

*Short communication*

## Optimal Preservation and Storage Regimes of Total RNAs from Different Fish Tissues

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### ABSTRACT

Standardized and optimized methods in preservation and storage of RNA samples on three different tissues were elucidated on fish tissues. The treatments used included excision of fresh tissues, preservation of tissues by immersing completely in RNAlater followed by immediate freezing at  $-80^{\circ}\text{C}$  prior to RNA assays. Current data suggests that preserving fish tissues in RNAlater with subsequent storage at  $-80^{\circ}\text{C}$  produced satisfactory amount of RNA from dorsal fin and gonad, but not from muscle tissues. The optimized method will provide an alternative storage method option and enables greater usage of RNAlater for preserving RNA from animal cells.

*Keywords:* Fish tissues, preservation, RNA, RNAlater, storage

### INTRODUCTION

The advent of molecular techniques has increased the number of researches using DNA and RNA in bulk. The RNA, which is closely related to protein synthesis, is

widely used in studies related to genomics and proteomics. RNA can be assayed from different parts of any organism either through conventional method or by using readily available kits (Deng et al., 2005; Junttila et al., 2009). The RNA extraction kits, for instance are usually optimized to enhance the quality and amount of RNA being assayed. High quality of RNA is essential and is a prerequisite for many downstream applications such as microarray analysis (Copoys et al., 2007), gene expression (Shabihkhani et al., 2014) and other nucleic acid-based technologies.

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It is well known that RNases, which is ubiquitous, are very aggressive and can rapidly digest significant amounts of RNA (Escobar & Hunt, 2017). Therefore, sample preparation, preservation and storage free from RNases are critical for determining the quality of RNA. Traditional method to deactivate the activity of RNases is usually through deep freezing of samples with liquid nitrogen. However, fast snap-freezing facilities are often not available in certain sampling locations which are remote (Gorokhova, 2005). Moreover, freezing sampling materials with liquid nitrogen are very often do not deactivate RNases activity efficiently, resulting RNA fragmentation (Shabihkhani et al., 2014). An alternative method will be the usage of *RNAlater* during RNA assay.

*RNAlater* is a storage reagent that stabilizes and protects RNA by rapidly permeating into the tissues and stabilizing cellular RNA. Manufacturer's recommendation on the usage on animal tissues preservation with *RNAlater* in general are not plausible. According to manufacturer's instruction, *RNAlater* is not suitable for stabilization of RNA in animal tissues, whole blood, plasma, serum and tissues with high abundance of fat (QIAGEN, 2006). Despite this, the effectiveness of stabilizing effect in animal tissues should be tested to gain the optimum preservation and storage regimes for RNA, especially for samples which are difficult to obtained or rare in nature. Previous work demonstrated that preserving fresh microcrustaceans (*Artemia*) samples

with *RNAlater* with subsequent storage at 5°C and room temperature (19 to 22°C) were effective to maintain a huge amount of RNA in crustacean for at least eight months and one month after preservation respectively. Moreover, this study also showed no significant RNA degradations were detected when samples were deep freezed immediately into a -80°C freezer at any time of the experiment (Gorokhova, 2005). Besides that, study had shown that high RNA quality could be achieved by optimizing procurement and storage methods specifically for each cell type (Shabihkhani et al., 2014).

Thus, the objective of this study was to evaluate the applicability of optimal regime for preserving RNA from different animal tissues (using an ornamental fish) by applying optimal preservation (soak in *RNAlater*) and storage condition (freeze at -80°C).

## MATERIALS AND METHODS

### Sample Preparation and Preservation

Fish samples from subgenus *Poecilia* were collected from local breeding aquaria. The fish samples collected comprised of six sexually matured males and six sexually matured females ( $2.5 \pm 1.23$  cm, standard length). The fishes were euthanized in crushed ice for 10 minutes with subsequent tissues excision from dorsal fin, muscle and gonad from each sample. Approximately 10 mg of each tissue were dissected, sliced thinly and immediately soaked completely in 100 µl of *RNAlater*. The *RNAlater* volume used was in accordance to the

ratio suggested by the manufacturer (10  $\mu$ l reagent per 1 mg tissues) (QIAGEN, 2006). A total of 36 samples were prepared for RNA assay (12 replicates per tissue type).

### Storage

Preserved tissues samples were stored immediately into a  $-80^{\circ}\text{C}$  freezer. The storage regimes applied were in accordance to the optimized storage condition as described by Gorokhova (2005), which exhibited high amount of RNA yield even after eight months of storage.

### Extraction Procedure

Prior to RNA assay, samples were left to thaw at room temperature and subsequent transfer to a new 1.5 ml appendorf tube. Excess RNA<sub>later</sub> solution was dried from the samples by using paper tissues. Next, approximately 350  $\mu$ l of lysis buffer were immediately added in each tube. Subsequent extraction procedures were in accordance with RNeasy Mini kit (cat. #74104, QIAGEN, Germany) handbook with minor modifications on lysate homogenization. In detail, homogenization of tissues in lysis buffer (RLT buffer) was carried out by passing the lysate through a blunt 20-gauge needle (0.9 mm diameter) for at least 20 times, until a murky solution was formed. Murky solution is an indication of well mixed lysate. Purified RNA was quantified immediately prior to freeze storage at  $-80^{\circ}\text{C}$ .

### Nucleic Acid (RNA) Quantification

Quantification of purified RNA was performed by using Qiagen's QIAexpert.

Spectrophotometric absorbance measurements performed were: 230, 260 and 280 nm. Each purified RNA sample was loaded into 16 wells QIAexpert slide and scanned for measurements.

### Statistics

Data were presented as comparisons between RNA amount in ng (mean  $\pm$  SD) and ratio of  $A_{260}/A_{280}$ , and between RNA amount in ng (mean  $\pm$  SD) and ratio of  $A_{260}/A_{230}$  among different tissues. One-Way Independent ANOVA was performed using IBM SPSS Statistics 24 analysis software to investigate significant differences of obtained data and significance was accepted when  $P < 0.05$ .

## RESULTS AND DISCUSSION

Protein contamination was generally low as indicated by  $A_{260}/A_{280}$  ratio, which is greater than 2.0 in all 36 purified RNA samples (Table 1). The  $A_{260}/A_{280}$  ratio of 2.0 and above indicates high purity of RNA in the eluent (Escobar & Hunt, 2017; QIAGEN, 2011). However, the  $A_{260}/A_{230}$  ratios, which were less than 1.7, indicates carry over of salts and other contaminants during purifications (Escobar & Hunt, 2017; QIAGEN, 2011) (Table 1). Possible examples of contaminants could be urea, EDTA, carbohydrates and phenolate ions, which were the ingredients commonly used in extraction buffers. According to the manufacturer's handbook, presence of contaminants could be due to mishandling of spin column, which accidentally contacted the flow through after centrifugation (QIAGEN, 2012).

Table 1  
Amount of RNA in ng (mean  $\pm$  SD), ratios of  $A_{260}/A_{280}$  and  $A_{260}/A_{230}$  absorbance among different tissues

Tissues	n	mean $\pm$ SD		
		Amount of RNA/ng	$A_{260} / A_{280}$	$A_{260} / A_{230}$
Dorsal fin	12	119.65 <sup>a</sup> $\pm$ 101.35	2.00 $\pm$ 0.09	1.17 $\pm$ 0.71
Muscle	12	19.30 <sup>c</sup> $\pm$ 16.57	2.51 $\pm$ 0.48	0.03 $\pm$ 0.04
Gonad	12	72.80 <sup>b</sup> $\pm$ 97.50	2.03 $\pm$ 0.16	0.74 $\pm$ 0.64

Different superscript alphabet indicates significant difference at  $P < 0.05$

Total amount of RNA yield extracted from dorsal fin was the highest ( $119.65 \pm 101.35$  ng), followed by gonad ( $72.80 \pm 97.50$  ng) and RNA yield extracted from muscle produced the least amount ( $19.30 \pm 16.57$  ng) comparatively (Table 1). Samples exhibited negligible degradation of RNA (Figure 1). From our empirical results, it is reasonable to assume that the proposed preservation and storage regime is more efficient for preserving RNA from fin rays and gonad compared to muscle tissue. Perhaps due to different cells types which are having different composition and structural integrity have varied penetration affinity towards RNA later. With respect of tissue type, fins in fishes composed of stiff rays covered by skin, which comprised stratified squamous epithelium of various thickness (Bone et al., 1995). On the other hand, gonadal tissues consist of synchronous germ cells from oogenesis or spermatogenesis (Huang et al., 2002). In addition, muscle tissue comprised mainly of muscular cells only. To demonstrate further, in microcrustacean such as *Artemia*, *Daphnia* and copepods, the presence of an exoskeleton could be a barrier for the diffusion of aqueous sulfate salt solutions such as RNA later (Gorokhova, 2005). In

addition, exoskeleton permeability may vary according to different species, ontogenetic stages and molting cycle of the crustacean. These biological conditions could impeded the penetration of preservative into tissues, causing different level of nucleic acids degradation prior to extraction. Further to this, low amount of RNA in muscle cells discovered in this study could be also attributed by low tolerance of this cell type towards freeze-thaw stress. For instance, degradation of 28S rRNA was prominent from samples extracted from snap-frozen autopsy tissues (Auer et al., 2014; Ross et

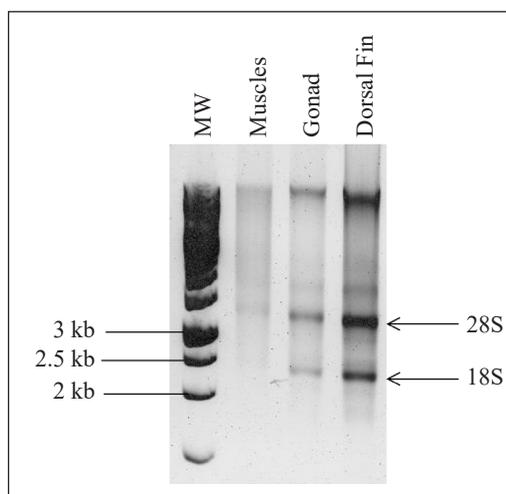


Figure 1. Analysis of RNA isolated from muscle, gonad and dorsal fin tissues using Qiagen's RNeasy Mini kit. MW: Molecular weight (1 kb)

al., 1992) and soil microbial (Pesaro et al., 2003). In the context of qPCR, samples with high amount of RNA are preferred to ease normalization of RNA, as low volume is required, dilution is carried out during generation of standard curve.

## CONCLUSION

Current data suggests that preserving fish tissues in RNAlater with subsequent storage at -80°C produced satisfactory amount of RNA from dorsal fin and gonad, but not from muscle tissues. Further studies are required to evaluate the effectiveness of RNAlater and storage condition for stabilizing RNA in animal cells from other tissues type as well as from other animal species.

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## COMPLIANCE WITH ETHICAL STANDARDS

The authors declared that there is no conflict of interest involved. All applicable local institutional guidelines for the care and use of animals were followed. This article does not contain any studies with human participants performed by any of the authors.

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