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TITLE:

Tissue Collection and RNA Extraction from the Human Osteoarthritic Knee Joint

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SUMMARY:

Primary tissues obtained from patients following total knee arthroplasty provide an experimental model for osteoarthritis research with maximal clinical translatability. This protocol describes how to identify, process, and isolate RNA from seven unique knee tissues to support mechanistic investigation in human osteoarthritis.

ABSTRACT:

Osteoarthritis (OA) is a chronic and degenerative joint disease most often affecting the knee. As there is currently no cure, total knee arthroplasty (TKA) is a common surgical intervention. Experiments using primary human OA tissues obtained from TKA provide the capability to investigate disease mechanisms *ex vivo*. While OA was previously thought to impact mainly the cartilage, it is now known to impact multiple tissues in the joint. This protocol describes patient selection, sample processing, tissue homogenization, RNA extraction, and quality control (based on RNA purity, integrity, and yield) from each of the seven unique tissues to support disease mechanism investigation in the knee joint. With informed consent, samples were obtained from patients undergoing TKA for OA. Tissues were dissected, washed, and stored within 4 h of surgery by flash freezing for RNA or formalin fixation for histology. Collected tissues included articular cartilage, subchondral bone, meniscus, infrapatellar fat pad, anterior cruciate ligament, synovium, and vastus medialis oblique muscle. RNA extraction protocols were tested for each tissue type. The most significant modification involved the method of disintegration used for low-cell, high-matrix, hard tissues (considered as cartilage, bone, and meniscus) versus relatively high-cell, low-matrix, soft tissues (considered as fat pad, ligament, synovium, and muscle). It was found that pulverization was appropriate for hard tissues, and homogenization was appropriate for soft tissues. A proclivity for some subjects to yield higher RNA integrity number (RIN) values than other subjects consistently across multiple tissues were observed, suggesting that

underlying factors such as disease severity may impact RNA quality. The ability to isolate high-quality RNA from primary human OA tissues provides a physiologically relevant model for sophisticated gene expression experiments, including sequencing, that can lead to clinical insights that are more readily translated to patients.

INTRODUCTION:

The knee is the largest synovial joint in the human body, comprising the tibiofemoral joint between the tibia and the femur and the patellofemoral joint between the patella and the femur[1]. The bones in the knee are lined with articular cartilage and supported by various connective tissues, including menisci, fat, ligaments, and muscle, and a synovial membrane encapsulates the whole joint to create a synovial fluid-filled cavity[1,2–3] (**Figure 1**). A healthy knee functions as a mobile hinge joint that allows frictionless motion in the frontal plane[1,3]. Under pathological conditions, movement can become restricted and painful. The most common degenerative knee joint disease is osteoarthritis (OA)[4]. A variety of risk factors are known to predispose to OA development, including older age, obesity, female sex, joint trauma, and genetics, among others[5,6]. There are currently an estimated 14 million people in the USA with symptomatic knee OA, with the prevalence increasing due to rising population age and rates of obesity[7,8]. Initially considered to be a disease of the cartilage, OA is now understood as a disease of the whole joint[9]. Commonly observed pathological changes in OA include articular cartilage erosion, osteophyte formation, subchondral bone thickening, and inflammation of the synovium[9,10]. Since there is no known cure for OA, treatments primarily focus on symptom (e.g., pain) management[11,12], and once OA has progressed to end-stage, joint replacement surgery is often indicated[13].

Joint replacement surgeries can either be partial or total knee replacements, with total knee arthroplasty (TKA), including replacing the entire tibiofemoral articulation and the patellofemoral joint. As of 2020, approximately 1 million TKAs are performed in the USA each year[14]. During TKA, an orthopedic surgeon resects the upper portion of the tibial plateau and the lower femoral condyles (**Figure 2A,2B**) to be fitted with prosthetic implants. Sometimes misinterpreted by patients, in a TKA, only 8–10 mm is resected from the end of each bone, subsequently capped or resurfaced, with metal. An interposed polyethylene liner forms the bearing surface (i.e., padding) between the two metal implants. In addition, several soft tissue components of the joint are fully or partially excised to achieve proper joint balance. Among these tissues are the medial and lateral menisci (**Figure 2C**), infrapatellar fat pad (**Figure 2D**), anterior cruciate ligament (ACL; **Figure 2E**), synovium (**Figure 2F**), and vastus medialis oblique muscle (VMO; **Figure 2G**)[15]. Though TKAs are generally successful for OA treatment, around 20% of patients report reoccurrence of pain post-surgery[16]. Along with the high cost and relative invasiveness of the procedure, these limitations point to the need for further research to identify alternative treatments to mitigate the progression of OA.

To explore disease mechanisms in OA that may present new avenues for therapeutic intervention, experimental systems, including cells, tissue explants, and animal models can be used. Cells are typically cultured in monolayer and are derived from primary human or animal tissues (e.g., chondrocytes isolated from cartilage) or immortalized cells (e.g., ATDC5[17] and

CHON-001[18]). While cells can be useful for manipulating experimental variables in a controlled culture environment, they do not capture conditions of the natural joint which are known to impact cell phenotypes[19]. To better recapitulate the complex cascade of chemical, mechanical, and cell-to-cell communication underlying OA, an alternative is found in primary human or animal tissue samples, whether used fresh or cultured *ex vivo* as explants, to preserve tissue structure and the cell microenvrionment[20]. In order to study the joint *in vivo*, small (e.g., mouse[21]) and large (e.g., horse[22]) animal models for OA (e.g., through surgical induction, genetic alteration, or aging) are also useful. However, translation from these models to human disease can be limited by anatomical, physiological, and metabolic differences, among others[23]. Considering the advantages and disadvantages of experimental systems, the key strengths of being species-specific and maintenance of the extracellular niche offered by the primary human OA tissues maximize the translational potential of research findings.

Primary human OA tissues can be readily obtained following TKA, making the TKAs performed at a high rate a valuable resource for research. Among potential experimental applications are gene expression and histological analyses. To realize the potential of primary human OA tissues for these research approaches and others, outlined are the following key considerations. First, the use of patient specimens is subject to ethical regulation, and protocols must meet Institutional Review Board (IRB) approvals[24]. Second, the inherent heterogeneity of human primary diseased tissues and the influence of variables such as age and sex, among others, create the need for careful patient selection (i.e., application of eligibility criteria) and data interpretation. Third, the unique biological properties of different tissues in the joint (e.g., low cellularity of cartilage and mensicus[25]) can present challenges during experiments (e.g., isolating high quality and quantity of RNA). This report addresses these considerations and presents a protocol for patient selection, sample processing, tissue homogenization, RNA extraction, and quality control (i.e., assessment of RNA purity and integrity; **Figure 3**) to encourage the use of primary human OA tissues in the research community.

PROTOCOL:

This study protocol was approved and followed institutional guidelines set by the Henry Ford Health System Institutional Review Board (IRB #13995).

1. Patient selection

1.1. Identify the patients from among those scheduled to undergo total knee arthroplasty with an orthopedic surgeon.

1.2. Select the patients based on the eligibility criteria defined by the study protocol. Examples of inclusion criteria include being 18 years of age or older and having a confirmed diagnosis of knee osteoarthritis. Examples of exclusion criteria include undergoing partial knee replacement or having a confirmed diagnosis of rheumatoid arthritis.

1.3. Contact the patients to obtain informed consent prior to the surgery.

2. Sample processing (for RNA)

NOTE: Perform all tissue processing in a class II biosafety cabinet and follow sterile techniques. Always wear appropriate PPE (nitrile gloves, lab coat, safety goggles) when processing human samples. Several bone fragments are produced during TKA, a large amount of bone/cartilage will potentially be available for dissection. Due to disease progression, articular cartilage degeneration may be more severe on some bone portions, which can be factored into experimental design. Only tissues that mandate electrocautery for resection have thermal edge damage, and a concerted surgical effort is made to procure most tissues with a scalpel to minimize damage. Resected tissues must be kept hydrated at all times with sterile PBS.

2.1. Disinfect all work surfaces and equipment with 70% ethanol, RNase decontaminant, DEPC-treated water, and again with 70% ethanol. Wipe away residual liquid with clean, lint-free tissues. Forceps, bone cutters, and scalpels are either autoclaved or soaked in 70% ethanol for at least 10 min prior to use. Keep equipment submerged in 70% ethanol when not in immediate use.

2.2. Pre-label at least three cryovials with de-identified sample name and aliquot number for each tissue (e.g., TKA-1 Cartilage 1).

2.2.1. Identify each tissue type from the specimen based on the differences in size, shape, color, and texture as shown and described in **Figure 2**. Identify the cartilage (**Figure 2A** arrow), bone (**Figure 2B** arrow), meniscus (**Figure 2C**), infrapatellar fat pad (**Figure 2D**), anterior cruciate ligament (**Figure 2E**), synovium (**Figure 2F**), and the Vastus medialis oblique muscle (**Figure 2G**).

2.3. Isolate the articular cartilage.

2.3.1. Select a bone portion with minimal cartilage degradation.

2.3.2. Using a No.10 scalpel, cut through the cartilage depth as far as possible to dissect the full thickness of the cartilage layer.

NOTE: A No.10 scalpel will only penetrate cartilage, not bone.

2.3.3. Using the full thickness of the cartilage, dissect three equal-sized portions (~2 cm^2 each).

2.4. Isolate the subchondral bone.

2.4.1. Use the same bone section from which cartilage was collected.

2.4.2. Using a No.10 scalpel, scrape any remaining cartilage and residual tissues from the bone surface.

2.4.3. Hold the bone portion to be cut with forceps and use the bone cutters to cut three equal-

sized portions (~2 cm^2 each).

2.5. Isolate the meniscus.

2.5.1. Identify a relatively undamaged portion of either medial or lateral meniscus.

2.5.2. Using a No.10 scalpel and forceps, cut the tissue into three equal-sized and homogeneous portions (~2 cm^2 each).

2.6. Isolate the infrapatellar fat pad.

2.6.1. Identify the fat pad.

2.6.2. Using a No.10 scalpel and forceps, cut the yellow portion of the tissue into three equal-sized and homogenous portions (~500 mg each).

2.7. Isolate the anterior cruciate ligament (ACL).

2.7.1. Identify the ACL.

2.7.2. Using a No.10 scalpel and forceps, cut the ligament into three equal-sized and homogenous portions (~2 cm^2 each).

2.8. Isolate the synovium.

2.8.1. Identify the synovium.

2.8.2. Using a No.10 scalpel and forceps, isolate the pink cellular portion of the membrane as much as possible by scraping away fat tissue.

2.8.3. Cut the synovial tissue into three equal-sized and homogenous portions (~2 cm^2 each).

2.9. Isolate the vastus medialis oblique muscle (VMO)

2.9.1. Identify the VMO.

2.9.2. Using a No.10 scalpel and forceps, remove any fat tissue from the specimen, leaving only the red muscle tissue.

2.9.3. Cut the muscle tissue into three equal-sized and homogenous portions (~1–2 cm^2 each).

NOTE: The initial size of the tissue limits the size of the portions.

2.10. Rinse the tissue portions with sterile PBS to remove any residue or debris.

2.11. To perform histology, fix each tissue portion as described in section 3.

2.12. To perform RNA extraction, cut each of the three tissue portions into smaller pieces (~1–2 mm^2).

2.12.1. Transfer the smaller pieces to a 2 mL cryovial, secure caps tightly, flash freeze by submerging in liquid nitrogen for 30 s, and then transfer to a -80 °C freezer for long-term storage (up to 4 months tested in the current protocol). Repeat this for all the aliquots of all the tissues.

2.12.2. Continue to homogenize the tissue as described in section 4.

3. Sample processing (for histology)

CAUTION: Formalin is a hazardous chemical, only use in a chemical fume hood.

3.1. Fill pre-labeled 15 mL conical tubes with 10% formalin solution.

3.2. Using forceps, transfer the tissue section to the formalin-filled tubes.

3.3. Fix the tissues in formalin for 1 week at room temperature while shaking/agitating if possible.

3.4. After 1 week, discard the formalin into proper chemical waste disposal, rinse the tissues with PBS, and then transfer to a fresh 15 mL conical tube containing 70% ethanol for long-term storage at 4 °C until samples are embedded for sectioning.

NOTE: For bone/cartilage only, perform decalcification as follows.

3.5. After 1 week, discard the formalin into proper chemical waste disposal, and rinse the tissues with PBS.

3.6. Transfer the tissue to a 50 mL conical tube with 45 mL of 10% EDTA solution (pH 7.4).

3.7. If shaking/agitation is not possible, invert the tubes 10–15 times once daily.

3.8. Discard and replace the EDTA solution once weekly.

3.8.1. Twice weekly, test the texture with an instrument (i.e., spatula) or gloved finger to confirm decalcification by observing deformation when pressure is applied. Time required to decalcify bone tissue will vary between samples from 4–6 weeks.

3.9. Once decalcified, rinse the tissue with PBS and transfer to a fresh 15 mL conical tube containing 70% ethanol for long-term storage at 4 °C until samples are embedded for sectioning.

4. Tissue homogenization

CAUTION: The protocol uses the hazardous chemical phenol. Work with phenol must be performed in a chemical fume hood.

NOTE: Thoroughly clean all equipment and surfaces to be used with 70% ethanol (soak for a minimum of 10 min), followed by RNase decontaminant (soak for a minimum of 10 min), rinse with DEPC-treated water, wipe with a clean, lint-free paper towel, and then respray or soak with 70% ethanol.

4.1. **Hard tissue homogenization (articular cartilage, subchondral bone, meniscus)**

4.1.1. Before homogenization, chill the mortar, pestle, and spatula using liquid nitrogen. These must be kept as cold as possible to prevent sample thaw.

4.1.2. Process the samples one at a time, keeping the other samples at -80 °C until used.

4.1.3. Transfer the tissue sample to mortar using a chilled spatula; pour additional liquid nitrogen on top of the tissue and allow it to evaporate. Crush the tissue using the pestle. Repeatedly add more liquid nitrogen to the tissue sample, allow it to evaporate, and then continue grinding with the pestle to make a fine powder.

4.1.4. After powdering the tissue as much as possible, transfer to a pre-chilled 1.5 mL microcentrifuge tube.

4.1.4.1. Pre-chill tubes by submerging in liquid nitrogen for 30 s prior to tissue transfer.

4.1.5. Add 1 mL of the acid-guanidinium-phenol solution to each tube and keep it on ice.

4.1.6. Repeat steps 4.1.3–4.1.5 for each hard tissue sample.

4.1.7. After each tissue, clean the mortar, pestle, and spatula with 70% ethanol, RNase decontaminant, DEPC-treated water, and an additional soak in 70% ethanol. Wipe any residual liquid with a clean, lint-free tissue.

4.1.8. Incubate the samples on ice for an additional 20 min.

4.2. **Soft tissue homogenization (infrapatellar fat pad, ACL, synovium, VMO)**

4.2.1. Disinfect the homogenizer by running tubes of 70% ethanol, RNase decontaminant, DEPC-treated water, and an additional 70% ethanol wash, each for 30 s. Wipe any residual liquid with a clean, lint-free tissue. Repeat this between each sample.

4.2.2. Pre-label 5 mL round bottom tubes for each sample. Add 1 mL of the acid-guanidinium-

phenol solution to each tube.

4.2.2.1. Work on one sample at a time, keeping other samples to be processed at -80 °C until use.

4.2.3. Transfer the tissue to a pre-labeled 5 mL tube with acid-guanidinium-phenol.

4.2.4. Homogenize the tissues in 30 s pulses, keeping on the ice during and between pulses. Repeat until the tissue is visually dissolved or for a maximum of five 30 s pulses.

NOTE: Some fibrous tissues (i.e., muscle) may not thoroughly homogenize.

4.2.5. Incubate the dissolved tissue on ice and move to the next sample.

4.2.5.1. Clean the homogenizer as described in step 4.2.1. Ensure tissue chunks do not remain in the teeth of the probe; remove with sterile forceps if needed.

4.2.6. After homogenization of all the samples, incubate on ice in acid-guanidinium-phenol for an additional 20 min.

4.2.7. Transfer the samples from round bottom tubes to pre-labeled and pre-chilled 1.5 mL microcentrifuge tubes.

5. RNA extraction from tissues

CAUTION: This protocol uses hazardous chemicals such as phenol, chloroform, and isopropanol. Perform all the work in a chemical fume hood.

NOTE: Equipment and reagents are reserved for RNA work only and must be of proper chemical grade for molecular applications (i.e., sterile, nuclease-free). This protocol succeeds both **Parts** 4.1 and 4.2 tissue homogenization protocols. Thoroughly clean all equipment and surfaces to be used with 70% ethanol (soak for a minimum of 10 min), followed by RNase decontaminant (soak for a minimum of 10 min). Rinse with DEPC-treated water, wipe the residual liquid with a clean, lint-free paper towel, and then respray or soak with 70% ethanol.

5.1. Centrifuge the microcentrifuge tubes at 10000 x *g* for 10 min at 4 °C to pellet the debris.

5.2. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube.

NOTE: For fatty tissues, a lipid layer will sometimes be present on the top. Avoid transferring this by piercing the layer on the side of the tube with a pipette tip.

5.3. Add 200 µL of chloroform per 1 mL of the acid-guanidinium-phenol solution to each sample. Shake the tubes vigorously by hand for 30 s to mix. Then, incubate on ice for 2 min.

5.4. Centrifuge samples at 10000 x *g* for 12 min at 4 °C.

NOTE: After centrifugation, three layers will have formed: an aqueous phase containing RNA, a white DNA interphase, and a pink protein phase at the bottom.

5.5. Transfer ~500 µL of the upper, aqueous phase to a fresh 1.5 mL microcentrifuge tube.

NOTE: Do not disturb the interphase and the protein fractions. Store these phases at -80 °C for future DNA or protein isolation.

5.6. Add an equal volume of acid-guanidinium-phenol solution to the transferred aqueous phase, mix by inverting the tube 8–10 times. Incubate the tube on ice for 20 min.

5.7. Add 200 µL of chloroform per 1 mL of the acid-guanidinium-phenol solution to each sample. Shake vigorously by hand for 30 s to mix and then incubate on ice for 2 min.

5.8. Centrifuge samples at 10000 x *g* for 12 min at 4 °C.

5.9. Transfer <500 µL of the aqueous phase to a fresh microcentrifuge tube, careful not to contaminate the sample with the other phases.

CAUTION: Discard the remaining phases with appropriate hazardous material disposal methods.

5.10. Add an equal volume (as aqueous phase) of 100% isopropanol to each sample. Mix by inverting the tube 8–10 times. Incubate on ice for 5 min.

5.10.1. Add 1 µL of glycogen coprecipitant to each sample to aid in locating the RNA pellet post-centrifugation.

5.11. Centrifuge the samples at 12000 x *g* for 25 min at 4 °C.

5.12. Locate the pellet in the tube (if using coprecipitant, it will appear blue). Carefully pour off the supernatant.

5.13. Wash the pellet by adding 1 mL of 75% ethanol to each sample, vortex to dislodge the pellet from the bottom of the tube.

NOTE: Prepare 75% ethanol using molecular grade pure ethanol and nuclease-free water.

5.14. Centrifuge at 7000 x *g* for 5 min at 4 °C. Carefully pour off the supernatant.

5.15. Repeat steps 5.13 and 5.14 two more times.

5.16. Quick spin (<2000 x *g* for 5 s) to bring any residual liquid to the bottom of the tube. Use a P20 pipette to remove any residual ethanol from the bottom of the tubes.

5.16.1. Avoid touching the RNA pellet with a pipette tip. Change tips between samples.

5.17. With tube caps open, air dry samples at room temperature for 10 min.

NOTE: Pellet may become translucent as it dries. Ensuring all residual ethanol has evaporated will improve RNA purity.

5.18. Add 25 µL of nuclease-free water to each tube to dissolve the pellet.

5.19. Incubate at room temperature for 5 min.

5.20. Gently pipette up and down to mix the RNA.

5.21. Aliquot 5 µL of the sample into a fresh tube for quality control analysis (section 6). Store the remaining 20 µL at -80 °C for gene expression assays.

6. Quality control

6.1. Determine the concentration and purity of RNA using a spectrophotometer according to the manufacturer's instructions.

6.2. Determine the RNA integrity using an electrophoresis device according to the manufacturer's instructions.

NOTE: Dilute the RNA to be within the range of the chip detection limits.

REPRESENTATIVE RESULTS:

Seven unique human knee joint tissues are available for collection from patients undergoing TKA for OA (**Figure 1**). In this protocol, each of these tissues were identified and processed within 4 h of surgical removal (**Figure 2**). Following the steps outlined in **Figure 3**, portions of each tissue were formalin-fixed for histological assessment (**Figure 4**), while other portions were flash-frozen for RNA isolation. Separating hard tissues from soft tissues by method of disintegration (pulverization versus homogenization, respectively), RNA of high integrity and purity was extracted from each tissue type, with representative results shown in **Table 1** (High quality columns). Notably, some subjects yielded lower quality RNA across multiple tissues (**Table 1**, Low quality columns), suggesting that despite an optimized method, external factors (e.g., disease severity) may be impacting RNA quality across tissue types.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic of the human knee joint showing a lateral cross-section. Each of the seven labeled tissues are collected during TKA and used for research purposes as described in this

protocol. VMO = vastus medialis oblique muscle. Image accessed and modified from OpenStax College under a creative commons license[26].

Figure 2: Representative gross images for each of the seven tissues obtained from patients undergoing TKA. (**A**) Anterior femoral bone cut with arrow pointing to articular cartilage to be collected. Cartilage is identified by a whitish layer found on the surface of the bone. (**B**) Anterior femoral bone cut with arrow pointing to subchondral bone to be collected. (**C**) Meniscus. Avoid collecting burnt sections caused by electrocauterization during TKA. (**D**) Infrapatellar fat pad (yellow in color). (**E**) Anterior cruciate ligament (white, fibrous, spongy tissue). (**F**) Synovium. One side will appear light colored and fibrous, often containing fat tissue, while the opposite side will appear pinkish and less fibrous. The pink side of the membrane contains the synovial lining. (**G**) Vastus medialis oblique muscle (red). This may often be the smallest tissue portion (5–10 cm^2) and may contain some fat tissue. Scale bar = 2 cm.

Figure 3: Overview of steps taken to collect primary human OA tissues for histology and RNA. This protocol describes patient selection, sample processing for histology and RNA, tissue homogenization for hard tissues and soft tissues, RNA extraction, and quality control for seven primary human knee OA tissues.

Figure 4: Histological sections for each of the seven tissues obtained from patients undergoing TKA. Hematoxylin and eosin-stained sections are shown at 6x magnification in panels **A–F** with inset magnified to 40x in panels **A'–F'** and **A"**. (**A**, **A'**) Articular cartilage; (**A**, **A")** subchondral bone; (**B**, **B'**) meniscus; (**C**, **C'**) infrapatellar fat pad; (**D**, **D'**) anterior cruciate ligament; (**E**, **E'**) synovium; (**F**, **F'**) vastus medialis oblique muscle. Scale bars = 400 µm for A–F and 50 µm for A'–F' and A".

Table 1. Quality and quantity of RNA isolated from OA tissues collected from TKA patients. RIN = RNA Integrity Number. Data presented as mean ± standard deviation for RIN, A_{260}:A_{280} and A_{260}:A_{230} ratios, and mean (range) for RNA concentration values. High quality samples consist of patients from which all tissue types yielded RNA with RIN > 6 (n = 8–10). Low quality samples consist of patients from which multiple tissue types yielded RNA with RIN < 6 (n = 3–4).

DISCUSSION:

The protocol presented has proved successful for collecting seven primary human OA tissues for RNA extraction (**Table 1**) and histological processing (**Figure 4**). Prior to collecting patient samples, it is necessary to establish an IRB-approved protocol, ideally in collaboration with a surgeon or surgical team. Applying a standardized protocol for specimen collection (e.g., resection from consistent *in situ* locations) is essential for maximizing experimental reproducibility. Tissue samples should be transported to the lab in sterile containers and processed within 4 h of the surgery to avoid degradation. During tissue dissection and processing, all tissues are kept hydrated in sterile PBS and are rinsed in fresh, sterile PBS to remove potential surface contaminants such as biofluids and other unwanted debris prior to being flash-frozen for RNA extraction or formalin-fixed for histology. A useful application of histological analysis is confirmation of the tissue types and the disease severity since these can be distinguished by cell number, distribution, and morphology, among other factors observable by standard stains such

as hematoxylin and eosin (**Figure 4**).

Primary human OA tissues can present challenges for extracting RNA of sufficient quantity and quality, as defined by purity and integrity[27]. RNA quantity is a function of the overall cellularity of the tissue, and in the knee joint, there are low-cell, high-matrix tissues such as the cartilage, bone, and meniscus, and relatively high-cell, low-matrix tissues such as the fat pad, ACL, synovium, and VMO. For example, both articular hyaline cartilage and meniscal fibrocartilage[28] are characterized by low cellularity, with the extracellular matrix containing varying quantities of collagens, proteoglycans, and other glycoproteins[28,29]. Having fewer cells results in less RNA per volume of tissue (reducing quantity) and having more protein results in co-purification with RNA (reducing purity)[25,30]. RNA purity can be determined by spectrophotometry where A_{260}:A_{280} and A_{260}:A_{230} values of <1.5 reflect the presence of organic contaminants (e.g., protein) and values of ~2.0 reflect pure RNA[31]. RNA integrity reflects the level of degradation, whether caused by experimental conditions (i.e., shearing forces) or by enzymatic digestion (e.g., nucleases), and is often determined by electrophoretic analysis. An RNA Integrity Number (RIN) of 1 reflects degraded RNA and a RIN of 10 reflects intact RNA[31,32]. For RNA-sequencing, a minimum RIN of 7 is often recommended[33–35]. Data presented in **Table 1** reveals that these A_{260}:A_{280}, A_{260}:A_{230}, and RIN thresholds were met across all tissues from the patient samples in the High quality RNA group compared to patient samples in the Low quality RNA group, except for some A_{260}:A_{230} values, which may reflect protein contamination of RNA in the low-cell, high-matrix tissues. While there are many factors that could be contributing to the quality of RNA isolated from a given patient sample, among them may be the level of disease severity. The diseased nature of OA tissues suggests degradative processes are occurring through increased levels of enzymes that can digest tissues, but also RNA, thereby reducing quality.

This protocol for RNA extraction aims to maximize RNA quantity and quality from primary human OA tissues. The most critical step pertained to whether the tissues were disintegrated by being pulverized or homogenized, and this was found to correlate with the tissue cellularity and matrix composition. Initially, all seven tissues were subjected to the same protocol where tissues were first pulverized by mortar and pestle using liquid nitrogen, then transferred to acid-guanidinium-phenol solution, and further homogenized using a hand-held tissue homogenizer. This method produced favorable RNA yield, purity, and integrity for the fat pad, ACL, synovium, and VMO (collectively soft tissues; also, relatively high-cell, low-matrix), but unfavorable results for cartilage, bone, and meniscus (collectively hard tissues; also, low-cell, high-matrix). Based on these observations, the seven tissues were divided into two groups for further protocol refinement. It was observed that the additional homogenization had minimal effect on further disintegrating the hard tissues after they had been pulverized into a fine powder. Conversely, dissociation of the soft tissues was successfully achieved with homogenization alone and did not require pulverization. Therefore, the homogenization of the hard tissues and pulverization of the soft tissues was eliminated. This was beneficial for minimizing shearing forces, processing time, and temperature fluctuation, all of which can improve RNA integrity. Two rounds of phenol/chloroform phase separation for all seven tissues were performed, as this has been reported to improve RNA purity without reducing yield[31].

A potential limitation of this protocol is the batch effect that may arise from separating the tissues into two groups if the experimental design requires comparison among all tissues. The use of pulverization versus homogenization methods may alter technical (e.g., processing time) and environmental (e.g., temperature fluctuations) conditions that can introduce variability[36]. A second limitation is the potential inconsistency in identifying, dissecting, and orienting (for histological sectioning) the tissues within and across subjects. A third limitation is our inability to confirm potential correlations between patient disease severity and RNA quality in the current report. A fourth limitation is the lack of availability of healthy control tissues for comparison. Though control samples may be available from cadavers, these are less readily available than OA tissues from TKA. An experimental strategy to circumvent this is to use each subject as their own control, whether making comparisons across tissues or within tissues comparing treatment to control or lesioned to preserved areas. Finally, using tissue explants for RNA extraction does not permit gene expression analysis of the individual cell types that comprise the tissues (e.g., synovial fibroblasts versus synovial macrophages[37]).

Despite the noted limitations, primary human OA tissues are a valuable resource for research, offering advantages over other experimental systems for OA, including preserving the cell niche[23]. However, primary human OA tissues may be underutilized in research due to logistic or technical challenges. This protocol describes the patient selection, sample processing, tissue homogenization, RNA extraction, and quality control to support the use of the samples obtained from TKA. Following sample processing, several experimental approaches can be pursued, including gene expression and histology, among others. Most relevant to the rapidly evolving omics field is the ability to isolate sufficient quantities of high-quality RNA for applications such as RNA-sequencing[38,39]. Molecular profiles can be compared within and across tissues from subjects with specific disease phenotypes (e.g., based on age, sex, and other OA risk factors). Insights gained may inform new therapeutic avenues that can be more readily translated back to the OA patient population.

ACKNOWLEDGEMENTS:

The authors thank the study participants who made this research possible and dedicate this report to new scientists in the osteoarthritis field.

DISCLOSURES:

The authors declare no conflicts of interest.

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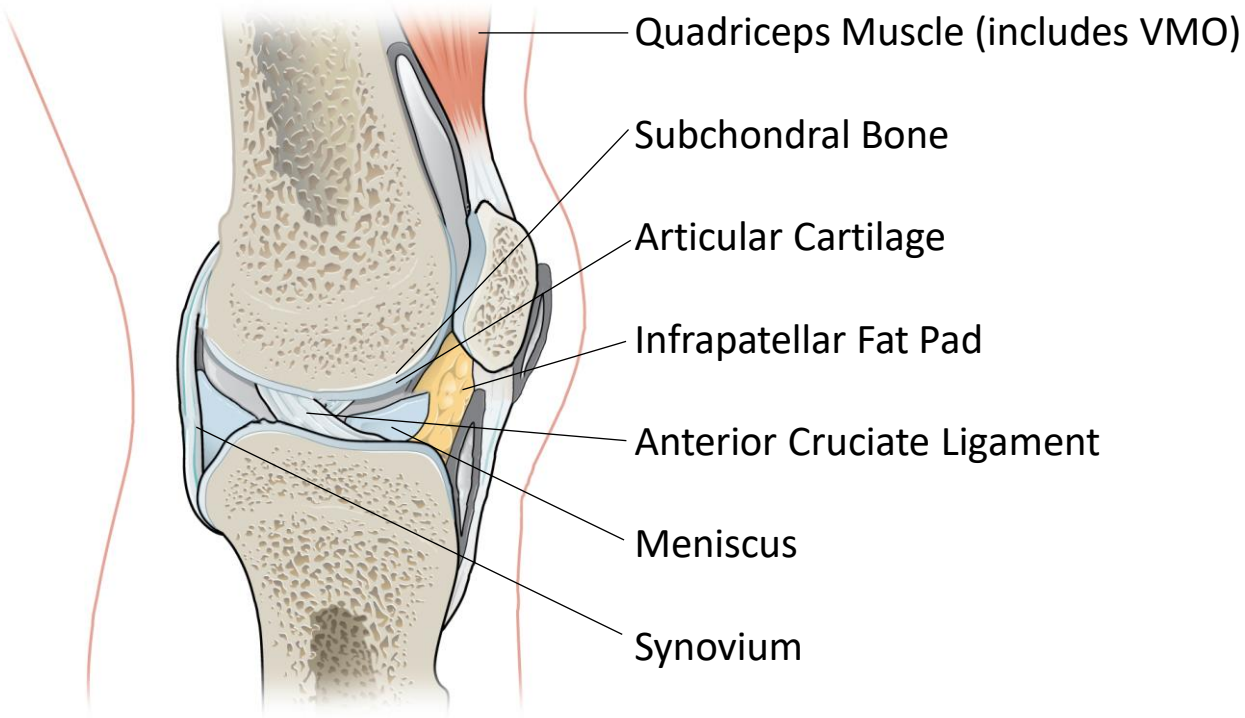

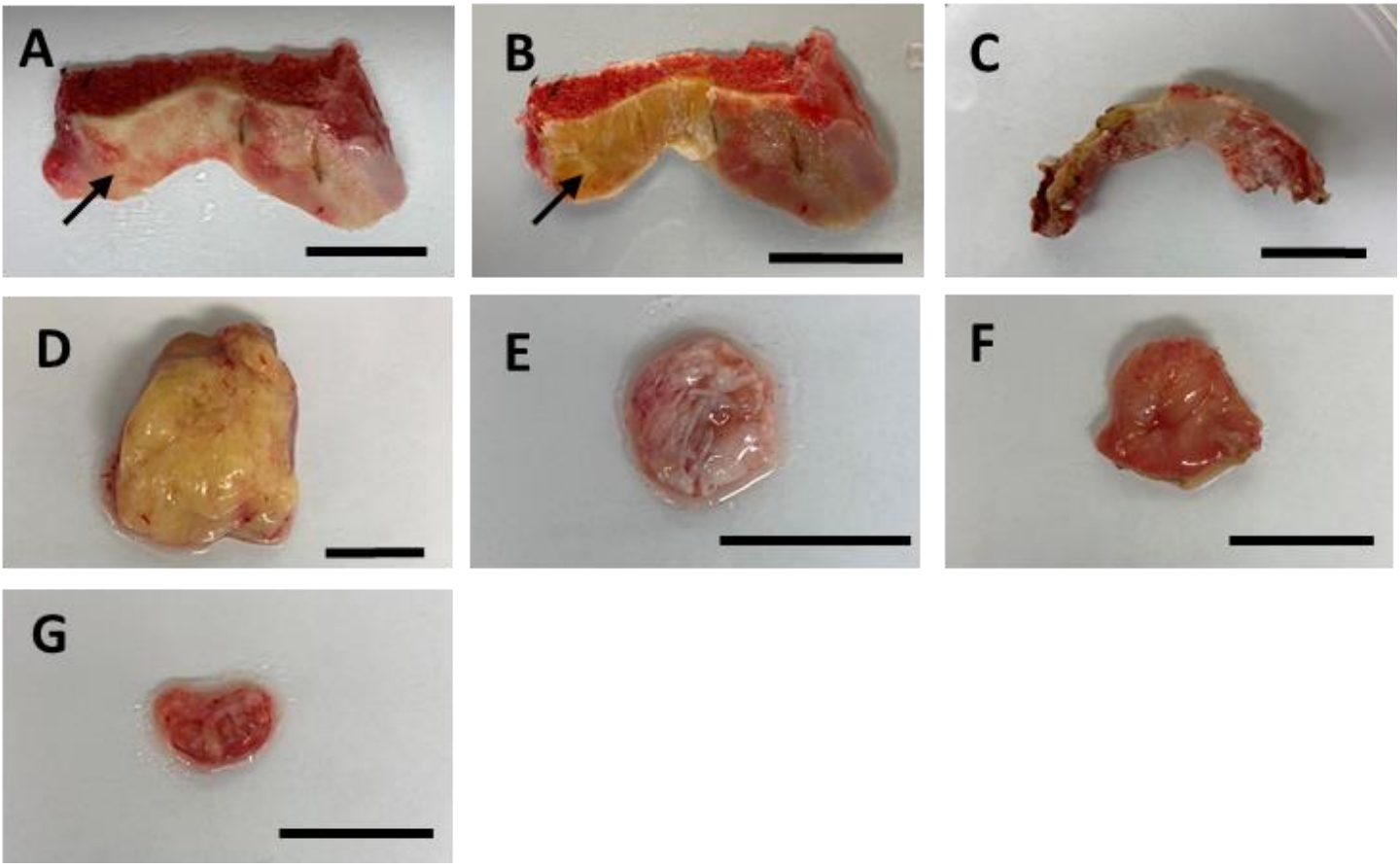

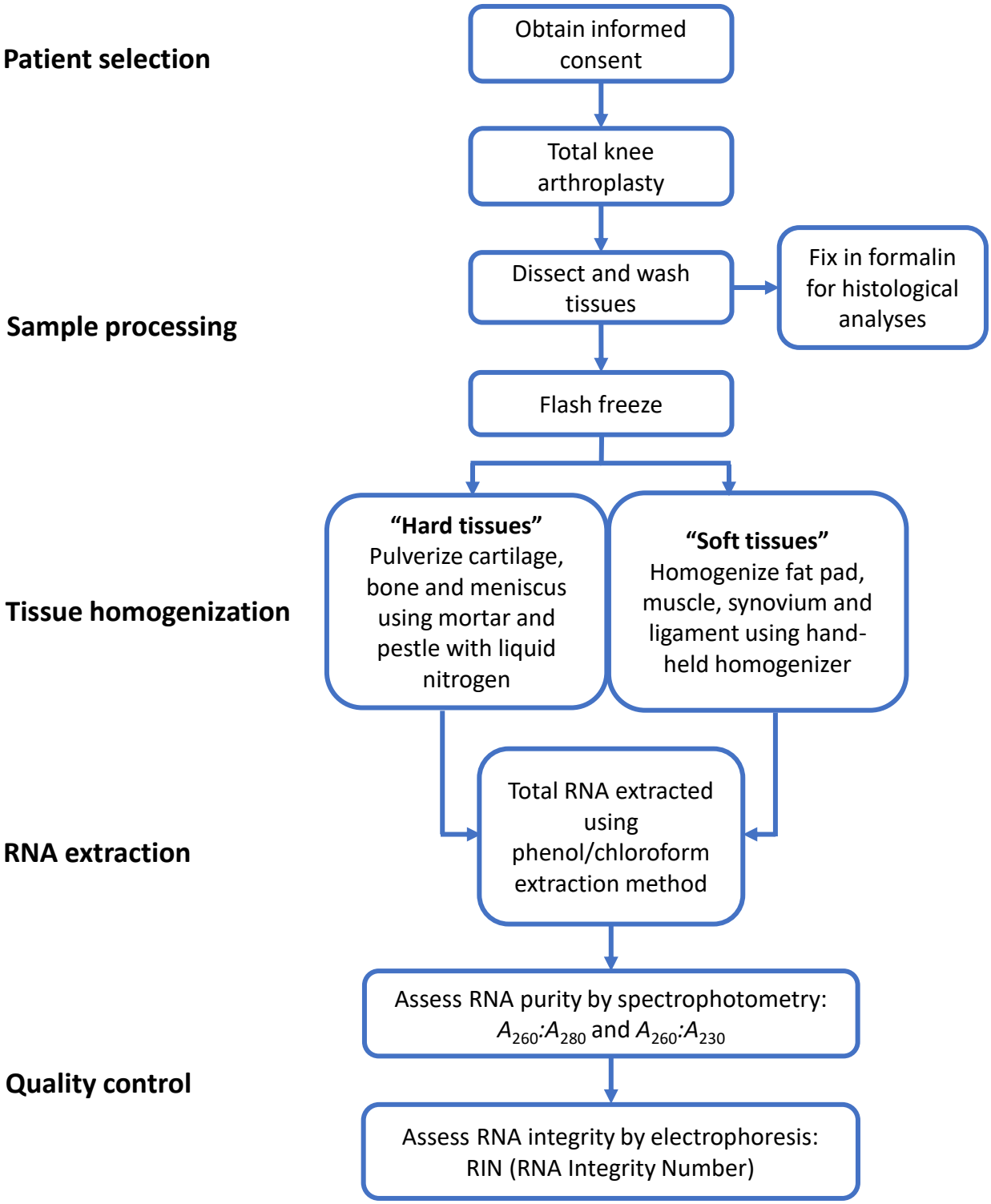

Figure4

Click here to access/download;Figure;Wilson_JoVE_Figure4.pdf

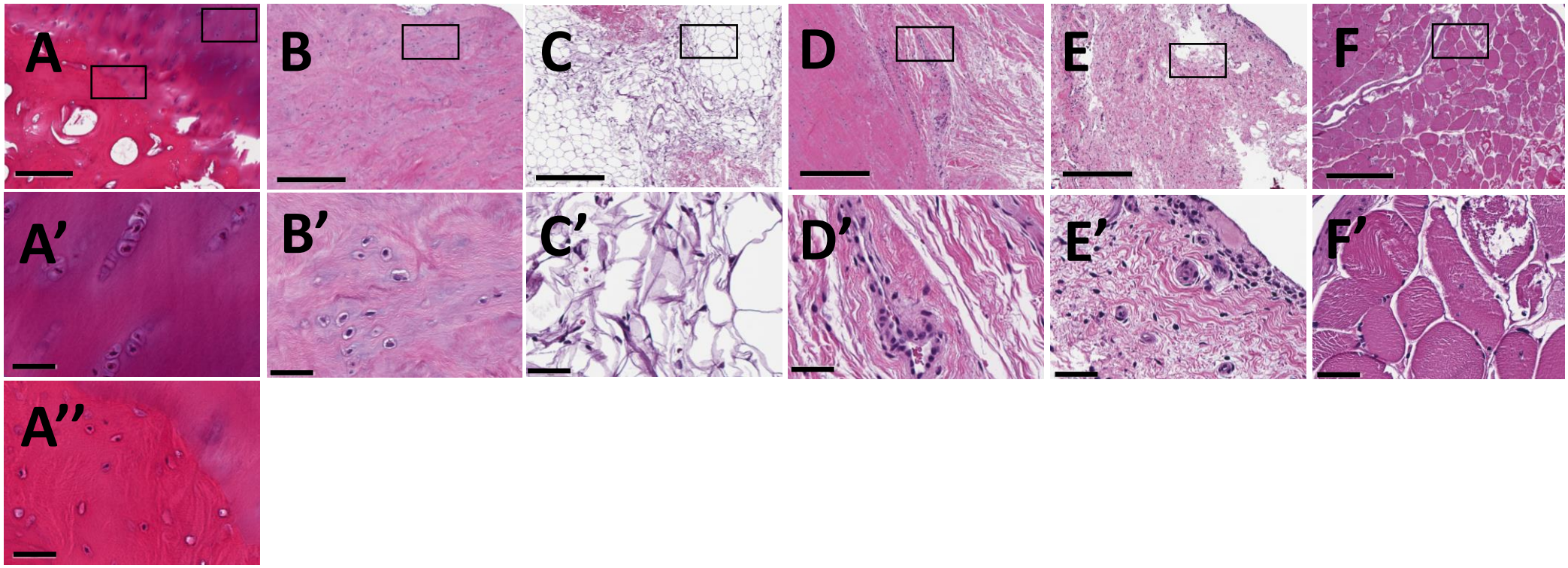

Table1

Tissue	N		RIN		[RNA] (ng/µL) in 25 µL		A_{260}:A_{280}		A_{260}:A_{230}	
	High quality	Low quality	High quality	Low quality	High quality	Low quality	High quality	Low quality	High quality	Low quality
"Hard tissues"										
Articular Cartilage	10	4	7.0 ± 0.8	1.3 ± 1.2	135 (36-243)	46 (26-78)	1.80 ± 0.09	1.47 ± 0.34	1.40 ± 0.36	0.45 ± 0.26
Subchondral Bone	10	4	7.8 ± 0.6	3.6 ± 1.1	514 (181-1586)	342 (122-769)	1.96 ± 0.05	1.90 ± 0.17	1.72 ± 0.27	1.12 ± 0.62
Meniscus	10	4	7.5 ± 0.6	2.4 ± 0.3	242 (38-1629)	95 (60-318)	1.91 ± 0.05	1.71 ± 0.15	1.41 ± 0.47	0.77 ± 0.59
"Soft tissues"										
Infrapatellar Fat Pad	10	3	8.5 ± 0.6	6.1 ± 1.4	1905 (668-5100)	1151 (381-2306)	1.99 ± 0.01	2.02 ± 0.03	2.09 ± 0.17	1.47 ± 0.71
Anterior Cruciate Ligament	8	3	7.4 ± 0.4	5.2 ± 1.9	1836 (613-8456)	727 (97-1479)	1.97 ± 0.03	1.91 ± 0.18	1.88 ± 0.20	1.35 ± 0.70
Synovium	9	3	8.5 ± 0.6	6.2 ± 3.1	2239 (401-4100)	1897 (902-3366)	1.99 ± 0.04	2.00 ± 0.03	2.05 ± 0.11	1.91 ± 0.20
Vastus Medialis Oblique	9	3	8.5 ± 0.6	8.4 ± 0.7	1002 (377-1715)	1097 (308-2138)	1.96 ± 0.03	1.99 ± 0.03	1.82 ± 0.11	1.62 ± 0.27

Name of Material	Company	Catalog Number
1.5 mL microcentrifuge tubes	Eppendorf	05 402
10% Formalin	Cardinal Health	C4320-101
100% Chloroform (Molecular Biology Grade)	Fisher Scientific	ICN19400290
100% Ethanol (Molecular Biology Grade)	Fisher Scientific	BP2818500
100% Isopropanol (Molecular Biology Grade)	Fisher Scientific	AC327272500
100% Reagent Alcohol	Cardinal Health	C4305
15 cm sterile culture dishes	Thermo Scientific	12-556-003
15 mL polypropylene (Falcon) tubes	Fisher Scientific	14 959 53A
2 mL cryovials (externally threaded)	Fisher Scientific	10 500 26
5 mL round-bottom tubes	Corning	352052
50 mL polypropylene (Falcon) tubes	Fisher Scientific	12 565 271
Bioanalyzer	Agilent	G2939BA
Biosafety Cabinet	General lab equipment	
Bone Cutters	Fisher Scientific	08 990
Chemical Fume Hood	General lab equipment	
Disposable Scalpels (No.10)	Thermo Scientific	3120032
EDTA	Life Technologies	15-576-028
Forceps	Any vendor	
Glycoblue Coprecipitant	Fisher Scientific	AM9516
Kimwipes	Fisher Scientific	06-666
Liquid Nitrogen	Any vendor	
Liquid Nitrogen Dewar	General lab equipment	
Mortar and Pestle	Any vendor	
Nanodrop Spectrophotometer	Thermo Scientific	ND-2000
Nuclease-free/DEPC-treated water	Fisher Scientific	
PBS (Sterile)	Gibco	20 012 050
Pipettes (2 µL, 20 µL, 200 µL, 1000 µL) &	Any vendor	
Plasma/Serum Advanced miRNA kit	Qiagen	217204
Refrigerated Centrifuge 5810R	Eppendorf	22625101
RNAlater	Thermo Scientific	50 197 8158
RNAse Away/RNAseZap	Fisher Scientific	7002
Spatula (semimicro size)	Any vendor	
Tissue homogenizer	Pro Scientific	01-01200

TRIzol Reagent	Fisher Scientific	15 596 026

Comments/Description

Sterile, nuclease-free. Reserved for RNA work only.

Store in chemical cabinet when not in use.

Sterile, nuclease-free. Reserved for RNA work only, store in chemical cabinet when not in use.

Sterile, nuclease-free. Reserved for RNA work only, when diluting use DEPC/nuclease-free wate

Sterile, nuclease-free. Reserved for RNA work only, store in chemical cabinet when not in use.

Diluted to 70% with dH_2O for cleaning purposes.

Sterile, nuclease-free.

Sterile, nuclease-free.

Sterile, nuclease-free.

Sterile, nuclease-free. Reserved for RNA work only.

Sterile, nuclease-free.

For RNA integrity measurement.

Sterilized with 70% EtOH.

Sterile, nuclease-free.

10% solution with dH_2O.

Sterilized with 70% EtOH.

Reserved for RNA work only, store at -20 °C.

Reserved for RNA work only, sterilzed per protocol.

For RNA purity and yield measurements.

Sterile, nuclease-free. Reserved for RNA work only.

Sterile, nuclease-free.

Sterile, nuclease-free.

Sterile, nuclease-free.

Reserved for RNA work only.

Reserved for RNA work only, sterilzed per protocol.

Sterile, nuclease-free. Reserved for RNA work only.

er.

May 13, 2021

To the Reviewers,

Thank you for reviewing our manuscript entitled "**Tissue collection and RNA extraction from the human osteoarthritic knee joint"** for the *Journal of Visualized Experiments (JoVE)*. We have carefully considered each comment and concern that was raised, and revised the manuscript accordingly. Below we outline each of the editors' and reviewers' comments in red and our detailed responses in black.

We hope you find our improved manuscript suitable for publication in *JoVE*.

Editorial comments

We thank the editor for preparing this detailed list of feedback and apologize for any formatting oversight in our original submission. We have carefully revised the manuscript to meet all standards outlined below.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Thank you for the opportunity to proofread our manuscript. We confirm that there are no spelling or grammar issues to the best of our knowledge.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. For example: Falcon, TRIzol, RNAse Away, Glycoblue, Qiagen, miRNeasy, Nanodrop, Pro Scientific 200, etc.

We apologize for including commercial language in our original submission. We have now removed all commercial language and brand names from our manuscript and figures and replaced these with generic terms. Specifically, "Falcon" is now referred to as "conical", "TRIzol" is now referred to as "acid-guanidinium-phenol", RNAse Away is now referred to as "RNAse decontaminant", Nanodrop is now referred to as "spectrophotometer", Bioanalyzer is now referred to as "electrophoresis device", "Pro Scientific 200" is now referred to as "hand-held homogenizer", and Glycoblue is now referred to as "glycogen coprecipitant".

3. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Numbering throughout the Protocol has been adjusted to follow the specified guidelines (see tracked changes). Accordingly, we have removed all instances of bullets or dashes.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All phrases previously containing personal pronouns have been revised to avoid use of these terms. For example, in the Abstract on Lines 32-33, where we previously stated, "We found pulverization appropriate for "hard tissues" and homogenization appropriate for "soft tissues".", we now state, "It was found that

pulverization was appropriate for “hard tissues” and homogenization appropriate for “soft tissues”.”. Similar edits were made throughout the manuscript.

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution’s human research ethics committee.

The ethics statement previously included as point 1 in Part 1 has now been moved to the top of the numbered protocol steps as follows, “This study protocol was approved and follows institutional guidelines set by the Henry Ford Health System Institutional Review Board (IRB #13995)”.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All text in the protocol section has been carefully revised to the imperative tense, using complete sentences. Several notes have been removed or consolidated in order to use notes sparingly. We include safety procedures and use of hoods on Lines 152-154, 267, 302-303, 370-371 and 414-415. For example, Lines 152-154 states, “NOTE: All tissue processing must be performed in a class II biosafety cabinet and sterile technique followed. Appropriate PPE (nitrile gloves, lab coat, safety googles) must always be worn when processing human samples.”

7. For time units, please use abbreviated forms for durations of less than one day when the unit is preceded by a numeral throughout the protocol. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

All time units have been updated based on these guidelines (see tracked changes).

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion (Line 198-200/214-215/222-224/230-231)

Non-action items have been reworded or removed from the protocol section. For example, the discussion previously found on Lines 198-200/214-215/222-224/230-231 have been removed and are now described in the Figure 2 legend as follows, “(A) Anterior femoral bone cut with arrow pointing to articular cartilage to be collected. Cartilage is identified by a whitish layer found on the surface of the bone. (B) Anterior femoral bone cut with arrow pointing to subchondral bone to be collected. (C) Meniscus. Avoid collecting burnt sections caused by electrocauterization during TKA. (D) Infrapatellar fat pad (yellow in color). (E) Anterior cruciate ligament (white, fibrous, spongy tissue). (F) Synovium. One side will appear light colored and fibrous, often containing fat tissue, while the opposite side will appear pinkish and less fibrous. The pink side of the membrane contains the synovial lining. (G) Vastus medialis oblique muscle (red). This may often be the smallest tissue portion (5-10 cm^2) and may contain some fat tissue. Scale bar = 2 cm.”

9. Line 325: Please include more details about homogenization. How long is it performed? Are there any other specific conditions to be maintained?

We apologize for not previously including this detail. Additional information regarding homogenization has been included on Page 9, Line 352-353 as follows, "Homogenize tissues in 30 s pulses, keeping on ice during and between pulses. Repeat until tissue is visually dissolved or for a maximum of five 30 s pulses."

10. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

We now include a one-line space between each protocol step and have highlighted key steps in the protocol that could be visualized in the video, including identification of seven distinct tissues (Lines 173-188) and methods of homogenization for each tissue type (Lines 310-366). This is within the 3-page limit.

11. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The legend for Figure 1 states the following, "*Image accessed and modified from OpenStax College under a creative commons license.*" The relevant copyright information can be accessed at this website: https://creativecommons.org/licenses/by/3.0/legalcode [Source: OpenStax College (https://commons.wikimedia.org/wiki/File:908_Bursa.jpg), „908 Bursa", Modified from original.] If this is not sufficient for use in this publication, please advise and we will revise the figure.

12. Please do not use the &-sign or the word "and" when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.

Our original reference formatting utilized the JoVE settings on EndNote. We have now revised the reference formatting according to the guidelines specified here.

13. Figure 3: Please remove all commercial language from the image and use generic terms instead (Qiagen, Nanodrop, Pro Scientific 200, etc.)

Commercial language has been removed from Figure 3. Specifically, "Qiagen" and "NanoDrop" were removed and Pro Scientific 200 is now referred to as "hand-held homogenizer".

Reviewers' comments

Reviewer #1

Manuscript Summary:

This manuscript describes protocols to extract RNA from human osteoarthritic (OA) joint tissues. Since a joint is considered as a whole organ including articular cartilage, subchondral bone, meniscus, synovium

and that OA may affect surrounding tissues, several tissues with blood and synovial fluid have been processed for RNA extraction. Different protocols for hard or soft tissues are proposed.

Minor Concerns:
Since all the B tissues show RIN values inferior to A tissues, the authors suggest that the B patient is likely more affected by OA (e.g. degradative processes higher in B than in A). This is perhaps true (and interesting) but the authors compare only two subjects, and sampling may be another cause of difference (sample conservation). It would be interesting to add the OA score of A and B patients (international grading system, OARSI, ICRS...) to support the view that disease factors (or the degree of the disease) may impact RNA quality. Obviously, it would have been advantageous to collect more samples.

We thank the reviewer for thoroughly reviewing our manuscript and providing these useful comments for improvement. Since submitting our manuscript for consideration at *JoVE*, we continued to collect samples and perform RNA isolations. Therefore, we are now able to increase the number of samples shown in Table 1. Whereas we previously showed representative values for each of samples A and B, we now show average values for N=8-10 "High quality" samples and N=3-4 "Low quality" samples (Table 1). "High quality" samples consist of patients from which all tissue types yielded RNA with RIN > 6 and "Low quality" samples consist of patients from which multiple tissue types yielded RNA with RIN < 6.

We agree with the reviewer that the possibility of RNA quality correlating with the degree of disease severity is quite interesting and would require OA scoring to confirm. Unfortunately, we do not have access to sufficient histological sections that would enable OARSI or ICRS grading as suggested by the reviewer. We attempted to assess OA severity based on Kellgren-Lawrence (KL) radiographic grading (ranging from 0 as least severe to 4 as most severe), but since these are all end-stage knee OA patients, the range of grades was quite narrow (KL 3-4) as shown in the table below, and precluded any correlation analyses between disease severity and RNA quality.

High quality KL grades for N=10	**Low quality KL grades for N=4**
4	3
3	3
4	4
4	4
4	
4	
4	
4	
4	
4	

As noted by the reviewer, the degree of disease severity could be one of many factors that impact the quality of RNA, thus we have re-worded our discussion accordingly on Page 13, Lines 562-564, as follows: "While there are many factors that could be contributing to the quality of RNA isolated from a given patient sample, among them may be the level of disease severity."

This was also added as a potential limitation of the protocol on Page 14, Lines 591-593, as follows:

"A third limitation is our inability to confirm potential correlations between patient disease severity and RNA quality in the current report."

Through the manuscript: It is indicated that benchwork places, scalpels, ... should be washed by 70% ethanol then by water etc...It would be wise to indicate that any spot, instrument, to be in contact with tissues should be first cleaned by water/detergent, DEPC water, etc... then with ethanol 70%. Ethanol 70% will precipitate (and not wash away) any DNA/RNA already present on a surface/instrument, leaving therefore a source of contamination.

We thank the reviewer for this excellent comment regarding cleaning methods using 70% ethanol. This was an oversight on our part in the original submission. We have now added appropriate details regarding ethanol, which is used as both the first and last step in our cleaning methods. By first using ethanol to precipitate any DNA/RNA, we are then able to remove it by washing steps, and then finish with another ethanol step for disinfection of surfaces and instruments. We also wipe any residual liquids with Kimwipes prior to use. This is now specified on Page 4, Lines 164-168, as follows:

"Disinfect all work surfaces and equipment with 70% ethanol, RNAse decontaminant, DEPC-treated water, and again with 70% ethanol. Wipe away residual liquid with clean, lint-free tissues. Forceps, bone cutters and scalpels are either autoclaved or soaked in 70% ethanol for at least 10 min prior to use." Similar instructions are provided on Lines 305-308, 332-334, 340-342 and 377-380.

Reviewer #2

Manuscript Summary:

This manuscript provided the protocol to harvest and process the seven tissues and two biofluid components from total knee arthroplasty patients.

Major Concerns:

(1) Not enough replicates: This protocol only tested on two biological samples, which were subject A & subject B.

We thank the reviewer for reviewing our manuscript in detail and providing these helpful comments for improvement. Since submitting our manuscript for consideration at *JoVE*, we continued to collect samples and perform RNA isolations. Therefore, we are now able to increase the number of samples shown in Table 1. Whereas we previously showed representative values for each of samples A and B, we now show average values for N=8-10 "High quality" samples and N=3-4 "Low quality" samples (Table 1). "High quality" samples consist of patients from which all tissue types yielded RNA with RIN > 6 and "Low quality" samples consist of patients from which multiple tissue types yielded RNA with RIN < 6.

Based on table 1, there was only subchondral bone (8.7), fat pad (8.9), synovium (9.8) and vastus medialis oblique from subject A showed RIN more than 8.

Here we define RIN > 7 as sufficient integrity for RNA-sequencing which is consistent with previous reports by Li et al. (2014), Nazarov et al. (2017), and Madissoon et al. (2019). We acknowledge that other protocols require a RIN > 8, which we have been able to accomplish for most "soft tissues" as noted by the reviewer, but not consistently for the "hard tissues". Given the low cellularity and high matrix content of the "hard tissues", RIN > 7 can be considered high quality and sufficient for sequencing. We now describe this in the manuscript on Page 13, Lines 557-558 as follows:

"For the purposes of RNA-sequencing, a minimum RIN of 7 is often recommended[32-34]."

32. Li, S., et al. Multi-platform assessment of transcriptome profiling using RNA-seq in the ABRF next-generation sequencing study. *Nature Biotechnology.* **32** (9), 915-925, doi:10.1038/nbt.2972, (2014).

33. Nazarov, et al. RNA sequencing and transcriptome arrays analyses show opposing results for alternative splicing in patient derived samples. *BMC Genomics.* **18** (1), 443, doi:10.1186/s12864-017-3819-y, (2017).

34. Madissoon, et al. scRNA-seq assessment of the human lung, spleen, and esophagus tissue stability after cold preservation. *Genome Biol.* **21** (1), 1, doi:10.1186/s13059-019-1906-x, (2019).

Moreover, the subchondral bone from subject A showed poor A260:A230 (1.6) result, which indicated may have nucleic acid or phenol contamination. Was it because of a technical/protocol problem during the RNA isolation?

We agree with the reviewer that A_{260}:A_{230} values in the range of 1.8-2.2 are expected. While this is routinely achieved in our "soft tissue" isolations, there is considerable variability when isolating from the "hard tissues". As the reviewer noted, a low A_{260}:A_{230} ratio is caused by contaminants that absorb at 230 nm, whether residual phenol from the isolation or protein carryover from the starting sample. As this seems to occur primarily in the low-cell, high-matrix tissues, we suspect that the rich protein content of these tissues impacts this ratio greater than the presence of phenol contamination. To reduce protein contamination as best as possible (while maintaining RNA yield), we perform two rounds of phenol-chloroform phase separation. Our revised Table 1 now shows the average ratios we have been able to measure in each tissue from N=8-10 "High quality" samples and N=3-4 "Low quality" samples. In addition, we now discuss the A_{260}:A_{230} ratios observed for the "hard tissues" on Page 13, Lines 558-562, as follows:

"Data presented in Table 1 reveals that these A_{260}:A_{280}, A_{260}:A_{230}, and RIN thresholds were met across all tissues from the patient samples in the "High quality" RNA group compared to patient samples in the "Low quality" RNA group, with the exception of some A_{260}:A_{230} values, which may reflect protein contamination of RNA in the low-cell, high-matrix tissues."

Lines 521 & 522 have stated that "Comparing Subject B to Subject A in Table 1 reveals worse values across all 522 tissues, suggesting that disease factors may impact RNA quality." The data provided by this study are not sufficient to prove the efficacy of the developed protocol. Therefore, I will suggest recruiting three biological specimens (harvest the specimens from three similar demographic and severity of osteoarthritis patients). Each biological specimen should be repeated with three technical replicates. In total, at least to generate nine sets of data for comparison.

We thank the reviewer for this important comment and agree that the data provided in the original submission was insufficient to support the claim that disease factors impact RNA quality. Originally, Table 1 presented representative values for a "high quality" (Subject A) and a "low quality" (Subject B) patient. As suggested by the reviewer, we have now increased the sample size. In our revised Table 1, Subject A has been replaced by "High quality" samples which consists of average integrity and purity values for n= 8-10 patients for each tissue. Subject B has been replaced by "Low quality" samples which consists of average integrity and purity values for n= 3-4 patients for each tissue. Also noted by Reviewer 1, many

factors may be at play in determining the overall quality of isolated RNA so to clarify this point, we have now revised the Discussion on Page 13, Lines 562-564, as follows:
"While there are many factors that could be contributing to the quality of RNA isolated from a given patient sample, among them may be the level of disease severity."

We also added this point as a potential limitation of the protocol on Page 14, Lines 591-593, as follows:
"A third limitation is our inability to confirm potential correlations between patient disease severity and RNA quality in the current report."

(2) NO quality control for the two biofluid components: I don't understand why the authors want to isolate the RNA from the plasma? In fact, a high concentration of RNA can be easily isolated from the whole blood. Anyway, the authors can perhaps compare the proteins or extracellular vesicles in both plasma and synovial fluid as quality control.

We appreciate the reviewer's comment regarding the lack of quality control for our biofluid components. We agree that a higher concentration of total RNA can be isolated from whole blood versus plasma, but since we have not tested this, we have decided to remove the biofluid description from the current manuscript. We thank the reviewer for helping to focus the current manuscript on knee OA tissues.

Minor Concerns:
(1) No gene expression (qPCR and RNA-sequencing) was performed in this study, but it was stated in figure 3.

We thank the reviewer for pointing out this inconsistency between Figure 3 and the data presented in this manuscript. Since this is a methods manuscript focused on RNA isolation, we did not include qPCR and RNA-sequencing data. Therefore, we have now removed the "Gene expression" step from the original Figure 3 to avoid misleading readers. However, we have now conducted qPCR experiments to demonstrate the utility of the isolated RNA and present the Ct values for standard housekeeping genes ACTB and GAPDH below, obtained from "High quality" RNA from N=3 patients per tissue. The range of Ct values for ACTB were 23.1 to 27.2, and the range for GAPDH was 21.1 to 26.7, each with a standard deviation below 1 for all tissues, showing consistent expression of these reference genes as recommended by Stephens et al. (2011). If the reviewers suggest we include these qPCR data in the manuscript, we are happy to do so, but do not think it contributes significantly to the main focus of the manuscript as a description of an RNA isolation method.

Tissue	**Average ACTB Ct (SD)**	**Average GAPDH Ct (SD)**
Cartilage	27.2 ± 0.54	26.7 ± 0.42
Bone	23.1 ± 0.33	24.3 ± 0.31
Meniscus	24.4 ± 0.29	23.8 ± 0.62
Fat Pad	25.0 ± 0.74	26.2 ± 0.08
ACL	23.2 ± 0.04	23.5 ± 0.25
Synovium	23.9 ± 0.43	24.4 ± 0.18
VMO	25.1 ± 0.15	21.1 ± 0.17

Stephens, A. S., et al. Internal control genes for quantitative RT-PCR expression analysis in mouse osteoblasts, osteoclasts and macrophages. *BMC Res Notes.* **4** 410, doi:10.1186/1756-0500-4-410, (2011).

(2) How long for the "flash freeze" in liquid nitrogen?

Thank you for pointing out this missing information, we submerged the tissues in liquid nitrogen for 30 seconds prior to storage at -80C. We have added this information to the protocol on Page 7, Lines 258-261, as follows:

"Transfer smaller pieces to a 2 mL cryovial, secure caps tightly, flash freeze by submerging in liquid nitrogen for 30 s, then transfer to a -80°C freezer for long term storage (up to 4 months tested in current protocol)."

(3) According to the Thermo Fisher website:
(https://www.thermofisher.com/order/catalog/product/AM7021#/AM7021)
Advantages of using RNAlater RNA Stabilization Solution:
* Effectiveness—stabilize RNA for 1 day at 37°C, 1 week at 25°C, 1 month at 4°C, or indefinitely at -20°C
In the manuscript, line 245-246: Sample stored in RNAlater should not be flash frozen, instead store at 4° C for 1 week. After 1 week, discard RNAlater from vial and store the tissue at -80 °C.
I am not sure whether the tissues were stored using the correct method?

We acknowledge the reviewer's concern regarding correct use of RNAlater. Per the source that was provided by the reviewer, the effective times recommended by Thermofisher are their maximum guaranteed storage times at these conditions, removing the necessity for sample storage in a -80C freezer. As our general protocol entails freezing tissue samples immediately following processing, we added the use of RNAlater as a potential method to improve RNA stabilization prior to freezing.

Per the user guide (https://assets.fishersci.com/TFS-Assets/LSG/manuals/7020M.pdf), tissues can be "stored at 4C, followed by removal of RNAlater and long-term freezer storage". We increased the time spent at 4C from "overnight" as mentioned in this guide, to one week, to allow for extra time to penetrate the "hard tissues", and this time period is within the recommend maximum storage time at 4C (1 month as indicated by the source identified by the reviewer).

In our experiments, we found that tissues stored in RNAlater did not exhibit any marked improvement to RNA integrity or purity. To reduce the possible confusion that this may cause for readers, we have removed mention of RNAlater from the manuscript.

In addition, how long the tissues were stored at -80 °C prior to Part 3 (tissue homogenization)?

We thank the reviewer for identifying this missing detail. The longest duration these tissues were stored at -80°C before homogenization was approximately 4 months. In general, the time varies from a few weeks to a few months. We have not observed any decrease in RNA quality with longer storage time, however, our study has not existed long enough to do any longitudinal evaluations (i.e. 1 year+). We have now specified this in the protocol on Page 6, Lines 258-261 as follows:
"Transfer smaller pieces to a 2 mL cryovial, secure caps tightly, flash freeze by submerging in liquid nitrogen for 30 s, then transfer to a -80°C freezer for long term storage (up to 4 months tested in current protocol)."