reagent in freshly collected cells, because it contains high concentrations of guanidine thiocyanate and acid phenol to inhibit RNase activity. The advantages of using TRIzol rely on the absence of other foreign substances that could interpose in the subsequent procedures.

RNA extraction from fresh tissues is used in many cases with success (Gross et al. 1999; Matranga et al. 2003; Pinsino et al. 2008), but sometimes, field or laboratory conditions do not allow for direct extraction upon collection. We demonstrated here that the best option for preservation and storage of RNA from coelomocytes, when the direct extraction could not be performed, is the combination of preservation in TRIzol and storage at −80 °C for long periods. In this case, large variability in the concentration of RNA recovered should be taken into account.

When working in the field, sometimes freezers are not even available, and another strategy of preservation might be required. RNA later has been proved to be a reliable preservative for RNA in a wide array of tissues (Gayral et al. 2011; Hillyard & Clark 2012), although unadvised for animal cells and fluids. Unexpectedly, in fluids such as urine, and sperm, the addition of RNA later to the cell pellet improved the RNA yield (Medeiros et al. 2003; Das et al. 2010), thus providing a promising perspective for coelomocyte preservation. However, coelomocyte pellets preserved in RNA later yielded limited amounts of RNA, similar to the results obtained for human blood (Weber et al. 2010). Therefore, when large amounts of RNA (larger than 200 ng/μL) are needed, the use of RNA later as a preservative is unadvised when dealing with fluids containing phagocytic cells.

In conclusion, flash freezing is an adequate method of RNA preservation for solid tissues in echinoderms. For coelomocytes, extraction of freshly collected cell pellets rendered the best results in terms of quantity and quality of RNA. If direct extraction cannot be performed, the most reliable preservation method is the immersion of the coelomocyte cell pellets in TRIzol and subsequent storage at −80°C.

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