Recovery of RNA from avian nucleated whole blood stored under field conditions: an evaluation of commercial methods

C. Komini and A. Bounas

Department of Biological Applications and Technology, University of Ioannina, Ioannina, Greece

ABSTRACT
1. Whole blood can be used to gain insights on several molecular mechanisms involved in animal physiology. However, its use to extract RNA from organisms that carry nucleated red blood cells is limited due to its high nuclease content and instability under field conditions.
2. Here, we evaluate three commercially available RNA preservation and extraction methods applied to avian whole-blood samples kept in different storage conditions to identify the most suitable ones for use in field studies.
3. Whole-blood starting volume was the most important factor; only samples with a starting volume of 50 µl yielded RNA, whereas all 100 µl samples failed to provide any RNA regardless of the method used. RNA concentration decreased with storage time in every method employed. Samples stored at −20°C yielded higher RNA concentration than the ones stored at 4°C, although some possible effects of freeze-thaw cycles were observed.
4. For two of the three methods, it is likely that DNA contamination and/or extensive degradation of RNA could have taken place. Only whole blood stored and extracted using the Quick RNA Whole Blood kit provided high-quality RNA in every condition tested.

Introduction
Unravelling the mechanisms of the adaptive response of phenotypic traits to novel or changing environments presents an exciting opportunity to gain insight on how ecological processes and the environment can shape the phenotype. To this aim, advances in sequencing technology and powerful bioinformatics analyses have made it possible to quantify gene expression levels, thus providing a groundbreaking opportunity to explore genetic mechanisms facilitating adaptation in natural populations (Wang et al. 2009). To gain insight from these methods though, RNA of high quality and quantity is key, a challenging feat when working with non-model organisms. Many tissue types (e.g. brain, intestine and liver) cannot be collected without causing severe harm or death to the animal and that kind of sampling is not a choice for endangered and protected species or when repeated measures are required to tackle a research question (Jax et al. 2018). In such cases, depending on the research question, the least invasive and, at the same time, ample source of RNA is whole blood.

Although whole blood cannot be used as an alternative when examining processes in specific tissues (e.g. brain), it can be used to investigate molecular mechanisms influencing traits of interest in non-model organisms, mainly those involved in immune response or stress metabolism (Jax et al. 2018; van der Sjde et al. 2020; Watson et al. 2017). In addition, blood is easily collected with minimal harm to the animal. Due to its advantages, whole blood has been used as a source of RNA in several mammal species (Schwuchow et al. 2012), as well as in few bird species (Fudickar et al. 2016; Franchini et al. 2017). However, its use in bird studies and for other taxonomic groups is rather limited. Whole blood carries proteins in abundance that must be separated from the RNA to successfully extract it. Nucleated red blood cells as those of amphibians, reptiles and birds contain higher levels of DNA and endogenous nucleases making RNA degrade more rapidly (Chiari and Galtier 2011). Solutions to this situation including either manual protocols or commercial kits have been provided (Chiari and Galtier 2011; Mewis et al. 2014; Vomelova et al. 2009); however, challenges do not stop there.

RNA is a highly unstable molecule and can be easily degraded; thus, proper whole-blood stabilisation at the time of collection and storage until extraction is of utmost importance; changes in transcript abundance and potential loss of rare transcripts immediately after sample collection could hamper any gene expression study (Lorkowski and Cullen 2006; Rainen et al. 2002; Tanner et al. 2002). To date, snap-freezing of the sample in liquid nitrogen or dry ice has been the most effective preservation method (Rainen et al. 2002; Wolf 2013). However, in most cases when working with natural populations of non-model species, sampling may be performed under field conditions, making it difficult to timely and efficiently store the samples. Alternatively, commercial RNA-stabilising reagents could be used to immediately store the samples. This approach is much more suitable for fieldwork, as it can prolong the time from sample collection to freezing without compromising the research.

Literature that reviews, evaluates and improves either manual protocols or commercial kits to extract RNA from nucleated whole blood is scarce (Chiari and Galtier 2011; Mewis et al. 2014; Vomelova et al. 2009), while studies that consider field conditions are absent. Here, we evaluate three commercially available RNA preservation methods applied...
to different quantities of avian whole blood. Furthermore, samples were treated as if they had been collected in a field station; they were collected in an outdoor sampling station followed by mid- and long-term storage in room temperature (RT) or refrigerator/freezer temperatures.

Methods

Study design

To assess different methods of RNA extraction from avian blood samples, we conducted a $2 \times 2 \times 3 \times 3$ factorial experiment: two levels of whole-blood quantity (50 and 100 µl), two levels of storage duration (1 week and 1 month), three levels of storage temperature (RT = 20°C, 4°C or −20°C) and three commercial RNA preservation/extraction methods. The methods included the RNeasy Protect Animal Blood System kit (Qiagen), the NucleoProtect RNA reagent combined with Nucleospin RNA purification kit (Macherey-Nagel) and the Quick RNA whole-blood kit (Zymo Research). All factorial arrangements of the above-mentioned treatments were run, except for the following cases that were instructed otherwise by the commercial kit manufacturers: we did not evaluate any samples stored at RT using the RNeasy Protect Animal Blood System as the product sheet states that samples should be stored in RT for up to 48 h. Similarly, we did not evaluate samples stored at RT for the 1-month storage duration treatment using NucleoProtect according to the manufacturer’s guidelines (1 week maximum). Finally, we only processed samples with a quantity of 50 µl with the Quick RNA whole-blood kit as explicitly mentioned in the product guide. A graphical summary of the experimental design can be found in Figure 1.

Sample collection

This study was carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes of the Council of Europe (http://conventions.coe.int/Treaty/EN/Treaties/Html/123.htm). Every effort was made to minimise animal discomfort during blood sampling.

Blood was collected from two healthy chickens via puncture of the brachial vein using a 25-gauge needle not pre-treated with anti-coagulants. Puncture site was aseptically prepared using 70% ethanol. In total, approximately 2.5 ml of whole blood was collected from the birds and the appropriate experimental quantity (50 or 100 µl) was dispensed in each buffer-treatment arrangement. Whole blood was dispensed directly into the pre-designated RNeasy Animal blood tubes, whereas, for the other two methods, blood samples were diluted in a 1:20 ratio with the respective preservation buffers (Nucleoprotect RNA and DNA/RNA Shield, respectively).

RNA extraction and quality assessment

Total RNA was extracted from each sample following each kit manufacturer’s recommendations. For the blood samples preserved in the RNeasy Protect Animal Blood tubes (Qiagen), we followed the modifications previously proposed by Chiari and Galtier (2011) to increase RNA quality: samples were stored for 4 h at RT before extraction and incubated for 90 min (instead of the recommended 10 min) at 55°C in the proteinase K digestion step. Quality and quantity of the extracted RNA were evaluated using a Quawell Q3000 Spectrophotometer by estimating the concentration and the 260/280 RNA purity ratio (values of ~2.0 are indicative of ‘pure’ RNA). We also validated spectrophotometry readings and assessed RNA degradation by 0.9% agarose gel electrophoresis.

Results

RNA yield assessment

Amount of extracted RNA was severely impacted by whole-blood starting volumes. A starting quantity of 100 µl did not yield any measurable RNA, regardless of the method used. On the other hand, when we used 50 µl starting volume, most of the samples yielded even few nanograms in spectrophotometry readings (Table 1). However, we should note that overall RNA concentration measurements from samples stored in NucleoProtect RNA and RNeasy Protect Animal Blood were overestimated as we were not able to validate them visually using gel electrophoresis; thus, true RNA quantity should be much lower than reported (Additional file 1). Regarding the 50 µl samples, unsurprisingly, RNA concentration decreased with storage time in every method employed (Wilcoxon signed-rank test $W = 1, p < 0.05$; Figure 2(a)). Concerning storage temperature, as expected, samples stored at −20°C yielded higher RNA concentration than the ones stored at 4°C. However, samples stored in...
Table 1. Total RNA yield and 260/280 ratio for each sample in each experimental condition for each tested preservation buffer and extraction method.

| Blood quantity (µl) | Time       | Temperature | NucleoProtect/NucleoSpin RNA | RNeasy Protect Animal Blood System | Quick RNA whole blood |
|---------------------|------------|-------------|-----------------------------|-----------------------------------|-----------------------|
|                     |            |             | RNA yield (µg) | 260/280 ratio | RNA yield (µg) | 260/280 ratio | RNA yield (µg) | 260/280 ratio |
| 50                  | 1 week     | RT          | 5.20±*       | 1.05         | n/a           | 2.73          | 1.92          |
|                     |            | –20         | 1.81         | 1.03         | 0.22          | 1.98          | 1.89          |
|                     | 1 month    | RT          | 0.10±        | 0.70         | 0.38±         | 1.75          | 0.72          | 2.00          |
| 100                 | 1 week     | RT          | 0.00         | 0.00         | 0.00          | n/a           | n/a           | n/a           |
|                     |            | –20         | 0.00         | 0.00         | 0.00          | n/a           | n/a           | n/a           |
|                     | 1 month    | 4           | 0.00         | 0.00         | 0.00          | n/a           | n/a           | n/a           |

*Samples did not show any measurable RNA yield in the agarose gel electrophoresis.

**RNA fragmented, indicated by smear in the agarose gel electrophoresis.

RNeasy Protect Animal Blood performed less well when kept at −20°C. Furthermore, Quick RNA whole blood and NucleoProtect/NucleoSpin RNA methods yielded the highest RNA quantity for samples stored at RT even for the 1-month storage time treatment (Figure 2(b)). Overall, samples preserved/extracted with the Quick RNA whole-blood kit yielded the highest RNA concentrations ranging from 48.2 to 181.9 ng/µl, followed by the NucleoProtect RNA – Nucleopspin RNA purification combination with values ranging from 1.9 to 103.9 ng/µl. RNeasy Protect Animal Blood yielded from 1.1 to 35.7 ng/µl. Both the first two methods yielded >1 µg RNA per 50 µl whole blood, a quantity that should suffice for most downstream applications (Table 1; Figure 2).

**RNA quality assessment**

In all cases, RNA was measured by spectrophotometry and RNA quality was assessed by the 260/280 ratio. The RNA integrity was further validated with agarose gel electrophoresis. The agarose gel electrophoresis showed marked differences among methods used. Both RNeasy Protect Animal Blood and NucleoProtect/NucleoSpin RNA approaches showed lower RNA quality with high variation in ratio values, ranging from 0.22 to 2.12 and 0.7–1.14, respectively, indicating protein contamination. In addition, RNA extracted from RNeasy Protect Animal Blood was highly degraded showing large smear bands in the agarose gel electrophoresis (Additional file 1). For these two cases, it is likely that DNA contamination and/or extensive degradation of RNA resulted in the observed concentration measurements (Additional file 1). On the other hand, Quick RNA Whole Blood provided RNA with consistently high-quality values (1.84–2.00; Figure 3), which was also confirmed by agarose gel electrophoresis (Additional file 1).

**Discussion**

Here, we tested different commercial methods for RNA preservation and extraction that could be used in a field station, where samples need to be stored either at ambient temperature or best case in a freezer. Previous efforts have shown that blood stability can be achieved either by freezing at −80 or only for some hours in commercial buffers, both of which are impossible conditions to achieve for field studies. Several conditions on mammal blood tested so far have produced a wide range of results, from non-usable quantity and quality of RNA to enough quality and quantity for a range of high-throughput applications (Camacho-Sanchez et al. 2013; Rainen et al. 2002; Schwochow et al. 2012). Specifically for nucleated whole blood, either avian or reptilian, suitable methodologies have been developed (Mewis et al. 2014) and/or evaluated and modified to yield appropriate quality of
RNA (Chiari and Galtier 2011). However, a comprehensive study testing several limiting factors of field studies does not exist.

Our results reflect the main limitation of nucleated whole blood as a source of RNA: it carries higher DNA and nuclease content than non-nucleated blood, which leads to a rapid RNA degradation. Therefore, higher quantities of blood samples did not yield any RNA at all unlike the smaller quantity tested. However, this could be in part attributed to technical problems, such kit column overloading and subsequent clogging when using higher amount of starting material. For RNA to be an appropriate sample for real-time PCR, and further downstream applications, it must be of high quality without the presence of DNA, nucleases and proteins. In our case even when RNA was isolated, the extraction quality was found to be rather low in most cases, possibly as a result of DNA contamination and the presence of nucleases. Only whole blood stored in DNA/RNA Shield followed by Quick RNA Whole Blood kit extraction was able to provide high-quality RNA in every other conditions tested.

Some RNA degradation over time is to be expected due to its sensitive nature. Indeed, our results clearly show a significant decline in RNA concentration a month after collection, in every buffer tested. It should be noted, however, that despite the decrease in RNA quantity, Quick RNA Whole Blood kit yielded enough RNA for use in most applications (>1 μg). In relation to temperature, the −20°C storage condition provided better results in RNA yield than the storage at 4°C, although Quick RNA Whole Blood kit performed best at RT conditions even when samples were stored for a month, thus providing high-quality RNA. On the other hand, samples stored in RNeasy Protect Animal Blood performed worse when stored at −20°C. Freeze-thaw cycles may affect the quality of RNA, and in our case, samples stored in RNeasy Protect Animal Blood underwent two freeze-thaw cycles, one during the shipping of samples and a second prolonged one (4 h) as part of sample processing when following the modified protocol for RNA extraction (Chiari and Galtier 2011). Thawing of samples can allow endogenous nuclease to act and could therefore be the cause of such a discrepancy (Lorkowski and Cullen 2006; Townsend et al. 2020).

Overall, our results on the use of RNeasy Protect Animal Blood system support the conclusion of Chiari and Galtier (2011): modifications of the isolation protocol (samples were stored for 4 h at RT before extraction and incubated for 90 min rather than the recommended 10 min at 55°C when at the Proteinase K step) made it possible to recover even up to 0.64 μg of RNA. Similarly to Chiari and Galtier (2011), despite the extracted concentration, we obtained very low 260/280 ratios and the method only worked in lower volume of starting whole blood quantity. Nevertheless, RNeasy Protect Animal Blood system was developed for mouse blood and its use reflected the difficulties of nucleated whole blood even if at lower blood quantity. Furthermore, NucleoProtect RNA is inherently limited at storing whole blood samples as it is recommended for RNA stabilisation in white blood cells.

Although our results intend to inform field studies, and even though we treated samples as if they had been collected in a field station, our sampling involved domestic chicken whole blood as a source of RNA. Therefore, researchers involved in field studies should keep in mind that blood

![Boxplots of RNA quality as measured by 260/280 ratio across all samples extracted with each different commercial method.](image-url)
density could vary depending on a wild bird’s condition (Fair et al. 2007; Minias 2015); thus, the same starting amount of blood quantity could not provide the same amount of total RNA among samples, even when the same storage/isolation method is used.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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**ORCID**

A. Bounas [http://orcid.org/0000-0002-6724-0151](http://orcid.org/0000-0002-6724-0151)

**Data availability**

All data generated during this study are included in this published article and its supplemental material.

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