User-Developed Protocol

Intermountain Bone/Tooth Processing and Purification

This protocol was developed at Intermountain Forensics with the help of Sam Houston State University and describes the procedures associated with processing large bone fragments into bone powder via QIAGEN® TissueLyser II and the digestion and extraction of DNA from bone powder via QIAGEN EZ1® Advanced XL instrument. This protocol was adapted from the QIAGEN UDP Forensic "Quick Bone DNA extraction" clean-up protocol.

This protocol has not been thoroughly tested by QIAGEN.

IMPORTANT: Please read the "Safety Information" and "Important Notes" sections in the *EZ1 DNA Investigator® Handbook* before beginning this procedure.

IMPORTANT: The EZ1 DNA Investigator Kit is intended for research use only. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

Reference

QIAGEN: Forensic "Quick Bone DNA extraction" protocol using the EZ1 DNA Investigator Kit

EZ1 Advanced XL User Manual

EZ1 DNA Investigator Handbook

TissueLyser II User Manual

Intermountain Forensics: TissueLyser II and EZ1 DNA Investigator Kit Bone Extraction protocol

Equipment and reagents to be supplied by user

- BioRobot® EZ1, EZ1 Advanced, or EZ1 Advanced XL with appropriate DNA Investigator Protocol Card
- TissueLyser II (cat. no. 85300) and Grinding Jar Set, Stainless Steel (cat. no. 69985)
- EZ1 DNA Investigator Kit, cat. no. 952034
- 3M Sodium Acetate (NaOAc), pH 5.0
- Buffer ATL (cat. no. 19076)
- Buffer MTL (cat. no. 1023430)
- Thermomixer or shaker-incubator
- Variable speed rotary tool with appropriate cut off wheel
- Sandpaper*
- Sterile reagent grade water
- Commercial bleach
- Collection tubes, 2.0 ml (cat. no.19201)
- Ethanol* (96–100%) (do not use denatured alcohol, which contains other substances such as methanol or methylethylketone)

* Indicates optional equipment and reagents.

- Small cooler*
- Dry ice*
- Freezer* (−80ºC preferred but −20°C or 0°C can be used)
- ZipLoc® freezer bag
- Sterile, RNase-free pipet tips
- Disposable gloves
- Microcentrifuge tubes, 1.5 or 2 ml
- Conical tube, 50 ml
- Microcentrifuge (with rotor for 2.0 ml tubes) for centrifugation at 20–25°C
- Kimwipes

Notes

- This protocol does not utilize liquid nitrogen. Instead, an ethanol/dry ice bath is used to cool grinding jar and bone fragments. Alternatively, the jar and bone fragments can be cooled in a freezer.
- Buffer ATL is used as a lysis buffer, which is not included in the EZ1 DNA Investigator Kit. Buffer ATL, cat. no. 19076, may be purchased separately.
- The Large-Volume EZ1 Protocol is used and requires Buffer MTL, which is not included in the EZ1 DNA Investigator Kit. Buffer MTL, cat. no. 1023430, may be purchased separately.
- Buffer RLT contains guanidine thiocyanate and is therefore not compatible with disinfecting reagents containing bleach.
- Perform all steps of the procedure at room temperature (15–25°C). During the procedure, work quickly.

Things to do before starting

- Commercial bleach (6%) should be diluted in equal parts with deionized water to obtain a working solution for bone fragment decontamination.
- Carrier RNA (cRNA) solution should be prepared by adding 310 µl into the cRNA vial.
    - Recommended: Transfer entire volume into 20µl aliquots and store frozen.
- Warm MTL utilized in powder digestion step to 70°C (day 2).

* Indicates optional equipment and reagents.

Procedure

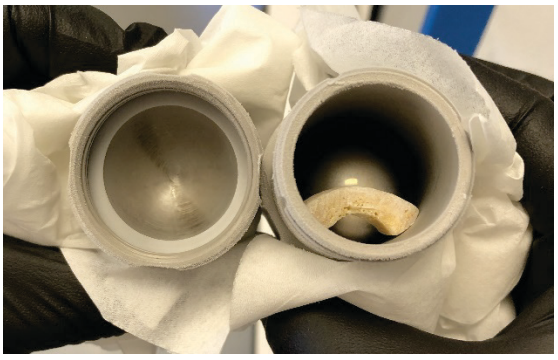
Bone/tooth preparation

1. If desired, remove the outside layer of the tooth or bone fragment(s) with a new, clean piece of sandpaper (or other rotary tool attachment).
2. An approximately 2 cm x 2 cm bone or tooth fragment(s) is need during the grinding step. If the bone fragment is larger than this size, cut the bone fragment using a rotary tool and new attachment. An example of acceptable bone fragment sizes is depicted in the image below:

3. Sanitize the outer surface of the bone or tooth fragment(s) by submerging the fragment(s) in the equal parts bleach solution previously prepared. Vortexing the bone or tooth in a bleach solution may be performed for especially dirty samples.
4. Rinse the bone or tooth fragment(s) by submerging in a sterile water bath for a total of 3 washes. Use fresh sterile water for each wash.
5. Allow the bone or tooth fragment(s) to air dry overnight in a ventilated hood.

Bone/tooth pulverization

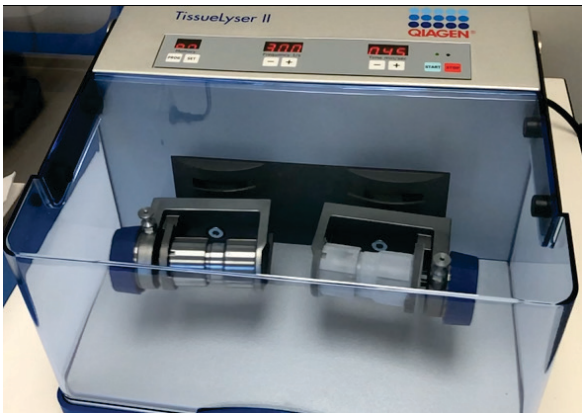
6. Place dried bone or tooth fragment into a stainless-steel grinding gar with one stainless steel grinding ball. See the image below:



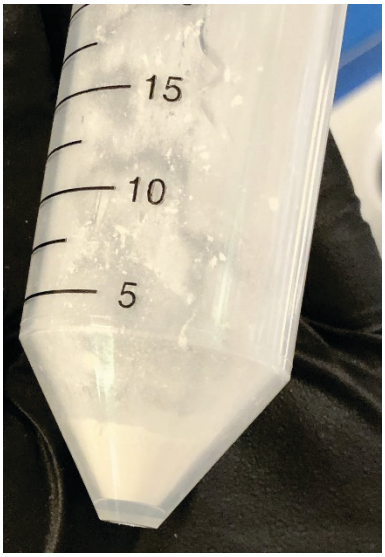
7. If an ethanol/dry ice bath is being utilized, prepare bath by pouring ethanol into a small cooler. Insert dry ice chips into ethanol bath until immediate evaporation of dry ice has ceased, which indicates that bath has reached desired temperature.
8. Place assembled grinding jar with bone or tooth fragment(s) into Ziploc freezer bag and submerge into bath for 10 minutes. Alternatively, the grinding jar assembly can be placed in the freezer for 20–60 minutes.

9. Place the grinding jar assembly into the clamp of the TissueLyser II. Rotate the handwheel clockwise with two fingers until the grinding jar is seated in the clamp appropriately. The jar should be level and not moving freely. Continue to rotate the handwheel clockwise until 6–8 audible clicks are heard; the locking pin will rise and fall with each audible click. Ensure that the locking pin is in the fully lowered position before starting the instrument.

   Note: Ensure both grinding jars are secured to the instrument for balance prior to operation. See the image below:



10. Grind the bone fragment(s) for 2 minutes at 30 Hz.
11. Transfer the bone powder into a clean tube for storage. The bone powder should be dry and have the consistency of fine dust or powder. See the image below:



12. Once powder has been transferred, clean the grinding assembly with bleach followed by ethanol.

Powder digestion

13. Transfer up to 200 mg of the bone powder into a 2 ml conical bottom tube.
14. Create a master mix by using 337.5 µl Buffer ATL, 37.5 µl Proteinase K, and 375 µl 0.5M EDTA, pH 8.0, for each sample/reagent blank. Mix thoroughly by vortexing and add 750 µl of master mix to each sample/reagent blank.

15. Vortex vigorously to ensure all bone powder is incorporated into solution.
    Note: This process can take several minutes of vortexing.
16. Place the tube in a thermomixer and incubate at 56°C overnight while shaking at max speed.
17. Centrifuge samples at max speed for 5 minutes to pellet the bone powder.
18. Transfer 500 µl of the supernatant to a clean 2 ml EZ1 sample tube (or other tube compatible with the EZ1 rack).
    Note: The bone powder will absorb some digest buffer.
19. Create a master mix using 400 µl warm MTL buffer, 30 µl 3M NaOAC, pH 5.2, and 1 µl cRNA for each sample/reagent blank, plus overage, and mix thoroughly by vortexing. Add 431 µl to each sample and reagent blank.

Sample purification

20. Continue with “Protocol: DNA Purification (Large-Volume Protocol)”.

Acknowledgements

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