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Improved method for isolating high-quality RNA from mouse bone with RNA*later* at room temperature

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ABSTRACT

Accurate gene expression analysis of bone requires the ability to isolate RNA of good quality. Isolation of intact RNA from frozen bone tissue is problematic since RNA rapidly becomes degraded after thawing. Since we are interested in assessing gene expression from both bone marrow and mineralized bone, we aimed to develop improved simple, robust and statistically validated methods providing high-quality RNA from both mouse femur shaft and femur marrow. RNA integrity was quantified by the RNA Integrity Number (RIN) measured on a TapeStation. While the RNA stabilization reagent RNA*later* is not commonly used or recommended for mineralized bone, we found that preservation methods with RNA*later* significantly improved the RNA quality with a mean RIN for the femur shaft of 8.0 and a mean RIN for femur marrow of 9.6. With RNA*later*, high quality RNA with a mean RIN of 9.3 could also be isolated from lumbar vertebral bone. A further advantage of using RNA*later* is that the tissue can be allowed to thaw to room temperature before TRI Reagent lysis without any loss of RNA integrity. A comparison of the TRI Reagent method with a hybrid method combining TRI Reagent lysis with RNeasy column purification showed no difference in RNA integrity. However, the hybrid method seemed to give femur shaft RNA with fewer impurities inhibiting qRT-PCR.

1. Introduction

Our laboratory conducts research into mechanisms whereby ethanol in combination with oxidative stress and hormonal changes affects bone structure and physiology (Alund et al., 2016; Watt et al., 2018; Ronis, 2018). An important part of this research is investigation of the transcriptome of bone tissue, both of the bone marrow and of mineralized bone, from mice of various genotypes. Getting high-quality RNA from bone tissue is challenging due to RNA degradation by RNases. When we, for example, have isolated RNA from frozen mouse bones by the TRI Reagent method without taking special care to keep everything frozen and ice-cold before TRI Reagent lysis, the resulting RNA has occasionally been so degraded, that an mRNA target like RANKL that is expressed in both mineralized bone and bone marrow (Streicher et al., 2017) could not be reliably quantified. While RNA can be isolated immediately upon dissection of a single bone from an animal, this is not a very efficient procedure, as each RNA isolation takes more than one hour. It is therefore common to flash-freeze bones in liquid nitrogen and store frozen bones at −80 °C before isolation of RNA. Keeping whole femur bone at near freezing conditions using liquid nitrogen and ice-cold TRI Reagent yielded RNA of good quality with RNA Integrity Numbers (RIN) between 6.7 and 9.2 (Carter et al., 2012). Apart from the hazards of working with liquid nitrogen, this method does not allow separation of bone shaft and bone marrow RNA as that would require thawing of the bone. Bone shafts and bone marrow should therefore be separated before preservation and freezing, e.g. by centrifugation as described by Kelly et al. (2014). In that paper, the quality of the RNA was only given for the shaft and was rather modest.

RNA degradation can be prevented by incubating tissues in an RNA stabilization reagent like RNA*later* from Qiagen. However, guidelines for the use of RNA*later* specifically suggest that bone is a tissue for which RNA*later* may be inefficient (Ambion by Life Technologies, 2011), and it seems rarely used for mineralized mouse bones. On the other hand, RNA*later* has been used for generating RNA of good quality from rat femur metaphyseal trabecular primary spongiosa and from mouse caudal vertebra trabeculae (Wasserman et al., 2013; Li et al., 2008). A comparison of RNA*later*-based preservation to other preservation methods for mouse bones has, to our knowledge, not been reported. RNA can be isolated from tissue by the simple TRI Reagent method with a phase separation step that separates RNA from the major

Abbreviations: RIN, RNA Integrity Number; qRT-PCR, Quantitative RT-PCR; C_T, Cycle threshold value

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part of the DNA and protein. The RNA is subsequently precipitated by isopropanol and ethanol and suspended in water or aqueous buffer. To provide better purity, the RNA can further be column purified. Hybrid methods can also be used where cells are lysed in TRI Reagent followed by applying the RNA-containing phase after phase separation to RNA purification columns (Carter et al., 2012; Kelly et al., 2014). Various laboratories may have validated that hybrid methods provide better RNA purity, but there is scarce documentation in the scientific literature.

We found that there was a need for an improved, simple, robust and statistically validated method giving high-quality RNA from both femur shaft and femur marrow, preferably without the need for working with liquid nitrogen. We tested various preservation methods and compared a TRI Reagent protocol to a hybrid RNA purification method. Our work successfully resulted in such methods that can even be conducted at room temperature.

2. Materials and methods

2.1. Animal dissection

The Institutional Animal Care and Use Committee (IACUC) of Louisiana State University Health Sciences Center, New Orleans, approved the animal work that was conducted according to the guidelines of the National Institutes of Health guide for the care and use of laboratory animals. Mice were euthanized by CO_2. The left leg was excised first, and the femur separated from the tibia. The femur was cleaned of muscle tissue by a scalpel and a final rubbing with gauze. With a scalpel, the proximal femur end including the metaphysis and epiphysis was separated from the diaphysis. The bone marrow was harvested by placing the remaining femur with the cut end down in a perforated 0.6 mL centrifuge tube inserted in a 1.5 mL centrifuge tube followed by centrifugation for 30 s at 5700 ×*g*. The shaft was subsequently separated from the knee end of the femur with a scalpel cut. The marrow and femur shaft were preserved separately. The two ends of the femur were preserved together in a single 1.5 mL tube. The right leg was excised next with preservation of the bone marrow, shaft, and the femur ends as above. Finally, the lumbar spine was removed, cleaned of muscle tissue with a pair of scissors and preserved. The tissues were stored at −80 °C prior to RNA isolation. The exact dissection protocol used in our laboratory is presented in Supplemental Information. To compare the quality of two preservation methods or two RNA isolation procedures, they were performed on the two legs of an animal. Between animals, the methods were alternated between the left and right leg to ensure that the order in which the legs were removed did not affect the results. Initial optimization was done on animals of various genotypes and ages. A systematic comparison of methods was done with C57Bl/6J female mice from the Jackson Laboratory (Bar Harbor, ME) at ages of 22–27 weeks.

2.2. RNA isolation

Total RNA was isolated by either a TRI Reagent protocol according to guidelines from the manufacturer of TRI Reagent (Molecular Research Center Inc., Cincinnati, OH) or a hybrid method combining tissue lysis in TRI Reagent with subsequent purification of the aqueous phase after phase separation on RNeasy Mini Kit columns (Qiagen, Hilden, Germany). Mineralized bone tissue was crushed with a steel bead in TRI Reagent using a TissueLyser II instrument (Qiagen) set at 30 strokes/s for 2–4 min. 1-Bromo-3-Chloropropane (Molecular Research Center Inc.) was used for phase separation. In the hybrid method, the aqueous phase was mixed with 70% (v/v) ethanol and applied to RNeasy columns. The exact protocols used in our laboratory are listed in Supplemental Information.

2.3. RNA assessment

RNA concentrations were determined by OD 260 nm measurements on a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). RNA integrity was assessed by the RNA Integrity Number (RIN) using a 2200 TapeStation Instrument with RNA ScreenTapes (Agilent Technologies, Santa Clara, CA). The RIN is a number on a scale from 1 to 10, where RIN = 10 indicates intact RNA and RIN = 1 indicates completely degraded RNA (Schroeder et al., 2006). Each TapeStation lane image were scaled separately with the TapeStation Analysis software option “Scale to Sample”.

2.4. Quantitative RT-PCR (qRT-PCR)

mRNA concentrations were determined with a Power SYBR Green RNA-to C_T 1-Step Kit (Thermo Fisher Scientific) and a LightCycler 480 II instrument (Roche). The genes with the corresponding forward and reverse primers were: *Icam4*, caatctcgacgggctagtgg, tgggcttaaagcgaggactg; *Actb*, agatgacccagatcatgtttgaga, ccagaggcatacagggacagc; *Tnfsf1*, cagcatcgctctgttcctgta, ctgcgttttcatggagtctca; *Bglap*, agccttcatgtccaaggcaggag, gactgaggctccaaggtagcg; *Dmp1*, cttgtgttcctttgggggct, gccaaatcacccgtcctctc; and *Mepe*, tcctgaaggtgaatgacgcc, gtcttcattcggcattggtgc.

2.5. Statistics

Paired-sample *t*-tests, two sample *t*-tests and split plot ANOVAs were used as appropriate. For a split plot ANOVA, the mouse is considered a whole plot with each leg a split plot. Microsoft Excel was used for calculating the statistics. Data are presented as means ± SD.

3. Results and discussion

Our first attempt to get good quality bone marrow RNA was to spin the marrow out of the femur by centrifugation for 30 s at 5700 ×*g* into an empty tube, re-suspend the pellet of bone marrow cells in a small volume (50 μL) of PBS, followed by immediate addition of 1 mL TRI Reagent and incubation for 5 min at room temperature to lyse the cells. The tubes with TRI Reagent lysates were frozen on dry ice and transferred to a −80 °C freezer for long-term storage. TRI Reagent denatures and inactivates RNases, and storage of TRI Reagent lysates is commonly used in our laboratory to obtain high quality RNA from cell cultures. Surprisingly, the RNA recovered from these samples was of low quality with RIN numbers < 7. This indicated that cells were damaged with release of RNases during the initial centrifugation and resuspension. We then tested whether centrifugation directly into a small volume of the RNase-inhibiting compound RNA*later* would protect the RNA from degradation. Centrifugation into RNA*later* deposits the cells on top of the RNA*later*. The cells were resuspended in the RNA*later*, TRI Reagent was added, and the TRI Reagent lysate stored at −80 °C. This led to essentially intact, undegraded RNA (Fig. 1A). To test whether the protection was due to the RNA*later* per se or simply due to cushioning of cells when they are centrifuged into a liquid rather than into the bottom of a centrifuge tube, we compared centrifugation into RNA*later* to centrifugation into PBS using femurs from C57Bl/6J mice. Under these conditions, PBS resulted in good RNA quality. Importantly, the RNA integrity was significantly higher ($P = 6.3 \times 10^{-5}$) when RNA*later* was used (Fig. 1B), while the total RNA yield was lower (Fig. 1C).

Mineralized bone is not amenable for storage as a TRI Reagent lysate, as TRI Reagent does not dissolve the bone. Instead, it is common to store bone tissue at −80 °C after flash freezing in liquid nitrogen (Watt et al., 2018; Carter et al., 2012). As an alternative, we tested whether RNA*later* could be useful for preservation of femur shaft and the femur ends. We incubated freshly dissected femur shaft and femur ends in 1 mL RNA*later* at 4 °C for 24 h. RNA*later* was removed and the tubes with tissues stored at −80 °C. Our equipment for tissue crushing is a TissueLyser II which is a bead crusher. Rather than pulverizing bone in

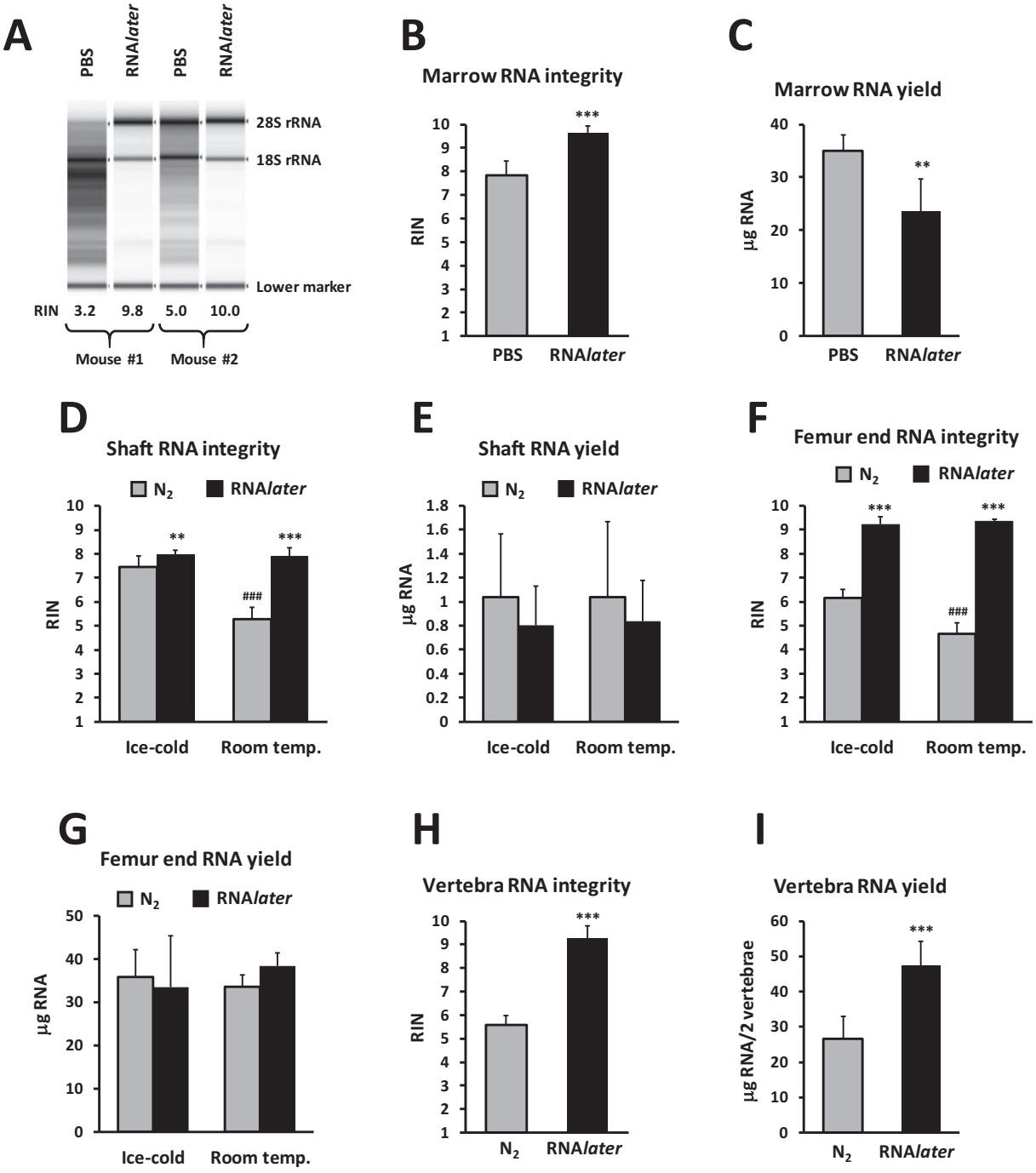

Fig. 1. Preservation of bone tissue with RNA*later* prevents subsequent degradation of RNA. (**A**) TapeStation of analysis of femur bone marrow RNA from two mice. Marrow from the left femurs was preserved as a TRI Reagent lysate after centrifugation and resuspension in PBS, whereas marrow from the right femurs was preserved as a TRI Reagent lysate after centrifugation of bone marrow cells directly into RNA*later*. RNA was isolated by the TRI Reagent method. (**B–C**) Bone marrow RNA integrity (**B**) and total yield of bone marrow RNA (**C**) were determined for femurs of 8 C57Bl/6J female mice. Marrows were preserved as TRI Reagent lysates after centrifugation into 80 µL PBS for one femur and 80 µL RNA*later* for the other femur of each mouse. RNA was isolated by the hybrid method. **, ***: $P < 0.01$, $P < 0.001$ vs. PBS by paired sample *t*-test. (**D–E**) RNA from femur bone shafts were isolated from 16 C57Bl/6J female mice. Femur shafts were preserved by flash freezing in liquid nitrogen (N_2) for one femur and by RNA*later* incubation for the other femur of each mouse. RNA was isolated by the hybrid method under ice-cold conditions (8 mice) or at room temperature conditions (8 mice) prior to TRI Reagent lysis. RNA integrity (**D**) and RNA yield (**E**) were determined. (**F–G**) RNA from femur ends was isolated from 16 C57Bl/6J female mice. Femur ends were preserved by flash freezing in liquid nitrogen (N_2) for one femur and by RNA*later* incubation for the other femur of each mouse. RNA was isolated by the hybrid method under ice-cold conditions (8 mice) or at room temperature conditions (8 mice) prior to TRI Reagent lysis. RNA integrity (**F**) and RNA yield (**G**) were determined. **, ***: $P < 0.01$, $P < 0.001$ vs. N_2 at the same temperature regimen; $^{\#\#\#}$: $P < 0.01$ vs N_2 at the ice-cold conditions by split plot design ANOVA. (**H–I**) The lumbar spine was preserved from 8 C57Bl/6 J female mice by flash freezing in N_2 and from 8 C57Bl/6J female mice by RNA*later* incubation. RNA from a pair of lumbar vertebrae was isolated by the hybrid method. RNA integrity (**H**) and RNA yield (**I**) were determined. ***: $P < 0.001$ vs. N_2 by two-sample *t*-test.

the presence of liquid nitrogen (Carter et al., 2012), we kept the shaft and femur ends frozen by keeping tubes on dry ice until the bone tissue was transferred to ice-cold TRI Reagent, immediately followed by crushing and lysis on the TissueLyser II. To assess the need for keeping tissue frozen until lysis, we also allowed some of the bone tissues to thaw for at least 5 min, and < 30 min, at room temperature (23 °C) before transfer to room temperature TRI Reagent. With RNA*later*, we obtained RIN numbers around 8.0 for the femur shaft and above 9.0 for the femur ends. The RNA integrity was significantly higher for samples treated with RNA*later* than for flash frozen bone samples (Fig. 1D and F). For flash frozen bone tissue, thawing to room temperature caused a significant decrease in RNA integrity, whereas exposure to room temperature did not compromise the quality of bone treated with RNA*later*. The yield of RNA was not significantly different between treatment groups for neither femur shaft nor femur ends (Fig. 1E and G).

The whole lumbar spine was also preserved by both flash-freezing in liquid nitrogen and storage in RNA*later* for 24 h at 4 °C before storage at −80 °C. Each spine was thawed for further cleaning of two vertebrae with removal of spinal cord and discs. Vertebrae were disrupted and lysed in TRI Reagent using the TissueLyser II instrument followed by RNA isolation by the hybrid method. Both the RNA integrity (Fig. 1H) and the RNA yield (Fig. 1I) were highly significantly increased by RNA*later*.

To demonstrate how the RNA integrity affects the qRT-PCR signals of some typical gene transcripts expressed in bone, we measured the expression of genes encoding RANKL (*Tnfsf11*), Osteocalcin (*Bglap*), osteocyte markers DMP1 (*Dmp1*) and MEPE (*Mepe*) (Bonewald, 2011), the erythroid cell marker ICAM-4 (*Icam4*) and β-Actin (*Actb*). The mRNA concentrations were expressed as the cycle threshold value (C_T) for samples diluted to 5 ng/μL. The lower the C_T, the higher the mRNA concentration with a difference in C_T of 1 corresponding to a 2-fold change in mRNA concentration. For bone marrow RNA, the higher integrity obtained with RNA*later* (mean RIN = 9.6) than with PBS (mean RIN = 7.8) was associated with small, but significant decreases in C_T values, i.e. significant increases in mRNA concentrations (Fig. 2A). The higher RNA integrity of shaft RNA from RNA*later*-treated bone shafts (mean RIN = 7.9) than from flash frozen bone shafts thawed to room temperature (mean RIN = 5.3) also gave significantly lower C_T values (Fig. 2B). As reported previously (Kelly et al., 2014), the shaft is enriched in Osteocalcin mRNA relative to the marrow, while the marrow is enriched in ICAM-4 mRNA. DMP1 mRNA and MEPE mRNA were also enriched in the shaft relative to the marrow. For femur ends thawed to room temperature after −80 °C storage where RNA*later* had the biggest effect on RNA integrity (mean RIN = 9.2 vs. mean RIN = 4.7), there were strong effects on the apparent mRNA concentrations in qRT-PCR (Fig. 2C). RNA*later* treated femur ends thus showed 5-fold higher ($P = 2.8 \times 10^{-6}$) and 28.6-fold higher ($P = 1.1 \times 10^{-9}$) concentrations of ICAM-4 and β-Actin mRNA, respectively, than flash-frozen femur ends. A spurious artifact caused by different mRNAs having different degradation rates was that the concentration of ICAM-4 mRNA relative to β-Actin mRNA appeared to be 5.7-fold higher ($P = 3.6 \times 10^{-8}$) in the flash-frozen than in the RNA*later*-treated femur ends.

We finally tested whether the TRI Reagent method and the hybrid RNA purification method gave differences in RNA integrity and in qRT-PCR responses (Fig. 3). RNA was isolated by the two methods from femur bone marrow samples and femur shafts after preservation with RNA*later*. The two methods did not show statistically significant differences for the RNA integrity (Fig. 3A and D), suggesting that the critical phase in protecting RNA from degradation occurs before TRI Reagent lysis. The RNA yields were not significantly different for the bone marrow (Fig. 3B), but the RNA yields of bone shafts were significantly lower with the hybrid method (Fig. 3E). In qRT-PCR, differences in C_T values for samples of the same RNA integrity diluted to the same concentration (5 ng/μL) becomes a measure of whether one sample contains more impurities inhibiting the qRT-PCR reaction than the other, as inhibition will shift amplification curves towards higher C_T values. The two methods did not show consistent qRT-PCR differences for bone marrow RNA (Fig. 3C), but C_T values were significantly lower for hybrid-purified femur shaft RNA for three of the four qRT-PCR targets (Fig. 3F). Thus, shaft RNA purified by the hybrid method seemed to exhibit less inhibition from impurities than the TRI Reagent method. The hybrid method may therefore be of advantage when the RNA yield of the tissue is relatively low, as is the case for the femur shaft.

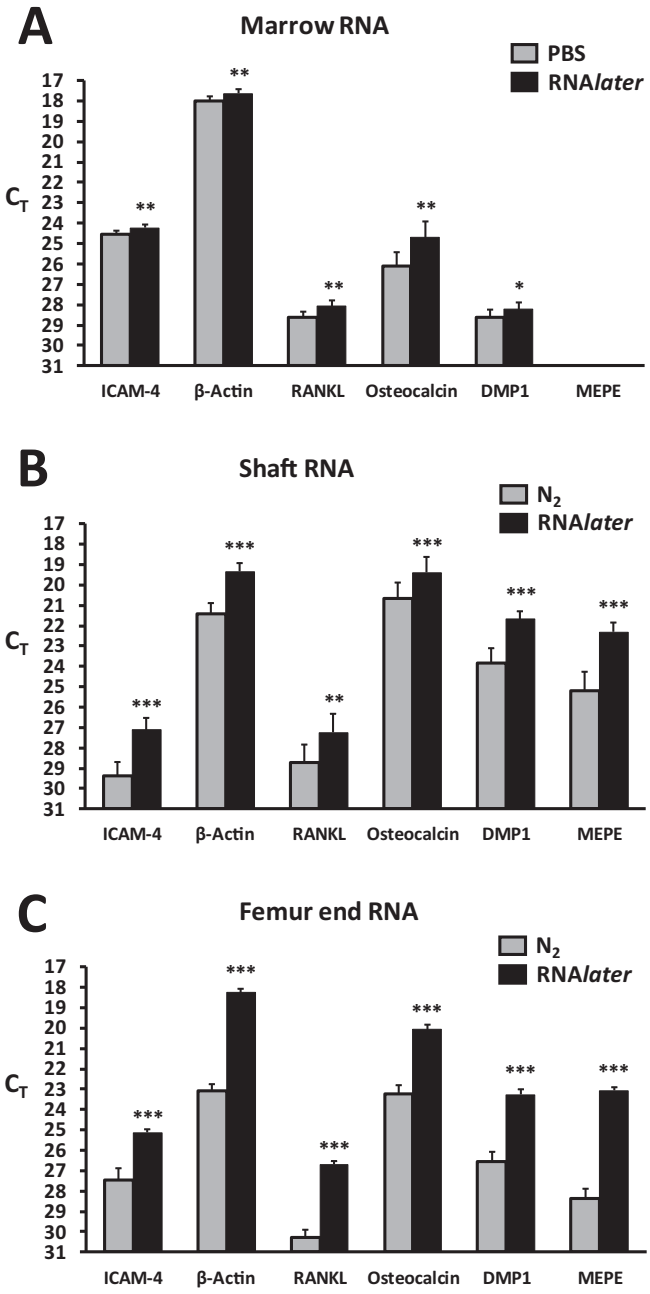

Fig. 2. Preservation of bone tissue with RNA*later* increases the amounts of mRNA detected in qRT-PCR. Femur marrow RNA (**A**), femur shaft RNA (**B**) and femur end RNA (**C**) diluted to 5 ng/μL were analyzed in qRT-PCR for the concentration of ICAM-4, β-Actin, RANKL, Osteocalcin, DMP1 and MEPE mRNA. The RNA was from femur tissue preserved in the absence (PBS resuspension or N_2 flash freezing) or presence of RNA*later*. RNA was isolated by the hybrid method. Femur shafts and femurs ends were thawed to room temperature prior to TRI Reagent lysis. Each column represents measurements from 8 femurs. *, **, ***: $P < 0.05$, $P < 0.01$, $P < 0.001$ in paired-sample *t*-tests.

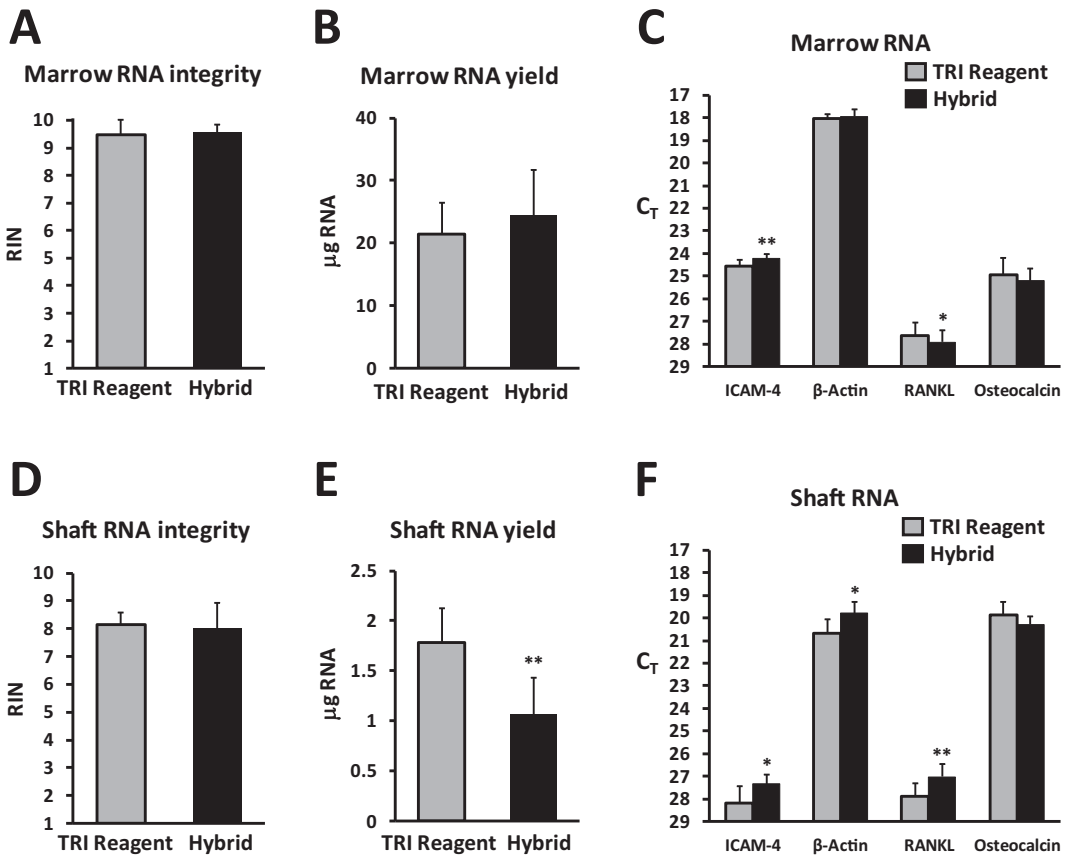

Fig. 3. Comparison of the TRI Reagent and the hybrid RNA isolating method. (**A–C**) Femur marrow was preserved from 7 C57Bl/6J female mice using RNA*later*. For each mouse, RNA was isolated from one femur by the TRI Reagent method and from the other femur by the hybrid method. The RNA integrity (**A**) and RNA yield (**B**) were determined. Four mRNA targets were quantified by qRT-PCR (**C**). (**D–F**) Femur shafts were preserved from 8 C57Bl/6J female mice using RNA*later*. For each mouse, RNA was isolated from one femur by the TRI Reagent method and from the other femur by the hybrid method with ice-cold conditions prior to TRI Reagent lysis. The RNA integrity (**D**) and RNA yield (**E**) were determined. Four mRNA targets were quantified by qRT-PCR (**F**). *, **: $P < 0.05$, $P < 0.01$ vs. TRI Reagent in paired-sample *t*-tests.

In this work, we did not attempt to remove DNA contamination as the primers for our qRT-PCR targets were designed to only allow amplification from mRNA. If DNA removal is necessary for downstream applications, a DNase treatment can be used. We have previously noted that the DNase treatment recommended by the manufacturer for use with RNeasy column is insufficient to remove all DNA. Instead, we have successfully used the Turbo DNA-free kit from Invitrogen in the past. RNeasy Plus kits from Qiagen contain so-called gDNA Eliminator spin columns for removal of genomic DNA. We have attempted to pass the aqueous phase from the TRI Reagent phase separation through the gDNA columns before continuing with the rest of the RNeasy protocol. TapeStation analysis of the resulting RNA did not show the two expected bands of 18S and 28S rRNA, but only a single band. The gDNA columns are therefore incompatible with the hybrid method in its current form.

A bead crusher such as TissueLyser II is convenient for isolating RNA from mineralized bone. However, a bead crusher is not necessary for obtaining high quality RNA from bone preserved with RNA*later*. As an example, we performed simple pulverization of 8 RNA*later*-preserved mouse femur shafts by immersing the shafts wrapped in aluminum foil in liquid nitrogen, immediately followed by crushing with a hammer. The crushed tissue was incubated in TRI Reagent followed by column purification of the RNA. No attempts were made to keep the bone tissue ice-cold before or after the pulverization. Yet, the RNA quality was as good as obtained with the TissueLyser II: RIN = 8.2 ± 0.6 (mean ± SD).

A key factor in acquiring high quality RNA was inclusion of RNA*later* in the preservation protocols. Interestingly, the RNA*later* guidelines suggest that RNA*later* may not be useful for bone tissue (Ambion by Life Technologies, 2011). While it is possible that penetration of RNA*later* into intact long bones like the femur may be insufficient to protect against bone marrow RNases, RNA*later* was highly efficient after cutting and separating the bone into shaft, marrow and femur ends. RNA*later* was also very effective in isolation of high-quality RNA from vertebral bone. Since observing the beneficial effects of RNA*later* on RNA quality, we have used the procedure with RNA*later* in our laboratory on a regular basis. As an indication of the robustness of the methodology, we have by now measured the RNA integrity of a total of 83 samples of bone marrow RNA isolated by the TRI Reagent protocol after preservation with RNA*later*. Of these, 74 samples (89%) gave a RIN number between 9 and 10. The great advantage with RNA*later* is that there is no need for using liquid nitrogen or maintaining ice-cold conditions prior to TRI Reagent lysis. In conclusion, we provide statistically validated methods allowing isolation at room temperature of high quality RNA from the femur shaft, femur bone marrow and vertebral bone.

Transparency document

The Transparency document associated with this article can be found, in online version.

Declaration of Competing Interest

The authors declare that they have no conflicts of interest.

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Author contributions

K.B.P. designed the study; K.B.P., J. W. and A.W. acquired the data; K.B.P. drafted the manuscript; all authors contributed to interpretation of the data and to critical revision and final approval of the manuscript.

Appendix A. Supplementary information

Supplementary information to this article can be found online at https://doi.org/10.1016/j.bonr.2019.100211.

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