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A Laser Capture Microdissection Protocol That Yields High Quality RNA from Fresh-Frozen Mouse Bones

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Corresponding Author:	Reinhold Erben University of Veterinary Medicine Vienna Vienna, AUSTRIA
Corresponding Author's Institution:	University of Veterinary Medicine Vienna
Corresponding Author E-Mail:	Reinhold.Erben@vetmeduni.ac.at
Order of Authors:	Ana Marek
	Christiane Schüler
	Maria Satue
	Barbara Haigl
	Reinhold G. Erben
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Veterinärmedizinische Universität Wien
Department für Biomedizinische Wissenschaften
Institut für Physiologie, Pathophysiologie und Biophysik
Veterinärplatz 1 · A-1210 Wien

Veterinärmed. Universität · Veterinärplatz 1 · A-1210 Wien

Editor-in-Chief
JoVE

Prof.Dr.Dr. Reinhold G. Erben

Telefon: +43-1-250 77 4550
Telefax: +43-1-250 77 4599
E-mail: Reinhold.Erben@vetmeduni.ac.at

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Manuscript submission

Dear Editor,

I am sending you the invited paper *entitled "A laser capture microdissection protocol that yields high quality RNA from fresh-frozen mouse bones"* by Ana Marek, Christiane Schüler, Maria Satue, Barbara Haigl, and Reinhold G. Erben for evaluation by *JoVE*. Please evaluate the manuscript for publication in *JoVE*. The manuscript has not been published and is not under current consideration elsewhere. There are no conflicts of interest.

Thank you for your time and consideration.

Sincerely,

Reinhold G. Erben, M.D., D.V.M.

TITLE:

A Laser Capture Microdissection Protocol That Yields High Quality RNA from Fresh-Frozen Mouse Bones

AUTHORS AND AFFILIATIONS:

Ana Marek[1], Christiane Schüler[1], María Satué[1], Barbara Haigl[1], Reinhold G. Erben[1]

[1]Department of Biomedical Research, University of Veterinary Medicine Vienna, Vienna, Austria

Email addresses of co-authors:

Ana Marek (ana.marek@vetmeduni.ac.at)
Christiane Schüler (christiane.schueler@vetmeduni.ac.at)
María Satué (maria.satue@vetmeduni.ac.at)
Barbara Haigl (barbara.haigl@vetmeduni.ac.at)

Corresponding author:

Reinhold G. Erben (Reinhold.Erben@vetmeduni.ac.at)

KEYWORDS:

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SUMMARY:

A laser capture microdissection (LCM) protocol was developed to obtain sufficient quantity of high-quality RNA for gene expression analysis in bone cells. The current study focusses on mouse femur sections. However, the LCM protocol reported here can be used to study gene expression in cells of any hard tissue.

ABSTRACT:

RNA yield and integrity are decisive for RNA analysis. However, it is often technically challenging to maintain RNA integrity throughout the entire laser capture microdissection (LCM) procedure. Since LCM studies work with low amounts of material, concerns about limited RNA yields are also important. Therefore, an LCM protocol was developed to obtain sufficient quantity of high-quality RNA for gene expression analysis in bone cells. The effect of staining protocol, thickness of cryosections, microdissected tissue quantity, RNA extraction kit, and LCM system used on RNA yield and integrity obtained from microdissected bone cells was evaluated. Eight-µm-thick frozen bone sections were made using an adhesive film and stained using a rapid protocol for a commercial LCM stain. The sample was sandwiched between a polyethylene terephthalate (PET) membrane and the adhesive film. An LCM system that uses gravity for sample collection and a column-based RNA extraction method were used to obtain high quality RNAs of sufficient yield. The current study focusses on mouse femur sections. However, the LCM protocol reported here can be used to study in situ gene expression in cells of any hard tissue in both physiological conditions and disease processes.

INTRODUCTION:

Tissues are made up of heterogeneous and spatially distributed cell types. Different cell types in a given tissue may respond differently to the same signal. Therefore, it is essential being able to isolate specific cell populations for the assessment of the role of different cell types in both physiological and pathological conditions. Laser capture microdissection (LCM) offers a relatively rapid and precise method for isolating and removing specified cells from complex tissues[1]. LCM systems use the power of a laser beam to separate the cells of interest from histological tissue sections without the need for enzymatic processing or growth in culture. This means that the cells are in their natural tissue habitat, and that tissue architecture including the spatial relationship between different cells is retained. Morphology of both the captured cells and the residual tissue is well preserved, and several tissue components can be sampled sequentially from the same slide. Isolated cells can then be used for subsequent analysis of their RNA, DNA, protein or metabolite content[2,3].

In order to analyze gene expression in different cell populations, or after different treatments, it is necessary to obtain mRNAs of sufficient quality and quantity for the subsequent analysis[4,5]. In contrast to DNA, RNAs are more sensitive to fixation and the use of frozen tissue is recommended when the objective is to study RNA. Since mRNAs are quickly degraded by ubiquitous ribonucleases (RNase), stringent RNase-free conditions during specimen handling and preparation and avoiding storage of samples at room temperature are required. In addition, rapid techniques without any prolonged aqueous phase steps are crucial to prevent RNA degradation[6]. The RNA yield and integrity can also be affected by the LCM process and the LCM system used[7,8]. Currently, four LCM systems with different operating principles are available[2]. The method of RNA extraction can also be important, since different RNA isolation kits have been tested with significant differences in RNA quantity and quality[7,8].

Any tissue preparation method requires finding a balance between obtaining good morphological contrast and maintaining RNA integrity for further analyses. For preparing frozen sections from bone, an adhesive film was developed and continuously improved[9]. The bone sections are cut and stained directly on the adhesive film. This adhesive film is applicable to many types of staining, and can be employed to isolate the cells of interest from bone cryosections using LCM[9–14]. All the steps including the surgical removal, embedding, freezing, cutting and staining can be completed within less than one hour. Importantly, cells such as osteoblasts, bone lining cells, and osteoclasts can be clearly identified[9–14]. This method has the advantage of being rapid and simple. An alternative method for generating bone cryosections is to use the tape transfer system[15]. However, the latter technique is more time-consuming and requires additional instrumentation, since the sections have to be transferred from the adhesive tape onto precoated membrane slides by ultraviolet (UV) cross-linking. Although the tape transfer system has been successfully coupled with LCM[16–19], it should be noted that the cross-linked coating can create a background pattern that can interfere with cell-type identification[20].

Typically, only small amounts of RNA are extracted from microdissected cells, and RNA quality and quantity are often assessed by micro-capillary electrophoresis[21]. A computer program is used to assign an index of quality to RNA extracts called RNA integrity number (RIN). A RIN

value of 1.0 indicates completely degraded RNA, whereas a value of 10.0 suggests that the RNA is fully intact[22]. Usually, indexes over 5 are considered sufficient for RNA studies. Gene expression patterns in samples with an RIN value of 5.0−10.0 have been reported to correlate well with each other[23]. Although the sensitivity of this method is high, since as little as 50 pg/µL of total RNA can be detected, it can be very difficult to obtain a quality assessment if the RNA concentration in the sample is very low. Therefore, in order to assess RNA quality, the tissue section remaining after the LCM is often used to extract RNA, by pipetting buffer onto the slide[24].

Although LCM has been used extensively on different frozen tissues, RIN values of extracted RNAs are rarely reported. Furthermore, there are no comparative studies to clarify the most appropriate method to study RNA in mouse bones. In the present study, frozen sections from adult mouse femurs were used to optimize sample preparation, LCM protocol and RNA extraction in order to obtain high quality RNAs. The present protocol was optimized particularly for the LCM system that uses gravity for sample collection.

PROTOCOL:

Bone tissue from mice was used in strict accordance with prevailing guidelines for animal care and all efforts were made to minimize animal suffering.

1. Animals and freeze embedding

1.1. House animals in conditions of constant room temperature (RT; 24 °C) and a 12 h light/12 h dark cycle with free access to food and water.

NOTE: Bone tissues in this study were obtained from 3-month-old male wild type C57BL/6 mice.

1.2. At necropsy, exsanguinate mice from the abdominal vena cava under general anesthesia (ketamine/xylazine, 100/6 mg/kg intraperitoneal).

NOTE: In order to avoid RNA degradation, use RNase-free instruments and materials, wear gloves and work quickly avoiding storage of samples at RT throughout the whole experiment.

1.3. Remove whole femurs rapidly, clean them of surrounding soft tissues using scalpel and paper towels and put them into the bottom of the embedding molds. Pour optimal cutting temperature (OCT) compound into the embedding mold and snap-freeze the samples in liquid nitrogen. OCT is transparent at RT and white when frozen.

1.4. Once entirely frozen, wrap the samples in foil and transfer them on dry ice to -80 °C. Store samples at -80 °C until further processing.

NOTE: The protocol can be paused here.

2. Section preparation

2.1. Set the temperature in the cryostat to -19 °C and of the block holder to -17 °C. Wipe the cryostat interior using 70% ethanol. Place the disposable blade for hard tissues, the glass slides and a fitting tool into the cryostat to cool. Keep them inside the cryostat for the duration of sectioning.

2.2. Transfer the frozen tissue block on dry ice to the cryostat and allow it to equilibrate for at least 10 min.

NOTE: Avoid repetitive thawing and frequent cycling of one block from -80 °C to -19 °C for cryosectioning.

2.3. Press the bottom of the embedding mold to push the OCT block out of the mold. Apply enough OCT medium to the block holder to adhere the block to it. Wait until the OCT medium is fully frozen.

2.4. Place the block holder in the object holder and tighten it in place. Adjust the blade position and trim the block using 15 µm cutting increments to remove the OCT covering the sample. If the sample has been cut earlier and the surface exposed to air, discard the first 2–3 tissue sections from the block.

2.5. Adjust the cryostat to generate 8 µm sections and cut 2–3 cryosections that will be discarded (the first few will typically be thicker than 8 µm). Place the adhesive film on the block and use a fitting tool to adhere the film to the block. Make the cut slowly and at a constant speed holding the section by the film.

2.6. Place the film (sample facing up) immediately on a precooled glass slide (on the cryobar within the cryostat) to avoid thawing of the sample. Use tape to fix the film to the glass slide for easier staining. Proceed immediately with the staining protocol.

3. Rapid staining protocol

3.1. Prepare 40 mL of the following solutions in 50 mL tubes and put them on ice: 95% ethanol, RNase-free water, 100% ethanol, 100% ethanol and 100% xylene. Perform all steps on ice (except the staining). For each experimental day, prepare all aqueous reagents fresh with RNase-free water.

CAUTION: Work in the hood to avoid intoxication by xylene.

3.2. Incubate sections for 30 s in 95% ethanol, then dip sections 30 s in RNase-free water to remove OCT carefully and completely which may interfere with LCM and downstream applications.

3.3. Dispense 50 µL of commercial LCM frozen section stain (**Table of Materials**) onto the section, incubate for 10 s at RT and drain it by placing the edge of the slide on absorbent tissue paper. Remove excess stain by rinsing 30 s in 100% ethanol.

3.4. Immerse bone sections in a second tube with 100% ethanol for 30 s and transfer them to 100% xylene for 30 s.

3.5. Put adhesive film (sample facing up) on dry glass slide as a support. Take care that the film is not impacted and placed as flat as possible. Do not allow the sample to dry completely.

3.6. Place a PET membrane frame slide on it. Shortly press a gloved finger on the membrane to attach it to the film. The sample is then sandwiched between the membrane and the adhesive film. The adhesive film should not be folded or wrinkled and there should be no air bubbles between the film and the membrane. Proceed immediately with the LCM.

4. Laser capture microdissection

4.1. Clean the stage and cap holder from RNase using surface decontaminant (**Table of Materials**). Load the slide and the caps in the slide holder and the cap holder, respectively.

4.2. Adjust the focus and acquire slide overview with 1.25x objective. Change to the 40x objective and adjust the focus. Choose the area of interest using the slide overview. Adjust laser parameters as follows: aperture, 7; laser power, 60; speed, 5; pulse frequency, 201; specimen balance, 20. Optimize these parameters for each objective.

4.3. If the laser fails to cut the sample, increase the laser power. Alternatively, the laser can be applied more than once. In addition, inspect the target for spots of incomplete cuts and re-drawn the line in these spots using the **Move and cut** option. Save laser settings for the dissection of subsequent slides.

4.4. Select osteoblasts, osteocytes and bone lining cells in distal femoral cancellous or cortical bone based on morphologic criteria. Draw a line for laser path further away from the target cells to minimize the damage by the UV laser. Perform all microdissections within less than 1 h after the staining.

4.5. Collect each cell type in a separate cap of a 0.5 mL tube.

NOTE: Dry capturing instead of liquid recovery may avoid RNA degradation. In addition, very small volumes of buffer contained in the cap of the microcentrifuge tube can either evaporate or crystallize (depending on its composition) during the LCM.

4.6. Confirm capture success by observation of the collection tube cap after the LCM where applicable. Proceed immediately with the RNA extraction.

5. RNA extraction

5.1. Dispense 50 µL of the lysis buffer containing β-mercaptoethanol into the cap of the collection tube and lyse the sample by pipetting up and down in the cap for 1 min. Spin down the lysate and add 300 µL of the lysis buffer containing β-mercaptoethanol into the tube. If several caps are going to be pooled, take care that the total volume of buffer is as recommended for the RNA extraction kit (**Table of Materials**).

CAUTION: β-mercaptoethanol must be added to protect RNA from degradation, but it is considered toxic. Wear protective clothes and gloves and work in the hood.

5.2. In addition, for each slide prepare one labeled 1.5 mL microcentrifuge tube with 350 µL of the lysis buffer containing β-mercaptoethanol. Use the sections remaining after the LCM to extract RNA. Carefully separate the film from the membrane and lyse the sample by slowly pipetting the lysis buffer onto the section several times.

NOTE: Total amount of lysis buffer should be 350 µL. Do not use all of it at once for digestion as it will flow off the slide.

5.3. Put the lysate samples on dry ice and store them at -80 °C.

NOTE: The protocol can be paused here.

5.4. Thaw the lysates at RT. Transfer lysates from LCM-harvested cells from collection tubes into the 1.5 mL microcentrifuge tubes. If more than one cap was used to harvest the sample, pool several lysates.

5.5. Extract RNA according to the manufacturer's instructions. Treat columns with DNase I to remove genomic DNA. Elute RNA with 14 µL of RNase-free water, resulting in a 12 µL eluate. Store RNA at -80 °C.

NOTE: The protocol can be paused here.

6. Measurement of RNA yield and integrity

6.1. Thaw RNA on wet ice. Measure the yield and integrity of isolated RNA using micro-capillary electrophoresis. Load total RNA (1 µL per sample) into a chip (**Table of Materials**) according to the manufacturer's instructions.

REPRESENTATIVE RESULTS:

An LCM protocol was developed to obtain sufficient quantity of high-quality RNA for gene expression analysis in bone cells of mouse femurs. In the optimized protocol, 8-µm-thick frozen bone sections were cut on an adhesive film and stained using a rapid protocol for a commercial LCM frozen section stain. The sample was sandwiched between the PET membrane and the

adhesive film. Mouse bone cells were microdissected using an LCM system that uses gravity for sample collection. A column-based RNA extraction method was used to obtain a high-quality RNA of sufficient yield. The yield and integrity of isolated RNA was measured using micro-capillary electrophoresis (**Figure 1**).

The difference in RNA quality and quantity obtained using different lysis protocols can be seen in the representative gel and electropherograms. When the sample was lysed by pipetting up and down in the cap for 1 min, it was possible to isolate approximately 8.5 ng of RNA from 1 mm^2 microdissected bone tissue (8-µm-thick section). RIN value was 8.60 (**Figure 2**).

Alternatively, LCM was performed using an LCM system that uses de-focused laser pulse, which catapults the material into the overhanging adhesive cap. RNA quality and quantity can be estimated in the representative gel and electropherograms. For fresh frozen bones, it was possible to isolate approximately 1.6 ng of RNA from 1 mm^2 microdissected bone tissue (8-µm-thick section). The RIN value was 1 (**Figure 3**).

FIGURE LEGENDS:

Figure 1: Flowchart of laser capture microdissection protocol for fresh-frozen bones.

Figure 2: Representative gel (top) and electropherograms (bottom) of RNA samples. LCM was performed using an LCM system that uses gravity for sample collection. RNA samples were retrieved from LCM-harvested tissue (samples 1, 3, 5, 7, and 9) and corresponding control sections (samples 2, 4, 6, 8, and 10, respectively). One RNA sample was retrieved from the control section that was stained but was not used for LCM (sample 11). A total area of 1 mm^2 was microdissected, and different lysis protocols were used. Sample 1: 350 µL of the lysis buffer containing β-mercaptoethanol were added into the tube. The cap was closed carefully, and the tube inverted. LCM-harvested cells were lysed by vortexing 1 min, incubating at RT for 10 min and vortexing 1 min. Sample 3: 350 µL of the lysis buffer containing β-mercaptoethanol was added into the collection tube, the cap was carefully closed, and the tube inverted. LCM-harvested cells were lysed by incubating collection tubes upside down for 30 min at RT. Sample 5: 50 µL of the lysis buffer containing β-mercaptoethanol was added into the cap of the collection tube and the sample was lysed by pipetting up and down in the cap for 1 min. The lysate was centrifuged and 300 µL of the lysis buffer containing β-mercaptoethanol was added into the tube. Sample 7: 350 µL of the lysis buffer containing β-mercaptoethanol was added into the tube. The cap was closed carefully, and the tube inverted. LCM-harvested cells were lysed by vortexing and inverting several times. Sample 9: 50 µL of the lysis buffer containing β-mercaptoethanol was added into the cap of the collection tube, and the sample was lysed by incubation for 5 min at RT in the cap. The lysate was centrifuged, and 300 µL of the lysis buffer containing β-mercaptoethanol was added to the tube.

Figure 3: Representative gel (top) and electropherograms (bottom) of RNA samples. LCM was performed using an LCM system that uses de-focused laser pulse, which catapults the material into the adhesive cap positioned above the section. RNA samples were retrieved from LCM-

harvested tissue (samples 5 and 6) and corresponding control section (sample 7). One RNA sample was retrieved from the control section that was stained but was not used for LCM (sample 8). A total area of 0.5 mm^2 or 1 mm^2 was microdissected (samples 5 and 6, respectively). 350 µL of the lysis buffer containing β-mercaptoethanol was added into the collection tube, the cap was carefully closed and the tube inverted. LCM-harvested cells were lysed by incubating collection tubes upside down for 30 min at RT.

DISCUSSION:

Both RNA quality and quantity can be affected negatively at all stages of the sample preparation such as tissue manipulation, LCM process, and RNA extraction. Therefore, an LCM protocol was developed to obtain sufficient amount of high-quality RNA for subsequent gene expression analysis.

For LCM, most laboratories use sections 7−8 µm thick[2]. Thicker sections would allow more material to be harvested. However, if they are too thick, this could reduce the microscopic resolution and the laser may not be able to cut through. It is likely that optimal tissue thickness depends on the LCM system (and the laser and slide type) used, as well as on the tissue in question[8,25]. In the present study, cryosections of different thicknesses (4, 8 or 12 µm) were tested; 8-µm-thick sections were found to be ideal for LCM, while 12 µm bone sections were more difficult to cut with the laser and 4 µm sections gave lower amounts of RNA.

The endogenous RNase activity varies between different tissues. Therefore, staining protocols developed for tissues with very low RNase activity could be unsuitable for other tissue types. Nuclear fast red[26] and cresyl violet[24] were suggested to be the best in terms of preserving RNA integrity. In addition, alcohol-based methods were superior to aqueous stains for maintaining RNA integrity. However, they suffered from irreproducible staining intensity[27]. Time is an important parameter to consider. On the one hand, the time required to search and identify cells of interest in the section, and to perform LCM is often relatively long. On the other hand, time available for LCM is limited, since, in some cases, 20% RNA degradation was reached after 30 min[28]. Therefore, different methods were applied in order to stabilize RNA, such as exposure of sections to argon flux during the microdissection[28], or use of buffered alcohol-based cresyl violet staining and keeping the humidity level in the laboratory low[27]. In addition, a maximum of 15 min for the microdissection step was suggested[2]. In this study, frozen bone sections were stained using a rapid protocol for a commercial LCM stain, and even after 1 h at RT, RIN values decreased from 8.3 to 9.1. Therefore, all microdissections were performed within less than 1 h after staining. In addition, different protocols for staining were tested (with shorter incubations in aqueous reagents or without the xylene step). No significant improvement in RIN values was achieved.

In LCM studies, two different types of RNA extraction methods have been compared: a phase separation method vs. a column-based method. It was found that the RNA extraction method based on columns led to better RNA quality and higher yield compared to the phase separation method[8]. In another study, a commercial kit that uses minimal digestion time and temperature (5 min at RT) resulted in superior RNA quality compared to an RNA isolation kit that needs

longer digestion time at higher temperature (30 min at 42 °C)[7]. In the present study, it was possible to extract high quality RNA (RIN > 8) of sufficient concentration from LCM samples using fresh-frozen mouse bones and a column-based RNA extraction method. Thorough lysis (1 min pipetting in the cap) is essential for good RNA yield. The effect of the RNA extraction method on the RNA yield and integrity obtained after LCM was investigated using several different RNA isolation kits according to the manufacturers' instructions. However, better quality RNAs and increased quantity were obtained with the kit used in the optimized protocol. In the pilot study, microdissected tissue regions of 1 mm^2 final area were cut, and it was possible to isolate approximately 8.5 ng of RNAs using 8-µm-thick bone sections (approximately 800 pg/µL). Typically, osteoblasts, osteocytes, and bone lining cells were captured in 2−3 sections per sample.

Direct comparisons between different LCM instruments are scarce. In one study, two common LCM instruments were tested, which differ in the type of laser used (UV and infrared [IR], respectively) and method of capturing tissue (adhesive isolation cap vs. caps coated with transparent thermoplastic film, respectively). It was found that for thinly-sectioned fresh-frozen mouse brain sections, the IR system resulted in modestly higher RNA quality[7]. In the present study, two LCM systems that allow contact-free sample collection were compared. In one system, the microdissected sample falls by gravity into the cap placed just below[29]. Another system uses a de-focused laser pulse, which catapults the material into the overhanging cap[30]. For fresh frozen bones, higher RNA amounts and RIN values were obtained when gravity was used for tissue capturing. In addition, the catapulting technology is not compatible with the "sandwich" composed of adhesive film, cryo-section, and PET membrane, due to the additional weight of the PET membrane.

LCM requires physical access to the tissue surface. Therefore, mounting and glass covering of the specimen is inapplicable, with the consequence of impaired visualization of morphology. To overcome this limitation, a fluid cover medium was developed[31]. Alternatively, 10−15 µL of ethanol can be added directly on the tissue section, enabling better morphological analysis before evaporation[2]. In the present study, frozen bone sections were cut on an adhesive film and, before the sample was allowed to dry completely, it was sandwiched between the PET membrane and the film. This "sandwich" method gave good morphological contrast and specific bone cells were clearly identified.

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DISCLOSURES:
The authors have nothing to disclose.

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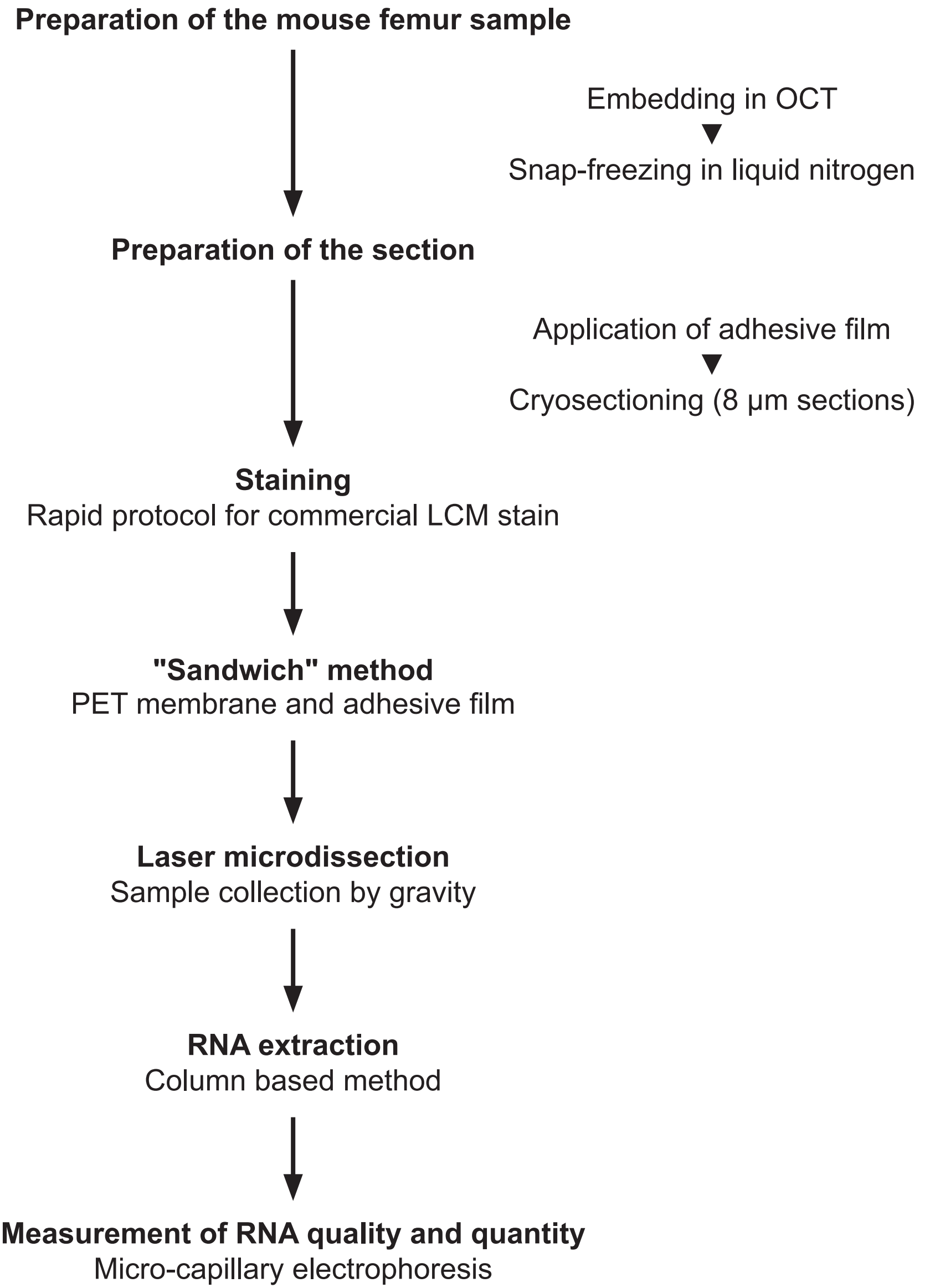

Figure 2

Click here to access/download;Figure;Figure 2.psd

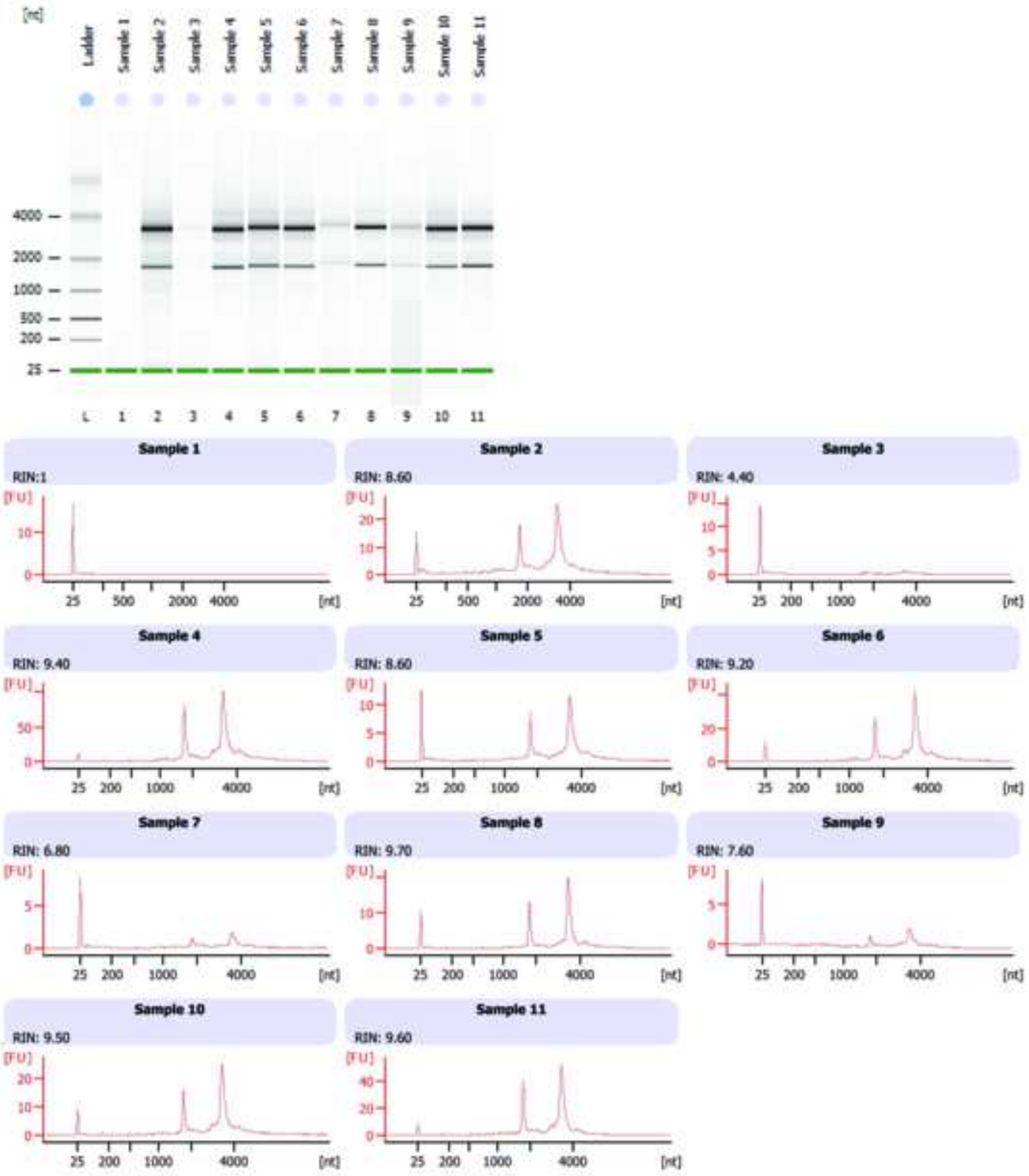

Figure 3

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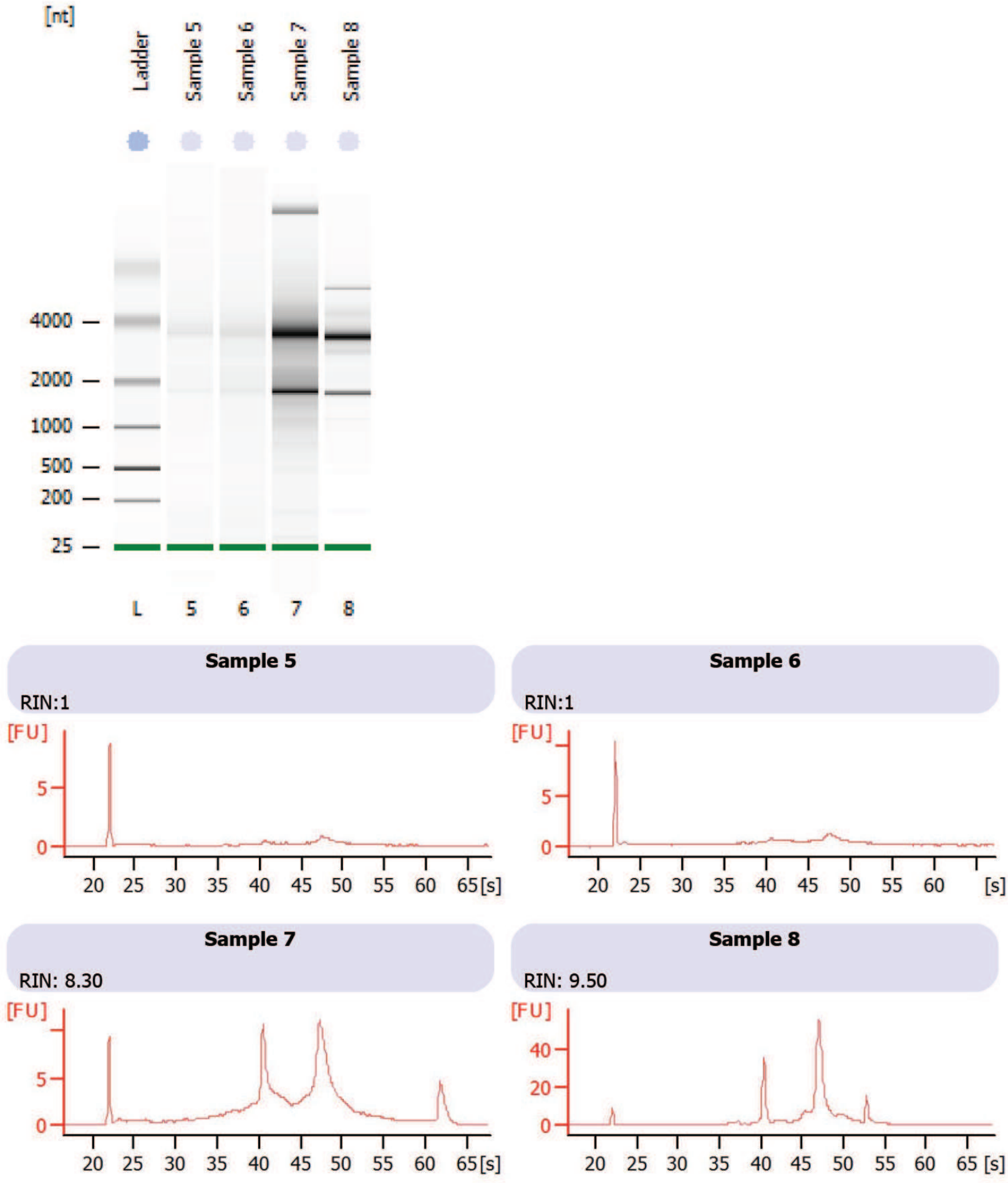

Table of Materials

Click here to access/download;Table of Materials;Table of Materials.xlsx

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2-Mercaptoethanol	Sigma	63689-25ML-F	
Absolute ethanol EMPLURA	Merck Millipore	8,187,601,000	
Adhesive film (LMD film)	Section-Lab	C-FL001	
Agilent 2100 Bioanalyzer System	Agilent Technologies		
Agilent RNA 6000 Pico Chip Kit	Agilent Technologies	5067-1513	
Arcturus HistoGene Staining Solution	Applied Biosystems	12241-05	
Cryofilm fitting tool	Section-Lab	C-FT000	
Cryostat Leica CM 1950	Leica Biosystems		
glass microscope slides, cut colour frosted orange	VWR Life Science	631-1559	
Histology tissue molds PVC	MEDITE	48-6302-00	
LMD7 Laser Mikrodissektion System	Leica Microsystems		
Low profile Microtome Blades Leica DB80 XL	Leica Biosystems	14035843496	
Nuclease-free water	VWR Life Science	E476-500ML	
PET membrane slides 1.4 mircon	Molecular Machines & Industries GmbH	50102	
RNase Away surface decontaminant	Molecular BioProduct	7002	
RNeasy Micro Kit	Qiagen	74004	
Tissue-Tek optimal cutting temperature (OCT) compound	Sakura Finetek	4583	
Xylene	VWR Life Science	28,973,363	

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Author(s):	Marek A, Schüler C, Satue M, Haigl B, Erben RG

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CORRESPONDING AUTHOR

Name:	Reinhold G. Erben
Department:	Dept. of Biomedical Sciences
Institution:	University of Veterinary Medicine Vienna
Title:	Professor of Physiology and Pathophysiology
Signature:	Reinhold Erben
Date:	April 28, 2019

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The Editor
JoVE

Vienna, June 05, 2019

RE: Manuscript no. JoVE60197

Dear Dr. Cao,

Please find below our point-by-point answers to the concerns and comments raised by the reviewers. The suggestions given by the reviewers are much appreciated, and were very helpful to revise the manuscript. All changes in the manuscript are highlighted using the track-changes tool.

Sincerely,

Reinhold G. Erben

Editorial comments:

Changes to be made by the author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Authors: We are very grateful to reviewer 1 for all the corrections and suggestions. All of the suggested changes were included into the paper.

2. Please revise lines 53-55 and 182-183 to avoid textual overlap with previously published work.

Authors: Changed accordingly (lines 57-58 and 189-191). We have re-worded the sentences.

3. Authors and affiliations: Please provide an email address for each author.

Authors: Changed accordingly (lines 9-12). The email addresses for all authors are now provided.

4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u and abbreviate liters to L (L, mL, µL) to avoid confusion.

Authors: Changed accordingly. We now use SI abbreviations throughout the manuscript.

5. Please specify all surgical tools used throughout the protocol.

Authors: Changed accordingly (lines 112 and 117-118). We specified the surgical tools used in the protocol.

6. In the JoVE Protocol format, "NOTE" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. Please consider moving some of the notes about the protocol to the discussion section.

Authors: Changed accordingly. Several "NOTES" within the text providing details about how to perform a particular step were included in the step itself. Some "NOTES" were deleted and some moved to the discussion section.

7. Please include single line spacing between each numbered step or note in the protocol.

Authors: Changed accordingly.

8. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Authors: Changed accordingly.

9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

Authors: Changed accordingly.

10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Authors: Changed accordingly.

11. Figure 2 and Figure 3: Please describe the top panel in the figure legend.

Authors: Changed accordingly (lines 271 and 291). It is now specified what is shown in the top and what in the bottom of Figures 2 and 3.

12. Table of Materials: Please sort the materials alphabetically by material name.

Authors: Changed accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The paper entitled « A laser capture microdissection protocol that yields high quality RNA from fresh-frozen mouse bones" describes a complete procedure of bone sample preparation in order to succeed RNA extractions after cell selection by laser capture microdissection. It is important to obtain good quality RNAs to be confident on subsequent data whatever RNA analysis procedure. Authors compare two procedures for bone cutting and microdissection, and different kits for RNA extraction. They evaluate RNA quality using micro-capillary electrophoresis.

Authors: We are very grateful to reviewer 1 for all the corrections and suggestions. All suggested changes were included into the paper.

Major Concerns:

Lines 160-163: the sentence is unclear: what is the message?

Authors: Since it was suggested in the editorial comments that some of the “NOTES” should be moved to the discussion section, this sentence is now part of the discussion, and has been re-worded (lines 327-329).

We conducted pilot experiments using different staining protocols. The bone was sectioned using an adhesive film, stained using Arcturus HistoGene Staining Solution, and RNAs were isolated directly from the section (without the LCM). RIN values were measured. We used the rapid protocol suggested by the manufacturer and compared it with our rapid protocol (for example the incubation steps in 75% ethanol and water were omitted). Also, the staining protocol for Zeiss LCM sample preparation suggests skipping the xylene step. The staining protocol that gave the best RNA quality in our hands is specified in the manuscript.

Minor Concerns:

Please find below suggestions to improve the paper. Typos are underlined.

Line 33: Definition of PET

Authors: Changed accordingly (line 38). We now defined this abbreviation upon first usage.

Line 35: RNAs (there are many different RNAs)

Authors: Changed accordingly (line 40).

Lines 55-56: mRNAs… for subsequent analyses. In contrast to DNA, RNAs are …

Authors: Changed accordingly (lines 58-59).

Line 57: Since mRNAs are quickly degraded…

Authors: Changed accordingly (line 60).

Line 65: … requires a striking balance.

Authors: Changed accordingly (line 68). We did not want to suggest that the balance is striking, but that it is necessary to strike a balance. Therefore, we have re-worded the sentence.

Lines 83-84: … considered sufficient for RNA studies. Gene expression patterns…

Authors: Changed accordingly (lines 86-88).

Lines 91 and 94: RNAs

Authors: Changed accordingly (lines 94 and 97).

Line 108: How do authors remove soft tissues?

Authors: The femurs are removed using a scalpel, and cleaned of surrounding soft tissues using paper towels. This is now specified in the protocol (lines 117-118).

Line 110: samples

Authors: Changed accordingly (line 119).

Line 122: replace "accelerate RNA degradation" by "alterate RNA integrity" as authors didn't study RNA degradation processes.

Authors: Changed accordingly (lines 135-136). Since it was suggested in the editorial comments that the "NOTES" should be concise and used sparingly, part of the text was included in the step itself and part was deleted.

Line 137: What is "a fitting tool"?

Authors: A fitting tool is a piece of wood covered with soft deerskin. It can be acquired by the same company that produces the adhesive film (LMD film) (Section-Lab). This was specified in the "Table of Materials" and explained in an earlier publication (Kawamoto et al. 2014) referenced in the reference list.

Line 138: 4-, 8- 12-µm

Authors: Changed accordingly (lines 310-312). Since it was suggested in the editorial comments that some of the "NOTES" should be moved to the discussion section, this sentence is now part of the discussion and has been re-worded.

Lines 147-149: Delete the sentences "Caution: The main…the hood", replace by "Work under the hood to avoid intoxication by xylene.

Authors: Changed accordingly (line 163).

Line 150: reagents

Authors: Changed accordingly (lines 160-161).

Line 154: … to remove carefully and completely OCT which may interfere…

Authors: Changed accordingly (lines 165-167).

Definition of OCT?

Authors: This abbreviation was defined upon first usage (lines 118-119).

Lines 160-163: the sentence is unclear: what is the message?

Authors: Changed accordingly (lines 327-329). This issue was already addressed under "Major concerns".

Line 174: replace "was" by "may be"

Authors: Since it was suggested in the editorial comments that the "NOTES" should be used sparingly, this part of the text has been deleted (line 187).

Line 177: …better quality RNAs and increased quantity were obtained…

Authors: Since it was suggested in the editorial comments that the “NOTES” should be used sparingly, this part of the text has been deleted (line 187).

Line 196: RNAs

Authors: Changed accordingly (lines 338-345). Since it was suggested in the editorial comments that some of the “NOTES” should be moved to the discussion section, this sentence is now part of discussion and has been re-worded.

Line 199: replace "RNAse activation" by "RNA degradation" since authors didn't study RNA degradation mechanisms.

Authors: Changed accordingly (lines 206-208).

Lines 2018-211: remove all characteristics of β-mercaptoehanol poisoning and just mention to manipulate it with caution due to its toxicity.

Authors: Changed accordingly (lines 221-222).

Line 224: remove "Resume"

Authors: Changed accordingly (line 238).

Lines 252-253: RNA quality and quantity can be estimated in the representative gel and electrophoregrams.

Authors: Changed accordingly (lines 264-265).

Line 305: cells

Authors: Changed accordingly (line 319).

Line 304: Add a sentence after "intensity": "Time is an important parameter to take into account. On one hand, the time required…

Authors: Changed accordingly (lines 317-319). A sentence was added as suggested by the reviewer.

Line 315: column-based method. It was …

Authors: Changed accordingly (line 331).

Line 316: replace "good" by "better"

Authors: Changed accordingly (line 332).

Line 332: Remove the sentence: "This is most likely due to…

Authors: Changed accordingly (line 355). The sentence was removed.

Table of Materials: Absolute ethanol…

Authors: Changed accordingly. The materials are now sorted alphabetically by material name.

Reviewer #2:

Manuscript Summary:

This manuscript describes how to use LCM to obtain RNA from femur for downstream application.

Authors: The suggestions given by reviewer 2 are much appreciated, and were very helpful to revise the manuscript.

Major Concerns:

In general this manuscript suffer from a lack of attention to details. There's no description for many materials being used (see more below), shockingly, didn't even say what LCM system they were using when they are trying to write a protocol for LCM. This is even worse than the materials and method section of a normal manuscript but this is supposed to be a method paper!

Authors: We completely agree that the reader of a Methods paper expects the brand names in the text. However, in the JoVE protocol format, the use of commercial language and the mention of company brand names of instruments or reagents should be avoided. Therefore, this information was not provided in the text. Rather, all materials and instruments used are listed in the Table of Materials, which is available to reviewers and future readers.

Without a video associated with the protocol, it is also somewhat difficult to know exactly how the authors perform some of the steps. For example, line 135-144, the authors mentioned using adhesive film and glass slides to obtain the sections from cryostat. It is quite difficult to picture how that was done without seeing it, be it a video or pictures. Isn't this supposed to be the journal of visualized experiments? Where is the visualization?

Authors: We fully agree. However, according to JoVE's guidelines, the first step of the process is to submit an original manuscript. Once the manuscript is accepted, JoVE's scriptwriters translate the manuscript into a video script, and afterwards the video is made and published online together with the article.

Specific Comments:

1. line 108-110: no information was provided about what part of the femur was used to prepare the OCT block. The whole femur? Or the distal femur? Did the author cut away some of the surrounding bone? How much trabecular and cortical bone did the authors keep in the block?

Authors: Changed accordingly (lines 117-118). The reviewer is right. We now specify that we use the whole femur to prepare blocks for cryosectioning.

2. Related to comment 1, assuming the authors kept the secondary ossification center of the femur in the OCT block, did that create a problem with sectioning? The presence of bone usually create problems in making good sections.

Authors: The reviewer is absolutely right that it was previously impossible to prepare good quality cryosections of mineralized tissue. This is exactly the reason why an adhesive film was developed for preparation of frozen sections from bone. Cryo-sectioning of bone with the help of a cryo-tape was described in an earlier publication (Kawamoto et al. 2014) referenced in the reference list. Actually, this technology works nicely.

3. What was the temperature of the cryostat set to? It wasn't specified in the protocol and the temperature could be adjusted and might make a difference in sectioning.

Authors: The temperature used for cryosectioning is now specified in the protocol (line 129).

4. Can the author explain why 12um sections are difficult to cut, compared to 8um? In general it is easier to make thicker section than thinner because there's lower chance of the section getting folded. Thicker section creates other problem when doing LCM, for example, because it is harder for the adhesive to pick up thick sections, and because you don't really know what you are getting in the middle of a thick section where you can't see the histology. But I have never heard of 12um sections being harder to make than a 8um section.

Authors: This is a misunderstanding. This sentence is now part of the discussion and has been re-worded to avoid confusion (lines 311-312). We did not want to suggest that it is harder to make 12-µm cryosections than 8-µm sections on the microtome. Rather, we noticed that it is more difficult to cut the 12-µm compared with the 8-µm bone cryosections with the cutting laser. It was often necessary to use the laser several times or to use a higher power for cutting. Since both the time necessary to perform LCM and the laser energy are important parameters for obtaining good RNA quality, we use 8-µm bone cryosections in our optimized protocol.

5. I am not sure if it is necessary to place all the tubes on ice while staining. Did the authors compared the difference when they place tube on ice versus not?

Authors: In all staining protocols for RNA sample preparation, it is suggested to keep solutions on wet ice in order to avoid RNA degradation. Although our staining protocol does not take long, we did not compare it to a staining protocol performed completely at room temperature.

6. Can the authors comment on how much RNA can be obtained from how many sections (or how many LCM dissected area combined)? This information will be useful to reader when planning their own experiments.

Authors: We agree and have changed the text accordingly (lines 341-345). In our study, RNA elution resulted in approximately 12 µL eluate (lines 239-240). Total RNA (1 µL per sample) was than loaded into a Bioanalyzer chip (lines 247-248). Using Bioanalyzer Pico chip, approximately 800 pg/µL could be measured in 1 µL RNA sample. Therefore, it was possible to isolate approximately 8.5 ng of RNA from 1 mm2 microdissected bone tissue (8-µm-thick section). RIN value was 8.60 (Fig. 2). This RNA can be used in downstream analyzes. Typically, osteoblasts, osteocytes and bone lining cells were captured in 2–3 sections per sample (lines 344-345).

7. Where can the reader find the list of materials used in the protocol? For example, where do you get the "commercial LCM frozen section stain" (line 155)? What is that "lysis buffer" (line 215)? Did the author used for example Arcturus PicoPure RNA extraction kit? Or the Qiagen RNeasy Micro Kit? These are very important information for the reader if one is to reproduce the results but none were found here. Line 224 said "Resume RNA extraction according to the manufacturer's instructions." But who is the manufacturer?

Authors: We agree. All materials and instruments used are listed in the Table of Materials. Indeed, we used the Qiagen RNeasy Micro Kit for RNA extraction. All the additional information important for reproducing the results is given in the protocol (lines 234-240).

8. What LCM system did the authors even used? How is it possible to omit that? There are different systems of LCM and depending on which system (like ArcturusXT uses the laser to make the membrane stick to the CAP, versus gravity assisted LCM where the laser-cut section drop down to a collection tube) and the protocol is specific for a certain system but not generally applicable to all.

Authors: We agree that this is a very important point. The issue was already addressed above.

9. There isn't a single image in the manuscript showing a LCM dissected section. Maybe the author should include that to illustrate how the section looks before and after the LCM.

Authors: We fully agree that images would be helpful. However, the film produced as part of this publication will illustrate all the steps of the procedure in a much better way than any single image could do.