Preparation and Extraction of Calcified Tissue Samples

Table of Contents

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1 INTRODUCTION

These procedures describe the process of chemical digestion and purification of deoxyribonucleic acid (DNA) from calcified tissue (i.e., teeth and bones) for nuclear and/or mitochondrial DNA testing.

2 SCOPE

These procedures apply to DNA personnel that extract DNA from calcified tissue and DNA personnel that perform the associated quality control procedures.

3 EQUIPMENT

3.1 Equipment/Materials

- General laboratory supplies (e.g., tubes, pipettes)
- Conical tubes (Sardstedt or equivalent), 15 mL and 50 mL
- Centrifuge (Sorvall™ Legend™ XT, Sorvall™ ST 16, Heraeus™ Megafuge™ 16, or equivalent) with 15ml conical tube inserts
- Centrifuge (Eppendorf Minispin Plus, Hermle MR-2, or equivalent)
- Freezer Mill (SPEX® 6750 or 6770, or equivalent)
    - Pulverization cylinder, end plugs, impactor bar
    - Extractor
- Hood Prefilter (Air Clean ACF PRE or equivalent)
- Isopropanol Wipes, 70%
- Incubator (Lab-Line Imperial III or equivalent) with nutator
- Rotary tool (Dremel® or equivalent)
    - Rotary tool accessories
    - Sanding disc(s) or barrel(s)
    - Cutting disc(s)
- Sonicator (Fisher Scientific FS-20 or equivalent) or nutator, if needed
- Vivacon® 2 concentrators, 50kDa/50,000 MWCO (ETO treated preferred)
- Qiagen® MinElute® spin columns

3.2 Reagents

- DNA-OFF™ or 10% Bleach Solution
- Liquid Nitrogen
- Demineralization/Extraction Buffer (Demin Buffer)
- 20mg/mL Proteinase K (ProK)
- Terg-a-zyme® (or equivalent)
- 25:24:1 Phenol/Chloroform/Isoamyl Alcohol (PCIA)
- Qiagen® Buffer PB
- Qiagen® Buffer PE (with Ethanol added)
- Purified water or equivalent, available at laboratory sinks
- Reagent Grade Water

4 STANDARDS AND CONTROLS

At least one extraction control (i.e., reagent blank [RB]) must be processed in parallel with each extraction sample.

For evaluation of the extraction controls, refer to the appropriate DNA interpretation procedure (i.e., BIO-570, BIO-571, BIO-572).

5 SAMPLING

If soft tissue, tooth pulp or bone marrow is present, DNA may be extracted using the appropriate DNA procedure for the extraction of DNA from body fluids and tissues. If several teeth are available for analysis, non-restored teeth are preferred over restored teeth, and molars are preferred over non-molars. If several bones are available, order of preference for bone selection for DNA extraction is generally as follows:

A. Long bone
B. Rib (mid-section)
C. Other (determined by Examiner)

6 PROCEDURE

Refer to the DNA Procedure Introduction (i.e., BIO-100) for applicable laboratory quality assurance and cleaning instructions. For previously pulverized samples, ensure sample is in appropriate tubes (refer to 6.2 step 7) and proceed to sample digestion (6.3).

Ensure the appropriate fields (i.e., instruments, reagents) in the Sample Tracking and Control Software (STACS) are completed, as necessary

6.1 Equipment Preparation

Supplies needed for sample preparation may include:

- Freezer mill sample vial assembly (cylinder, end plugs, impactor bar)
- Rotary tool and accessories (i.e., cutting disc(s) and sanding disc(s) or barrel(s))
- Balance
- Weigh paper or weigh boats
- 15 mL conical tubes and 15 mL tube rack
- Ruler
- Tweezers (optional)
- Screwdriver (optional)

1.	Prior to use, clean the rotary tool/accessories with bleach then wipe with isopropanol. Clean the freezer mill sample vial assembly: • Place cylinder, end plugs, and impactor bar in 50 mL conical tubes and agitate in DNA-OFF or 10% bleach for at least 20 minutes. • Rinse at least 3 times with water (purified or higher grade). • Agitate in reagent grade water for at least 20 minutes. • Wipe the cylinder, end plugs, and impactor bar with isopropanol. UV for at least 5 minutes.

6.2 Sample Collection and Preparation

Specimen may be photographed with label and ruler(s) before and after processing.

At the discretion of the examiner, steps 1 and/or 2 may be altered or not performed; for example, on a compromised (e.g., burned) or fragile item or sample. Similarly, additional cleaning before or after step 3 (e.g., additional Terg-a-zyme soak, additional water rinse) may be used as determined necessary for an item or sample. The case notes will reflect any alternative sample preparation steps or additional cleaning.

The following steps will be performed wearing a disposable lab coat.

1.	In an appropriate hood, use sanding disc or barrel to sand outer surface of sample around area to be excised. Clean sanded area with isopropanol.
2.	Use cutting disc to remove tooth root(s) or ~2 cm x 2 cm section of bone. Clean the cutting(s) with isopropanol.

When possible, avoid any previous bone cuttings or tooth restorations that may be present.

Sand interior of any excised portion, if necessary.

If marrow or pulp is present, remove with tweezers, place in a UV'd 1.5 mL tube, and process according to the appropriate procedure for the extraction of DNA from body fluids and tissues.

3.	If evidence will be consumed and/or if appropriate, perform additional cleaning: • Soak, nutate, or sonicate with 5% Terg-a-zyme solution for at least 15 minutes. • Soak, nutate, or sonicate with reagent grade water for at least 15 minutes. Allow sample to dry before proceeding.
4.	Place excised portion of bone or tooth into assembled freezer mill sample vial then insert into freezer mill.
5.	Ensure liquid nitrogen is to the fill line and pulverize the sample using the appropriate freezer mill program.

6750 Freezer Mill:

- T1: 5.0
- T2: 2.0
- T3: 0.1
- Rate: 15

6770 Freezer Mill:

- Cycles: 1
- Precool: 0 min.
- Run Time: 5 min.
- Cool Time: 2 min.
- Rate: 15 CPS

6.	When grinding is complete, open lid and remove cylinder.

If sample is not sufficiently pulverized, reinsert for additional grinding. Additional liquid nitrogen may be necessary.

7.	• Transfer ~0.2 g of pulverized sample to 15mL conical tube(s). Ensure tube(s) are appropriately labeled. • Create a total of 3 tubes of ~0.2 g of powder, if enough sample is available.

Store remaining powder, if applicable, in a conical tube at 4°C or colder. Case notes should indicate the approximate amount of powder remaining.

Powder sample(s) may be stored at 4°C *or colder prior to initiating extraction process.*

8.	The following may be done at the most convenient point after use: • Clean rotary tool with bleach and wipe with isopropanol. • Clean rotary tool accessories with Terg-a-zyme solution followed by bleach and isopropanol. • If reusing end pieces, bar, and/or cylinder, clean with Terg-a-zyme solution rinse with water, then repeat initial cleaning. • Clean hood with bleach and replace hood prefilter. UV for at least 5 minutes

Disposable lab coats used for sample preparation should not be worn in laboratory space used for extraction and amplification set-up procedures.

6.3 Pulverized Sample Digestion

Supplies needed for sample powder processing may include:

- Demin Buffer
- ProK (Do not UV)
- 15 mL conical tubes for RBs. *The number of RB tubes will be equal to or greater than the number of sample tubes*

1.	Add to each sample and RB tube: • 3 mL Demin Buffer • 200 µL ProK Vortex to suspend sample powder.
2.	Incubate tubes at 56°C with agitation for 4 to 48 hours.

Generally, samples are incubated for ~14-16 hrs which should be sufficient digestion for further processing.

6.4 PCIA/Vivacon Purification

Supplies needed for PCIA/Vivacon purification may include:

- 15 mL tubes and tube rack
- PCIA (Do not UV)
- Vivacon concentrators and tube rack
- Reagent grade water

The inside of the centrifuge should be wiped with isopropanol before use.

PCIA and all consumables that come into contact with PCIA (i.e., tips, tubes) must be disposed of in an appropriate waste container.

1.	• In a fume hood, add 3 mL PCIA to each tube. • Vortex briefly and centrifuge at ~4200 x g for 8 minutes.

If upper aqueous layer is still cloudy, additional centrifuge time may be required.

2.	• Transfer aqueous layer from each tube to a new, labeled 15 mL conical tube. Avoid transferring the interface. • In a fume hood, add 3 mL PCIA to each tube. *PCIA may be added to tubes prior to sample.* • Vortex briefly and centrifuge at ~4200 x g for 8 minutes.

If upper aqueous layer is still cloudy, additional centrifuge time may be required.

3.	• Add 700 µL reagent grade water to each labeled Vivacon concentrator. • Transfer ~1300 µL of the aqueous layer from each tube to the corresponding Vivacon. Avoid transferring the interface. • Gently pipette mix. • Centrifuge for 30 minutes.

For centrifuging the Vivacon concentrators:

- On the Sorvall™ Legend™ XT with fixed rotor, set at ~5860 x g.
- On the Heraeus™ Megafuge™ 16 or Sorvall™ ST16 with swinging-bucket rotor, set at ~5000 x g.

If the volume in the Vivacons is not low enough to add remainder of aqueous layer, centrifuge affected samples an additional ~5-15 minutes or add reagent grade water to all Vivacons, gently pipette mix, and centrifuge ~5-30 minutes.

4.	• Discard waste. • Add remainder of aqueous layer and/or reagent grade water to each corresponding Vivacon and bring volume to at least 2 mL. Gently pipette mix. • Centrifuge for 30 minutes or until volumes are ~700 µL or less (slightly above or anywhere below the "50K" on the Vivacon).

If the volume in the Vivacons is not ≤700 µL, centrifuge affected samples an additional ~5-15 minutes or add reagent grade water to all Vivacons , gently pipette mix, and centrifuge ~5-30 minutes.

5.	• Discard waste. • Add reagent grade water to each Vivacon to bring volume to ~2 mL. • Gently pipette mix. • Centrifuge for ~15 minutes to get volumes to ~100 uL or less.

If the volume in Vivacons is not ≤100 µL, centrifuge affected samples an additional ~5-15 minutes or if needed, add reagent grade water to all Vivacon(s), gently pipette mix, and then centrifuge an additional ~5-15 minutes.

6.	• Discard waste. • Add reagent grade water to bring volume to ~100 µL if necessary. • Invert Vivacons into labeled recovery caps. • Centrifuge at 2500 x g for 2 minutes.

Wipe centrifuge with isopropanol and clean centrifuge inserts with 10% bleach and rinse with water at most convenient point after use.

Lysates may be stored refrigerated for up to 6 days prior to purification. To prevent evaporation, lysates should be capped versus stored in the Vivacon assembly. Ensure that no precipitate remains in the lysates prior to continuing processing.

6.5 MinElute Purification

Supplies needed for MinElute purification may include:

- Buffer PB
- Buffer PE
- MinElute columns in collection tubes
- Reagent grade water
- 1.5 mL and/or 2 mL tubes

****NOTE: MinElute columns must be at room temperature prior to use.** (Generally takes about 30 minutes.)

1.	• Add 500 µL Buffer PB to each MinElute column. • Transfer the recovered volume (~100 µL) from one sample and one RB Vivacon recovery cap to the corresponding sample or RB MinElute column. Gently pipette mix. • Centrifuge at ~13000 x g for 1 minute. (Additional spins may be used, as needed.) • Discard waste. • If applicable return columns to collection tubes and repeat for each remaining sample(s) and RB(s) using the same corresponding MinElute column.
2.	• Add 750 µL Buffer PE to each MinElute column. • Centrifuge at ~13000 x g for 1 minute. • Discard waste
3.	• Centrifuge at ~13000 x g for 1 minute. • Transfer each MinElute column to a new, labeled tube.
4.	• Add 25 µL reagent grade water to each MinElute column. • Centrifuge at ~13000 x g for 1 minute.
5.	• Add another 25 µL of reagent grade water to each MinElute column. • Centrifuge at ~13000 x g for 1 minute. • Transfer sample and RB extracts to appropriate tubes for future processing.

If applicable, refer to the appropriate DNA procedure for concentrating extracted samples (i.e., BIO-520 or BIO-510).

7 LIMITATIONS

The quantity and quality of the DNA present within any biological material ultimately determines if a DNA extraction is successful.

8 SAFETY

- All evidence containing or contaminated with blood or other potentially infectious materials will be considered infectious regardless of the perceived status of the source individual or the age of the material.
- Refer to the FBI Laboratory Safety Manual for information on personal protection, the proper disposal of the chemicals used in these procedures, as well as the biohazardous wastes generated.
- Procedural Specific Chemical Hazards:
    - Solutions of Proteinase K can be irritating to mucous membranes. Use eye protection when handling.
    - PCIA is an irritant and is toxic. Its use will be confined to a chemical fume hood whenever possible.
    - Liquid nitrogen can be hazardous. Use appropriate PPE when handling.
    - Buffer PB spills should not be directly cleaned with bleach as a combination of the two can form highly reactive compounds. Spills should be absorbed prior to cleaning.

9 REFERENCES

Camps, F.E., editor. *Gradwohl's Legal Medicine*. Baltimore: Williams and Wilkins (1968).

Gaensslen, R.E. *Sourcebook in forensic serology, immunology, and biochemistry*. U.S. Department of Justice, National Institute of Justice, Washington, D.C. (1983).

Lee, H. C. Identification and grouping of bloodstains. Saferstein, R., ed., In: *Forensic Science Handbook*, Prentice-Hall, 267-337 (1982).

10 REVISION HISTORY

Revision	Issued	Changes
00	06/15/2022	Reformatted DNA 401-1 into new template and assigned new Doc ID. Minor edits to content.

11 APPENDIX A: QUALITY CONTROL PROCEDURES

11.1 Reagents

Refer to the DNA procedure for reagent purchasing, preparation and records (i.e., BIO-103) for additional requirements.

11.1.1 Contamination check of Demineralization Extraction Buffer

Each new lot of Demineralization Extraction Buffer should be evaluated for the presence of extraneous DNA prior to its use on evidentiary samples. Reagents may be concurrently tested or tested with an in-use lot of the counterpart reagent(s).

A. Extract, at a minimum, five reagent blanks (RBs) following sections 6.3 through 6.5 using the new lot of Demineralization Extraction Buffer.

B. Amplify the five reagent blanks, a positive control (HL60), and a negative control (reagent grade water) with a more sensitive region (usually HV1B) for 36 cycles and process through sequencing.

C. **Passing Criteria:**
   1. A majority of the five RBs and the negative control should result in no sequence data. If the RBs show possible contamination after sequencing and a source can be reasonably explained, the reagent may be put into use.
   2. The positive control must have the correct sequence.

D. If the contamination check of the Demineralization Extraction Buffer does not meet the above listed criteria, the process will be repeated. Alternatively, the lot may be discarded, a new lot made, and the process repeated on the new lot. If the results are still deemed unsuitable, then the Technical Leader will be consulted.