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A method for isolating RNA from canine bone V.2

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We use this protocol and it's working

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Abstract

Extracting sufficient quantity and quality RNA from bone is essential for downstream application, such as transcriptomic sequencing, to evaluate gene expression. Isolation of RNA from bone presents a unique challenge owing to the hypocellular, brittle and mineralized matrix, which makes homogenizing the tissue difficult and provides little RNA to work with. Removal of contaminating tissue, such as bone marrow and connective tissue, is essential for isolating RNA that is unique to osteoblasts, osteoclasts and osteocytes. This protocol establishes a method to effectively isolate RNA from normal canine bone cells using the second phalanx, without contamination from other tissue types, for downstream transcriptomic analysis.
This method combines physical manipulation to remove exterior tissue, washing and centrifugation to remove cells and fat within the diaphysis, homogenization using a mortar and pestle on dry ice prior to bead dissociation, followed by acid guanidinium thiocyanate-phenol-chloroform extraction and column purification to yield sufficiant quantity and quality RNA from canine phalanges. The second phalanx was chosen due to its size small enough to fit into a 1.7 mL microfuge tube, but large enough to provide enough RNA. Mean RNA obtained using this protocol was 14.7 ug of RNA per gram of frozen bone.

Attachments

PDF

btn-2019-0153.pdf

1.2MB

Materials

MATERIALS

KimWipes **Fischer Scientific**

TRI Reagent® **Molecular Research Center, Inc.**

RNase AWAY™ Spray Bottle, RNase in spray bottle; 475mL **Thermo Fisher Catalog #**7002

Ceria stabilized zirconium oxide beads 0.5 mm diameter **Next Advance Catalog #**ZrOB05

Bullet Blender **Next Advance Catalog #**Bullet Blender 24

Bromochloropropane (BCP) **Molecular Research Center, Inc. Catalog #**BP151

DNase I **Thermo Fisher Scientific Catalog #**18068015

RNeasy Micro Kit **Qiagen Catalog #**74034

Before start

Wipe all surfaces and materials with RNase Away to minimize potential degradation due to RNases. Prepare steel mortar and pestle RNA-free by wiping with RNase Away, wrapping in aluminum foil, and baking at 350 °F for 06:00:00 or Overnight. Freeze in -80 °C prior to RNA extraction.

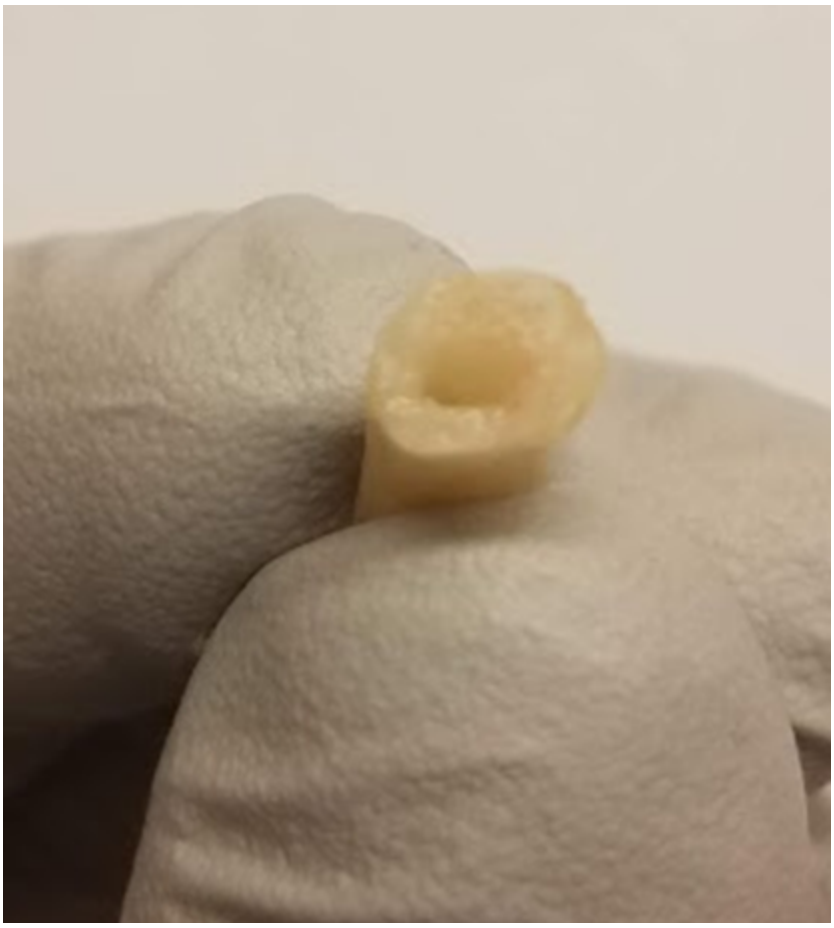
Bone Preparation

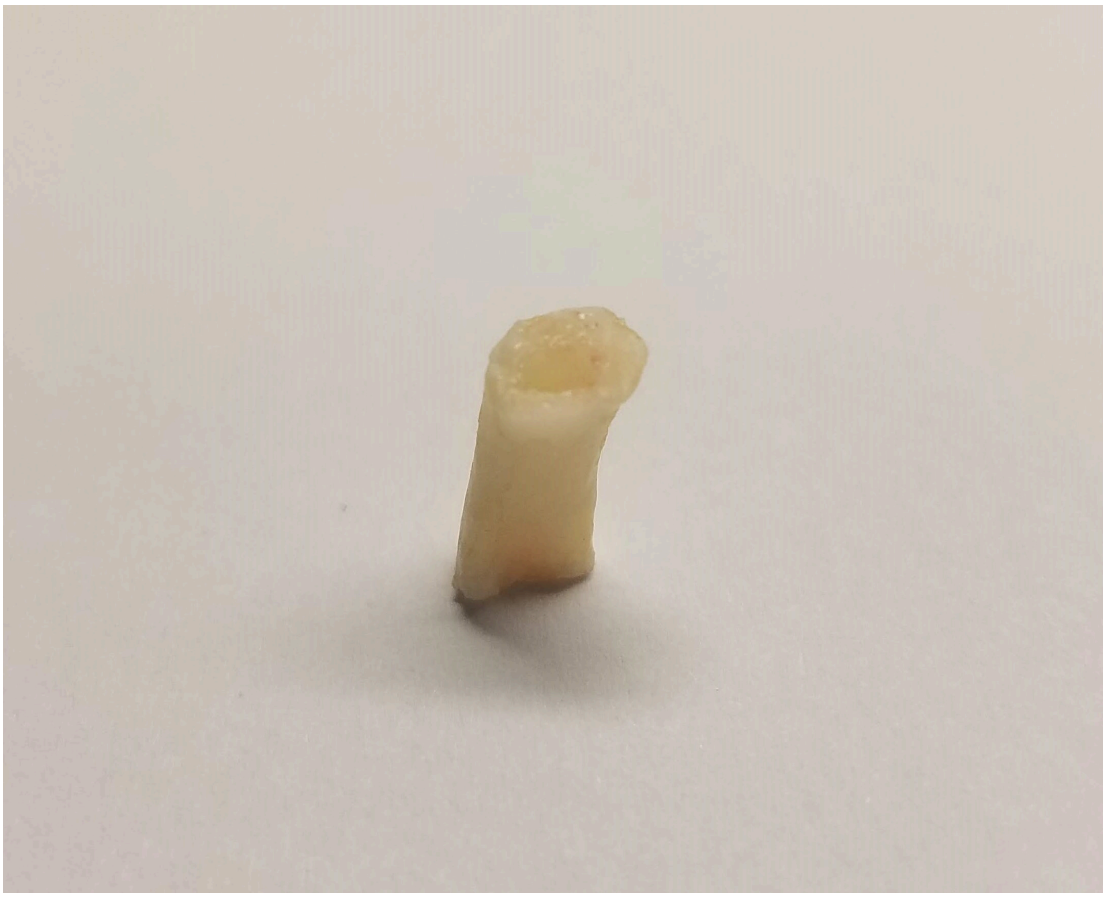
1 Remove all exterior soft tissue around disphysis using scissors/scalpel/KimWipes. Use a scalpel to scrape bone along diaphysis exterior to ensure complete removal of periosteum.

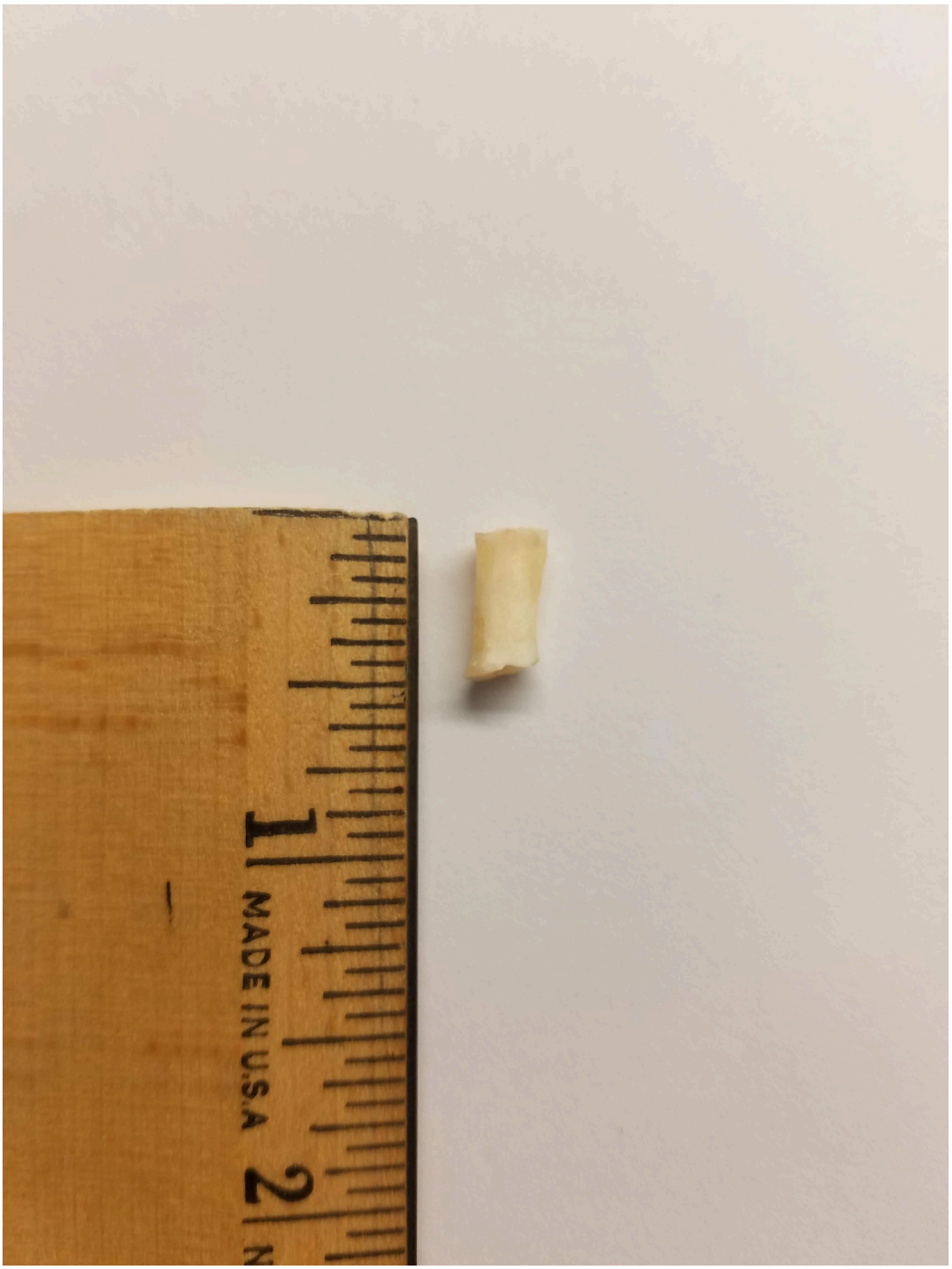
Note

**Isolating RNA that is unique to osteoblasts, osteoclasts, and osteocytes is dependent on sufficient removal of these 'contaminating' exterior tissues.

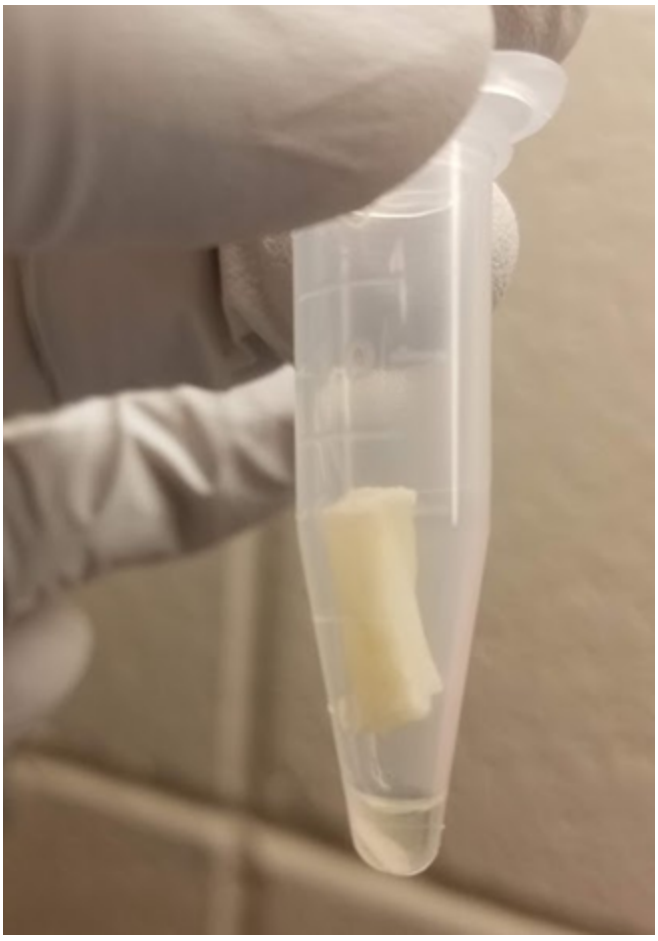
2 Cut and discard epiphyses using large bolt cutters, being careful to not disturb the tubular structure of the diaphysis.







3 Place diaphysis in a 1.7 mL microfuge tube and centrifuge at 10.000 x g, Room temperature, 00:10:00. Liquid in the bottom after centrifugation is the bone marrow/fat contained within the diaphysis.



4 Transfer diaphysis to a new 1.7 mL microfuge tube. Add enough 1x PBS (Phosphate Buffered Saline) to cover the bone completely. Centrifuge at 10.000 x g, Room temperature, 00:05:00 .

5 Transfer diaphysis to a new 1.7 mL microfuge tube. Add enough 1x PBS to cover the bone completely and centrifuge again at 10.000 x g, Room temperature, 00:05:00 .

6 Snap freeze bone in liquid nitrogen and store at -80 °C for up to 8 months until RNA extraction.

Bone Homogenization/RNA Extraction

7 Prepare two 1.7 mL Eppendorf Safe Lock** microfuge tubes, each containing 1 mL Tri-Reagent and approximately 0.5 g of zirconium oxide beads (0.5 mm diameter) and place tubes On ice .

Note

**Eppendorf Safe Lock tubes prevent sample leakage during subsequent Bullet Blender step.

8 Grind bone into a fine powder using a frozen RNase-free steel mortar and pestle** and hammer on top of a bed of dry ice, working quickly to avoid RNase degradation.

Note

**Mortar and Pestle can be prepared RNase-free by wiping with RNase Away, wrapping in aluminum foil, and baking at 350F for 6 hours or overnight. Freeze in -80 °C prior to RNA extraction.

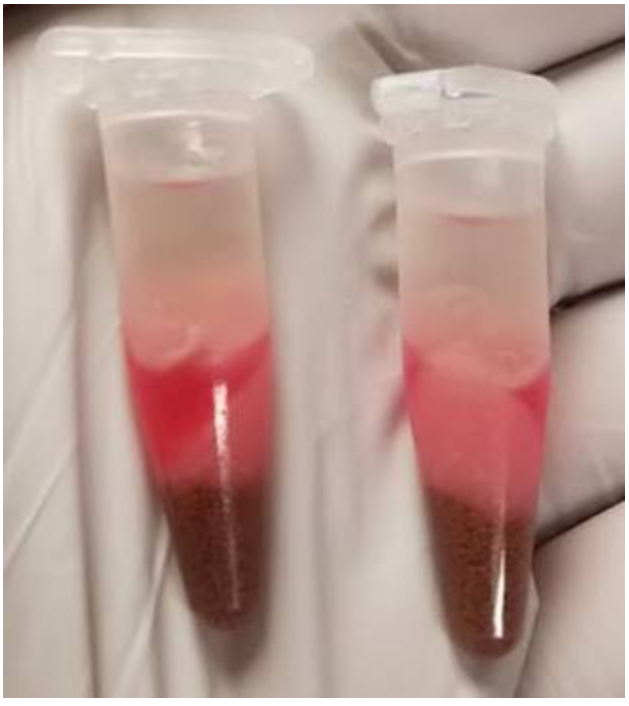
9 Divide the bone powder into the two previously prepared microfuge tubes containing 1 mL Tri-Reagent and 0.5 g zirconium beads .

10 Using a Bullet Blender at speed "5.5", subject samples to 4 rounds of a 00:00:30 spin in Bullet Blender with a 00:01:00 incubation On ice between rounds (to keep samples cool and minimize degradation).

11 Incubate samples for 00:10:00 at Room temperature .

12 Add 100 µL bromochloropropane (BCP) to each tube and vortex thoroughly.
Incubate samples for 00:05:00 at Room temperature .

13 Centifuge tubes at 20.000 x g, 4°C, 00:15:00 .



14 Carefully remove top aqueous layer and add 10 µL DNase I and 1/10 volume DNase I Reaction Buffer; Incubate 00:10:00 at Room temperature .

15 Inactivate DNase by adding 10 µL of 25 mM EDTA (included) and heat for 00:10:00 at 65 °C .

16 Add equal volume 70% ethanol and mix well by pipetting.

17 Apply sample to RNA column from RNeasy Micro Kit placed in a 2 mL collection tube and centrifuge 21.000 x g, Room temperature, 00:01:00 .

18 After discarding eluate, add 700 µL Buffer RW1 , incubate on the column for 00:02:00 at Room temperature , centrifuge 21.000 x g, Room temperature, 00:01:00 .

19 Discard flow through, add 500 µL Buffer RPE , incubate on the column for 00:02:00 at Room temperature , and centrifuge 21.000 x g, Room temperature, 00:01:00 .

20 Discard flow through, add 500 µL of 80% ethanol , incubate on the column for 00:02:00 at Room temperature , and centrifuge 21.000 x g, Room temperature, 00:03:00 .

21 Discard flow through and replace 2 mL collection tube and centrifuge the columns at 21.000 x g, 00:05:00 , with lids open .

22 Replace collection tube, add 17 µL of RNase-free water (preheated to 65 °C), incubate for 00:10:00 on the column at Room temperature , and centrifuge 21.000 x g, Room temperature, 00:05:00 .

23 Concentration and purity can then be determined using a Nanodrop spectrophotometer.