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# Storage by lyophilization – Resulting RNA quality is tissue dependent

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ABSTRACT

In today's highly collaborative scientific community there is a growing need to transport biological samples across the globe. Lyophilization is a cost-effective preservation method which avoids the use of hazardous chemicals, creating an appealing, yet essentially unexplored, prospect for the long-range transport of animal tissue samples. This study examined the integrity of RNA following its extraction from eel tissue (liver, spleen and ovary) that had been subjected to *i)* freezing only; *ii)* freezing and lyophilization, and *iii)* freezing, lyophilization and subsequent storage at ambient temperature for one week. Only small reductions in RNA integrity were identified in lyophilized, stored sample compared to that of flash-frozen or lyophilized sample not subjected to storage. Reductions in RNA integrity were most profound in ovary tissue, which has a notably higher lipid content (~35% of dry weight) than liver (~17%) or spleen (~15%). However, lowered RNA integrity numbers did not affect qPCR-estimated relative or absolute transcript copy numbers of two arbitrary target genes. These findings confirm that this method is a viable option for shipping animal tissue samples world-wide and could open up numerous benefits for the biological sciences as a whole.

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## 1. Introduction

The rapid evolution of molecular biology techniques has led to a growing divide between laboratories capable of carrying out advanced molecular analyses and those lacking the skills, financial resources and/or facilities to do so. Therefore, there is an increasing need to safely transport samples across the globe. Traditional methods of shipping biological samples involve dry ice or liquid nitrogen which make for expensive transport options. In addition, these avenues center around the use of tightly controlled hazardous chemicals and are further complicated by the risk that samples may defrost if shipments are delayed. Alternative options include the use of several aqueous preservatives (i.e. DNAgard and RNAlater) that have been developed to render DNA or RNA stable for use at a later time. However, these storage solutions are expensive (approx cost to preserve 100 mg of tissue: DNAgard = US$1.60; RNAlater = US$1.85) and can pose chemical hazards, again limiting their usefulness for long-distance shipping (see: Ambion MSDS# 4418289a). A recent development has seen the rise of vacuum-based refrigerated samples being transported [1], but refrigerated transport can be costly and risky over long distances.

In recent decades, scientists have largely overcome these expensive and dangerous transportation options for some types of tissues by lyophilizing extracted RNA and DNA samples prior to shipping. More recently, the lyophilization of whole plant tissues *prior* to RNA extraction produced good yields of high-quality RNA [2–4]. However, with the exception of a handful of previous studies, this method has not been evaluated for use in animal tissues. Two studies, the first carried out by Wu and colleagues [5] and the second by Mareninov and colleagues [6] provided very little validation of RNA integrity (the authors only reported optical densities of bands following RT-PCR and agarose gel electrophoresis; instead both studies provided a detailed analysis of extracted protein integrity following lyophilization. Pena and colleagues [7] reported an increased RNA yield from pig muscle and subcutaneous fat that was lyophilized immediately prior to RNA extraction, but that storing the samples at room temperature for five days following lyophilization resulted in a notable reduction in RNA integrity. Interestingly, the reduction in integrity was much more pronounced in fat than in muscle tissue. We sought to further unravel the effect of tissue type on applicability of lyophilization and subsequent storage at room temperature to allow for international shipping. Here, we report on RNA integrity following the lyophilization of three different animal tissues (eel liver, spleen and ovary) for the

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first time and provide information on the potential for this RNA to be used for downstream contemporary molecular analyses.

## 2. Methods

### 2.1. Animal husbandry

All experiments were approved by the University of Otago Animal Ethics Committee, in keeping with the guidelines from the Australian and New Zealand Council for the Care of Animals in Research and Teaching.

### 2.2. Experiment I

#### 2.2.1. Tissue collection and processing

Three wild-caught, early vitellogenic shortfinned eels were euthanized (0.30% benzocaine) and bled following tail removal. Three replicate portions of tissues with varying lipid content (liver, spleen and ovary) (known weights: < 100 mg) from each of the three fish were snap-frozen on dry-ice and stored at −70 °C. Two portions of each tissue from each of the three fish were lyophilized (VaCo 2-II: Zirbus Technologies) between −70 °C and −80 °C at < 0.014 mB for 24 h. Once dry, each piece of tissue was reweighed to allow the total amount of water removed to be calculated. Half of the samples were subjected to RNA extraction immediately after freeze-drying, whilst the other half were left to incubate at room temperature (22 ± 1.5 °C) for one week. Tissue was submerged in TRIzol reagent (Invitrogen) before the lost water volume was replaced. Tissues were then homogenized using a bullet blender (Next Advance) before standard RNA extractions were carried out following the manufacturer's instructions. Total RNA was also extracted from frozen tissue. The concentration and quality of isolated RNA were determined both via absorbance at 260/280 nm using a ND-1000 spectrophotometer (NanoDrop Technologies) and using an Agilent 2100 Bioanalyzer. The RNA integrity number (RIN number) was calculated by the Agilent 2100 Bioanalyzer software using an algorithm which incorporates the ratio of the 18S to 28S ribosomal RNAs as well as the entire electrophoretic trace [8]. A total of 5 μg of RNA was treated with TURBO DNA-*free* (Life Technologies) before 1 μg of treated RNA was used to synthesize cDNA (MultiScribe Reverse Transcriptase, 50 U/μl: Life Technologies).

#### 2.2.2. Quantitative PCR

All samples were subjected to qPCR analysis performed on an MX-3000P quantitative.

PCR machine (Stratagene) using SYBR *Premix Ex Taq* (Tli RNase H Plus) (Takara Clontech). Two target genes were chosen, one known to be highly expressed in the three tissues of interest (mitochondrial ribosomal protein L36: *l36*) and one with a lower level of expression (low density lipoprotein receptor: *ldlr*). Primer sequences have been published previously (*l36* [9]; *ldlr* [10]). A total of 40 cycles of amplification (95 °C for 5 s, 62 °C for 10 s, 72 °C for 5 s) were run before a melting curve analysis was performed to evaluate sudden changes in fluorescence and ensure primer specificity. Samples were run in single alongside a duplicate, six point, standard curve and no-template and RNA controls; runs were repeated three times (i.e., three measurements/sample) for each target, the positioning of samples on the plate changing each time. For each sample, the transcript copy numbers for each gene were averaged before the copy number of *ldlr* was divided by that of *l36* to control for any discrepancies in reverse transcription efficiencies.

### 2.3. Experiment II

#### 2.3.1. Tissue collection and processing

Six wild-caught, previtellogenic shortfinned eels were euthanized as above. Body weight and organ weight (liver, spleen and ovary) were recorded and used to calculate somatic indices (organ weight/body weight x 100). Total lipid content was determined from a portion of each tissue following the protocol described by Divers and colleagues [11].

### 2.4. Statistics

All data sets were analyzed in SPSS22 or Graphpad. Significant differences between the storage methods (frozen, lyophilized, lyophilized + holding at room temperature for 1 week) were identified using a two-way ANOVA (storage method: fixed variable; fish: random variable) (Experiment I). In addition, RIN number was plotted against each of the different extraction treatments and covariance analysis (ANCOVA, using increasing complexity of storage method as the co-variable) was performed (Experiment I). Differences in total lipid content were identified using a one-way

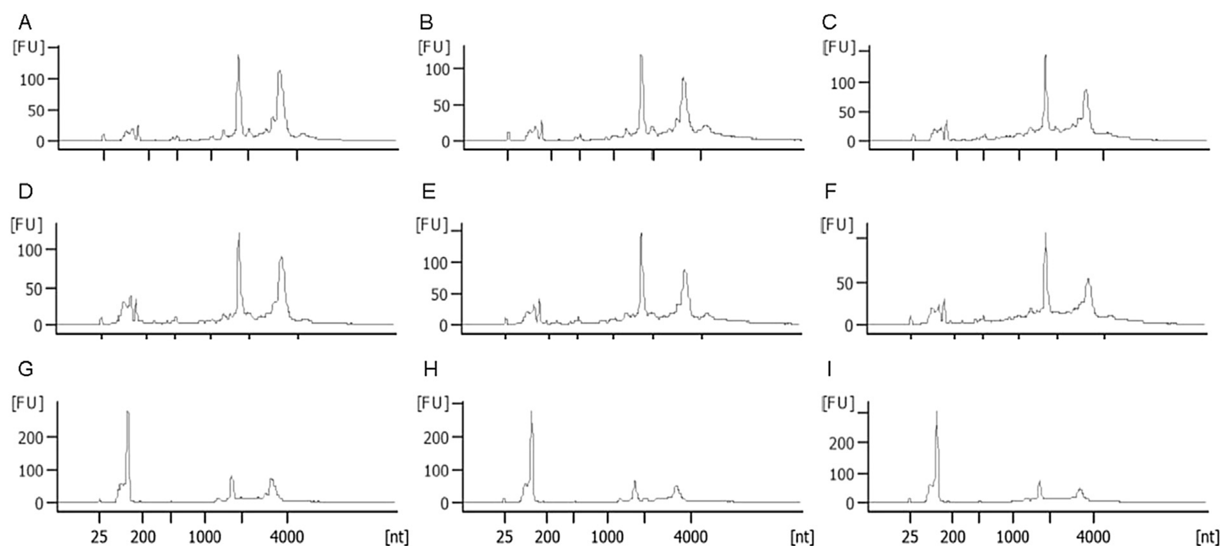


**Fig. 1.** Bioanalyzer RNA profiles from the liver (A–C), spleen (D–F) and ovary (G–I) of a single female shortfinned eel, *Anguilla australis*. Total RNA was extracted either from frozen tissue (A, D and G) or from tissue which had been lyophilized for 24 h and extracted immediately (B, E and H) or had been kept at room temperature for one week prior to extraction (C, F and I).

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