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Extraction of High-Quality RNA from Human Articular Cartilage

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Abstract

Extracting high-quality RNA from articular cartilage is challenging due to low cellularity and high proteoglycan content. This problem hinders efficient application of RNA sequencing (RNA-seq) analysis in studying cartilage homeostasis. Here we developed a method that purifies high-quality RNA directly from cartilage. Our method optimized the collection and homogenization steps so as to minimize RNA degradation, and modified the conventional TRIzol protocol to enhance RNA purity. Cartilage RNA purified using our method has appropriate quality for RNA-seq experiments including an RNA integrity number of ~ 8. Our method also proved efficient in extracting high-quality RNA from subchondral bone.

RNA sequencing (RNA-seq) utilizes high-throughput next-generation sequencing (NGS) to analyze differential gene expression in healthy and diseased tissues [1]. Data generated from RNA-seq have become an essential tool to discover mechanisms and signaling pathways involved in normal development and disease conditions [1]. A pre-requisite for a reliable RNA-seq experiment is a high-quality RNA sample that fulfills two criteria: *i*) high purity, and *ii*) high integrity. RNA purity is evaluated by determining $A_{260/280}$ and $A_{260/230}$ ratios, with $A_{260/280}$ ratios appreciably < 1.8 and $A_{260/230}$ ratios appreciably < 2 generally indicating contaminated RNA samples. RNA integrity is gauged by RNA integrity number (RIN) that ranges from 1 to 10, with 1 being the most degraded RNA. Samples with RIN values < 7 are usually considered inappropriate for RNA-seq since degraded RNA leads to biased RNA-seq results. Although conventional RNA extraction protocols and kits are routinely used to purify high-quality RNA from different cell lines and tissues, preparing high-quality RNA from particular tissues remains challenging. Articular cartilage is among

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the tissues that challenge conventional RNA extraction protocols due to its low cellularity and high proteoglycan content that compromise RNA purity and yield [2, 3]. Furthermore, the rigid and dense matrix structure of the articular cartilage necessitates harsh homogenization conditions, which compromise RNA integrity [2, 3]. Accordingly, obtaining cartilage RNA with an $A_{260/280}$ ratio ~ 1.8 , an $A_{260/230}$ ratio ~ 2 , and a RIN value ~ 7 has never been achieved with any of the thus far available protocols [2, 3]. Enzymatic digestion methods that isolate chondrocytes from articular cartilage tissues prior to RNA extraction have been developed to overcome this problem [4]. However, concerns over these methods are always raised due to changes that occur in transcriptomic composition during the lengthy cell isolation procedures. Lack of a reproducible protocol to extract high-quality RNA directly from the cartilage impedes efficient application of RNA-seq in studying cartilage homeostasis. Here we report a new method that bridges this significant gap in the field.

Knee cartilage and subchondral bone samples were harvested from osteoarthritic patients undergoing total knee replacement surgeries. The protocol for collection of these tissues was approved by the University of Rochester IRB (protocol # RSRB00042321) and informed consent was obtained from all patients. Following surgical exposure of the joint, a scalpel was used to shave 3-5 mm-thick slices of articular cartilage from each femoral condyle, with tissue being removed from sites of intact cartilage. Importantly, this was performed prior to excising other tissues for the arthroplasty procedure. Thus, the cartilage was harvested from living tissue, not from joint fragments that were removed prior to fixation of hardware. Subchondral bone tissue was also collected, with fragments of excised bone immediately denuded of remaining cartilage to minimize tissue cross-contamination. Harvested slices of articular cartilage and subchondral bone fragments were immediately flash-frozen in liquid nitrogen and thereafter stored in a liquid nitrogen tank until used.

Harvested tissues (cartilage and bone) were pulverized using a cryogenic mill (SPEX SamplePrep 6870 Freezer/Mill®). The optimized grinding program included a total of four 3-min cycles of grinding, each at a frequency of 15 cycles per second (cps) followed by 2 min of cool down time to ensure optimal brittleness of the tissue throughout the procedure. One hundred mg of pulverized tissue was then transferred directly to an Eppendorf tube containing 1 ml TRIzol and incubated for 15 min at room temperature on an end-to-end rotator. Samples were then centrifuged at $12,000 \times g$ for 5 min at 4°C to pellet undigested tissue. Cleared supernatant was transferred to a new tube and passed 10 times through a 22G needle. Extruded material was mixed with 200 μL of chloroform by vigorous shaking for 30 seconds, left to stand for 3 min at room temperature, and then centrifuged at $12,000 \times g$ for 15 min at 4°C . The aqueous layer was transferred to a new tube and a mixture of concentrated sodium chloride and sodium acetate was added to achieve final concentrations of 1.2 M and 0.8 M, respectively. RNA was then precipitated by addition of 0.3 volume of 100% isopropanol followed by a 10 min incubation at room temperature. Centrifugation at $12,000 \times g$ for 10 min at 4°C facilitated pelleting of the RNA precipitate, which was washed twice using 1 ml of 75% (V/V) ethanol, dried for 5-10 min at 37°C , and re-constituted in RNase-free water.

Our method purified cartilage RNA with an average RIN value of 7.9 ± 0.3 (Fig. 1A, B), an average $A_{260/280}$ ratio of 1.8 ± 0.11 , and an average $A_{260/230}$ ratio of 1.9 ± 0.23 (Fig. 1C, F).

The average yield was 2.33 μ g RNA per 100 mg cartilage tissue. RIN values were determined using an Agilent Bioanalyzer 2100. Yield and $A_{260/280}$ and $A_{260/230}$ ratios were assessed using the NanoDrop[®] Spectrophotometer ND-1000. Importantly, all the results presented here are from isolation procedures carried out on osteoarthritic cartilages, indicating that obtaining high-quality RNA from a cartilage undergoing a degenerative disease is feasible. These results also demonstrate that the low-quality cartilage RNA obtained in previous studies was, at least in part, due to sub-optimal RNA extraction protocols rather than disease-associated RNA degradation.

The high RNA yield and integrity achieved by our method were the outcome of optimizing collection, storage and homogenization procedures. Cartilage tissues were flash-frozen in liquid nitrogen inside the operating room within 1–3 min of their removal from the patient. This step was crucial to quench RNA degradation processes as evidenced by a significant reduction in RIN values when cartilages were kept in saline or even in the RNase inhibitor solution *RNAlater*[®] during transport from the operating room to the laboratory (Fig. 1D, E). Failure of *RNAlater*[®] to maintain RNA integrity may be due to inefficient diffusion into the dense cartilage tissue. We therefore do not recommend exporting or storing cartilages in RNase inhibitor solutions; they should instead be flash frozen and transported/stored in liquid nitrogen.

As mentioned, the homogenization step was also performed in liquid nitrogen to dissipate heat generated during the pulverization process that would otherwise compromise RNA integrity. It is of note that we did not let intact or pulverized cartilages warm to temperatures above that of liquid nitrogen until they were mixed with TRIzol. Importantly, we examined homogenizing the samples using the traditional mortar and pestle method or the Bullet Blender 24 Gold[®] as less expensive alternatives to the programmable SPEX SamplePrep 6870 Freezer/Mill. We kept the sample in the mortar under liquid nitrogen throughout the homogenization process, while the grinding program of the Bullet Blender included a total of five 2-min cycles of grinding at 4°C, each was followed by 2 min of immersing the sample in liquid nitrogen. None of the two methods generated acceptable RNA yield or quality (data not shown).

Our RNA extraction method involved modification of several steps in the conventional TRIzol protocol to make it compatible with the pulverization step and to enhance RNA yield and purity. First, after incorporating the pulverized material into TRIzol, sealed tubes were placed on a rotator to maximize tissue exposure to the action of this reagent. Second, we centrifuged the TRIzol-homogenized tissue mixture before addition of chloroform to prevent carryover of undigested tissues to subsequent extraction steps and, thus, to enhance RNA purity. Third, we added 1.2 M of sodium chloride and 0.8 M of sodium acetate to the aqueous layer prior to RNA precipitation. This is a typical modification to the TRIzol protocol for tissues with a high content of proteoglycan (3). We thoroughly investigated the importance of this modification in the context of purifying RNA from articular cartilage by comparing our method to two other protocols where the addition of high salt concentration was omitted. In the first protocol we followed the exact steps of our method except for the addition of high salt concentration (Our method/Low salt in Fig. 1F). In the second protocol we combined TRIzol and silica-membrane RNA purification columns as follows. We

followed the steps of our method until we separated the aqueous layer, to which we added 1 volume of 70% ethanol, loaded the solution on an RNeasy[®] spin column (Qiagen), and followed the manufacturer's protocol to purify RNA (TRIzol/RNeasy in Fig. 1F). Both protocols resulted in a significant reduction in RNA purity (Fig. 1F), which confirms the necessity of using a high concentration of salt to isolate pure RNA from articular cartilage homogenates. Notably, our method purifies cartilage RNA with appropriate purity even though it involves only one phase-separation step; unlike other methods that require two phase-separation steps prior to high salt RNA precipitation (3). The last modification to the TRIzol protocol was based on previous reports (3) and included washing RNA precipitates twice to remove traces of salt given the high salt concentration used in the precipitation step. It is noteworthy that using more than 100 mg of cartilage tissue in our method significantly reduced RNA yield and purity. If purifying RNA from more than 100 mg of cartilage is desired, we recommend scaling up the volume of TRIzol accordingly, or, alternatively, dividing the tissue into 100 mg portions followed by combining the resulting aliquots of purified RNA.

To further investigate whether our method is applicable to other tissues with high extracellular matrix content, we applied it to extract RNA from subchondral bones. One hundred mg of pulverized bone produced 10.13 μ g of RNA with an average RIN value of 8.3 ± 0.2 (Fig. 2A, B), and average $A_{260/280}$ and $A_{260/230}$ ratios of 1.93 ± 0.11 and 2.23 ± 0.09 , respectively (Fig. 2C). Notably, homogenizing subchondral bones in liquid nitrogen using a mortar and pestle yielded RNA with quality comparable to that obtained with the SPEX SamplePrep 6870 Freezer/Mill: an average RIN value of 8.5 ± 0.5 (Fig. 2D, E), and average $A_{260/280}$ and $A_{260/230}$ ratios of 1.96 ± 0.085 and 2.18 ± 0.17 , respectively. Accordingly, a mortar and pestle can be used in our method instead of the programmable Freezer/Mill to homogenize subchondral bones, but not cartilages, without sacrificing RNA quality. These data indicate that our method proves efficient in extracting high-quality RNAs from various tissues with a high content of dense extracellular matrix.

To investigate the impact of RNA integrity on the reproducibility of RNA quantitation results, we used reverse transcription coupled to real-time polymerase chain reaction (RT-qPCR) to analyze the expression of β -actin mRNA in RNA samples that had RIN values ranging from 3.5 to 8.0. β -actin mRNA was analyzed in each sample using 3 different primer pairs, where the first primer pair spanned sequences within the first exon, the second primer pair spanned exon 2–exon 3 junction, and the third primer pair spanned sequences within the last exon (exon 6) (Fig. 2F). We observed very high variability among the results obtained from the three primer pairs when β -actin mRNA was analyzed in RNA samples with low RIN values (Fig. 2G). On the other hand, samples with the highest RIN values showed the lowest variability (Fig. 2G). These data demonstrate how RT-qPCR results generated from RNA samples with low RIN values might change dramatically according to the location of the primer pair within the analyzed mRNA. Our results highlight the importance of high RNA integrity for the reliability of not only RNA-seq analyses but also routine RT-qPCR assays.

Overall, the method we have optimized and are presenting here opens the door for more reliable global transcriptome analyses of human cartilage, an area of current focus in the

osteoarthritis field [5]. This advance will support deeper understanding of cartilage homeostasis and causes of cartilage degenerative diseases including osteoarthritis, for which there is no accepted disease-modifying treatment [6].

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Fig 1.

Optimized collection and extraction protocols improve the quality of RNA extracted from human knee articular cartilage. (A) Electrophoretic profiles of two RNA samples isolated from flash-frozen human articular knee cartilage. The locations of 18S and 28S ribosomal RNA bands are indicated and the RIN value of each sample is given. (B) Electropherograms of samples analyzed in (A). (C) NanoDrop-generated UV spectra of samples analyzed in (A). $A_{260/280}$ and $A_{260/230}$ ratios calculated from each spectrum are shown. (D) is as in (A), except RNA was isolated from cartilages transported on saline or on RNAlater®. (E) is as in (B), except samples shown in (D) were analyzed. (F) Tabulation of $A_{260/280}$ and $A_{260/230}$ ratios calculated for RNA samples purified using the indicated extraction protocols. Data represent the mean $\pm$ SD; n=6 for Our method; n=3 for Our method/Low salt and TRIzol/RNeasy.

Fig 2.

Our optimized method purifies high-quality RNA from subchondral bone. (A) Electrophoretic profiles of two RNA samples isolated from flash-frozen human subchondral bone. (B) Electropherograms of samples analyzed in (A). (C) NanoDrop-generated UV spectra of samples analyzed in (A). (D) As is (A) except that samples were homogenized using a mortar and pestle instead of the programmable Freezer/Mill. (E) Electropherograms of samples analyzed in (D). (F) A diagram of the β -actin mRNA showing the location of the three primer pairs used in qPCR analyses. (G) RT-qPCR analysis of β -actin mRNA in 9 RNA samples with RIN values ranging from 3.5 to 8.0 was performed. In each sample, β -actin mRNA was analyzed using the three primer pairs shown in (F). One sample was set as a reference sample, and the difference in the cycle threshold value (C_t) between the 8 remaining samples and the reference sample was calculated and plotted against the corresponding RIN values. Each sample was run in triplicate; results represent the mean $\pm$ SD.