BONE DNA PURIFICATION PROTOCOLS FOR GENETIC ANALYSIS

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DISCLAIMER: *This procedure is still under development and results cannot be guaranteed. The University of Indianapolis shall not be held responsible for any tissue lost using these protocols.*

(1) SAMPLING OF BONE

A. Materials

1. bone specimen (compact bone, usually femur or humerus)
2. gloves, safety goggles, & lab coat (to protect clothing)
3. 10% household bleach (v/v)
4. paper towels
5. Dremel tool & blades
6. ziplock-style specimen bags
7. labeling pen

B. Procedure

1. Sampling is usually performed in a location that is separate from where genetic analysis will be performed.
2. Remove all items currently in the hood.
3. Wash down the hood with 10% bleach.
4. Don gloves and safety goggles.
5. Select an appropriate bone specimen.
6. Remove surface contamination by wiping down the exterior surface of the bone with a paper towel that is dampened with 10% bleach. Clean the Dremel tool and cutting blades in a similar fashion.
7. Remove a small section of about 1 in x 0.5 in using the Dremel tool. Be sure to change the cutting blades with each new bone specimen!
8. Place the removed section into a sterile plastic zip lock bag. Label the specimen bag.
9. Return the bone specimen to the appropriate location.

(2) PREPARING THE BONE FOR DNA EXTRACTION -- removing surface contamination (contemporary DNA) and "powderizing"

A. Materials

1. bone fragment from (1) above
2. razor blades (new, sterile, never used)
3. sterile petri dishes (2 per sample)
4. 2 sterile plastic bags, one smaller than the other
5. paper towels
6. hammer
7. coffee mill
8. 15 ml conical centrifuge tube (pre-weigh or tare the tube)

9. applicator stick or cotton swab (sterile, individually wrapped)
10. "DNA OFF" decontamination solution
11. top-loading balance
12. liquid nitrogen
13. tongs
14. styrofoam coffee cups
15. forceps (sterile, preferably baked)

B. Procedure

1. The bone sample should be handled in a room in which analysis of PCR products has not been conducted recently.
2. With a gloved hand, remove the bone sample from the bag and place it in a petri dish.
3. Unwrap a new sterile razor blade.
4. Carefully shave the outer surface (the surface most likely to have been touched; this does not include the interior area, which contained the marrow). Keep shaving until 0.5 to 1 mm of the outer cortical layer is removed, allowing the shavings to drop into the bottom of the petri dish.
5. Place the bone fragment in the second petri dish (to keep it clean). Discard the first dish.
6. The next step requires the use of liquid nitrogen -- very cold and hazardous!
7. Place a styrofoam cup into a styrofoam ice bucket or on a thick slab of wood (this protects the bench top from cracking if you should accidentally spill liquid nitrogen).
8. Carefully and slowly pour liquid nitrogen from the Dewar into a styrofoam cup until it is about 2/3 filled with N_2. At first, much of the N_2 may evaporate but if you pour slowly enough it will eventually fill.
9. Using sterile forceps, slowly immerse the bone sample in the cup. Allow to freeze for 5 to 10 minutes (or until the bubbling ceases).
10. Place the frozen bone into the smaller of 2 sterile plastic bags, then insert this bag into a slightly larger plastic bag. Then wrap the bags in several layers of paper towels.
11. Pound the packet with a hammer to fragment the bone into gravel size pieces.
12. Dump the bone pieces into a sterile coffee mill. Use your gloved finger to pull out fragments that have become embedded in the plastic bag.
13. Grind the bone to the consistency of fine sand. Use pulses at first. Pause to prevent overheating of the fragments. Tap gently to drop fragments from the lid into the chamber of the mill.
14. Working over the second petri dish (to collect spilled powder), carefully pour the powder into a tared (preweighed) 15 ml or 50 ml conical centrifuge tube. Use a cotton swab or applicator stick to guide the powder into the tube. Be sure to recover any you spilled into the petri dish.
15. Cap the tube. Weigh again and determine the mass of bone powder recovered by subtracting the tare weight from the total weight. Be sure to record this data.
16. Clean the coffee mill by wiping with several changes of soapy dampened paper towel. **Do not fill the mill with water or use a sopping, dripping wet towel**. If moisture seeps down the blade shaft into the motor, the mill will be ruined. Just wipe it many times until no signs of bone residue remain.
17. Wipe the coffee mill with several damp paper towels to remove the soap.
18. Wipe the coffee mill with DNA-OFF, then rinse it with DNA-free dH_20, drain, and allow it to dry upside-down on a paper towel. Then before using again, expose the inner surface of the grinder and its lid to short-wave UV for 60 minutes (about 30 to 40 cm from the UV lamp in a PLAS-Lab clean box).

(3) DECALCIFICATION OF THE BONE

NOTE: The jury is still out on the necessity of this step. Some argue it is unnecessary and that DNA is lost and/or further degraded during decalcification. Some argue that DNA yield may be less but greater success at amplifying rare alleles occurs with decalcification. We have had NO success amplifying ANY alleles without decalcification, but our DNA yields are 10x less than most scientists report.

NOTE: Think about each step as performed and be sure you are conducting the steps in such a way as to minimize adding your own DNA to the sample. That means:

1. wearing gloves to handle the sample when the tube is open, and changing gloves often if you touch anything that might be contaminated with DNA (yourself, benchtops, doorknobs, etc);
2. not touching pipettes to bench tops or other non-sterile surfaces;
3. handling pipettes only when wearing gloves;
4. replacing caps on stock reagents ASAP, to minimize aerosol contamination of these reagents;
5. discarding any reagents you know you have compromised.

A. Materials

1. bone powder (0.4 to 2 g, or as directed)
2. 0.5 M EDTA, pH 8.0
3. 10 ml pipettes & pipette pumps
4. rocker platform
5. transfer pipettes & bulb
6. discard container (beaker, paper cup, or styrofoam cup)
7. heavy duty centrifuge (Juoan refrigerated table top model)
8. saturated ammonium oxalate solution

B. Procedure

1. For each 2 g of bone powder, add 5 ml of 0.5 M EDTA.
2. Cap the tube and place it on the rocker platform (on its side) and set the rocker to a medium speed.
3. Allow it to incubate several hours or overnight.
4. Centrifuge the tubes at 1500 rpm for 5 minutes.
5. Carefully remove and discard the supernatant using a sterile transfer pipette.
6. Add another fresh 5 ml of 0.5 M EDTA.
7. Repeat steps 2 through 6.
8. After the third incubation with 0.5 M EDTA, remove the supernatant but save it in a sterile tube.
9. Add a few drops of saturated ammonium oxalate to the supernatant. If it turns very cloudy (calcium oxalate is insoluble), repeat the EDTA treatment. If it stays clear or is only faintly cloudy, then proceed with removing EDTA and deproteination.

(4) REMOVE (MOST) EDTA

A. Materials

1. decalcified bone powder with EDTA supernatant removed (left over from step 8 above)
2. DNA-free dH_2O
3. vortex mixer

4. centrifuge
5. transfer pipettes & bulbs
6. discard container

NOTE: DNA-free dH_2O can be obtained commercially at a high expense, or you can prepare distilled water that has been autoclaved then filtered through a 0.2 micron nitrocellulose filter which avidly binds single-stranded DNA. HPLC-grade water is also usually DNA free but not guaranteed by the companies that sell it (Beckman, Aldrich) so it is best to test each batch to be sure. We have never had DNA contamination in HPLC water but we do not guarantee that it can't happen.

B. Procedure

1. Add 5 ml of dH_2O to the decalcified bone powder.
2. Briefly vortex to mix.
3. Centrifuge for 5 min at 1500 rpm. Remove and discard the supernatant.

(5) DEPROTEINATE

A. Materials

1. proteinase K, 10 mg/ml (freshly made & aliquoted)
2. cell lysis buffer or CLB [10 mM Tris, pH 8.5, 10 mM EDTA, 10 mM NaCl, 0.1% Sodium sarcosine (a detergent that PK requires)]
3. 50 to 55^o C water bath with a plastic test tube rack immersed in water
4. 1 ml & 5 ml pipettes & pipette pumps

NOTE: Even though manuals say you can prepare aliquots ahead of time and freeze them, we have had MUCH better success by making our proteinase stocks as we need them, using them within 3 days.

B. Procedure

1. Add 5 ml of CLB to the decalcified bone powder.**
2. Add 50 ul of 10 mg/ml proteinase K.**
3. Place in a plastic rack in a water bath to incubate at least 2 hours at 50 to 55^o C.
4. If there is still insoluble material present, add another 50 ul of 10 mg/ml proteinase K.
5. Incubate another 2+ hours.
6. If the material is mostly solubilized, proceed to Organic Extraction below. If not, repeat the PK treatment one more time.
7. If the material is not completely solubilized after 3 PK treatments, it will not become solubilized after 10 treatments, so proceed to Organic Extraction.

**** Alternate procedure:** for each 2 g of bone powder, dispense 5 ml of CLB and 0.5 mg of proteinase K. Dissolve the PK, then add 5 ml per 2 g sample (see table below):

Grams of bone	CLB (ml)	PK (mg)
1	2.5	0.25
2	5	0.5
3	7.5	0.75
4	10	1
5	12.5	1.25
8	20	2
10	25	2.5
12	30	3
15	37.5	3.75
20	50	5
30	75	7.5

(6) ORGANIC EXTRACTION

A. Materials

1. water-saturated phenol (1/100 volume of 1 M Tris, pH 8.0 to 8.5, is added to buffer the normally acidic phenol)
2. chloroform:isoamyl alcohol (CI), 24:1 (24 ml $CHCl_3$ + 1 ml IAA)
3. phenol:chloroform:isoamyl alcohol (25:24:1), also called PCI (25 ml water-saturated phenol, 25 ml CI)
4. Tert-Butanol
5. sterile 15 ml tubes (3 per sample)
6. rocker platform (be sure that absorbant cloth diapers are placed on the platform to avoid damaging the rubber platform if chloroform leaks from the tubes)
7. GLASS pipettes (plastic pipettes dissolve in solutions containing chloroform)
8. pipette pump
9. discard container for waste PCI

B. Procedure

NOTE: perform these steps in the fume hood and WEAR GLOVES when handling organic solvents.

1. Using a GLASS pipette, add 5 ml of PCI to your deproteinized bone sample.
2. Cap and rock the sample for 5 minutes
3. Centrifuge at 5000 rpm for 5 minutes.

NOTE: there are TWO phases -- the top phase is aqueous and contains the DNA; the bottom phase is organic and contains PCI, lipids, and peptides.

4. Remove and save the supernatant (top phase) in a sterile 15 ml tube (it has the DNA). Be sure to avoid the interface.
5. Discard the bottom (organic phase, PCI) in a waste container.
6. Throw away the first tube (not the clean one with your aqueous DNA).

7. Add 5 ml of CI (chloroform:IAA) to the DNA.
8. Centrifuge at 5000 rpm for 5 minutes.
9. Remove and save the **supernatant (top phase)** in a sterile 15 ml tube (it has the DNA). Be sure to avoid the interface. Discard the organic waste and second tube.
10. Using a plastic pipette, add 5 ml of butanol to your sample (the top phase from step 9).

NOTE: Notice that this time the organic layer is on the top.

11. Cap, rock for 5 minutes, then centrifuge 5 minutes at 5000 rpm.
12. **Remove the bottom layer** (this time the top layer is butanol) and transfer it to a sterile 15 ml tube.
13. Proceed to desalting the DNA (removing excess salts, solvents, and equilibrating DNA to 10 mM TE and 1 mM EDTA).

(7) "DESALTING" THE ORGANICALLY EXTRACTED DNA

NOTE: During this step the DNA-containing solution is passed through a column that is made up of tiny beads. These beads have pores of a specific size such that molecules > 50,000 FW cannot enter the beads but molecules and ions < 50,000 FW can enter and get trapped in the beads. DNAs > 50 bp (or single stranded DNA > 100 bp) are excluded while DNAs < 50 bp and other small molecule (chloroform, phenol, EDTA) and ions (Ca+2 and Na+1) are trapped inside the beads. The column is equilibrated in TE buffer, pH 8 to 9 (10 mM Tris and 1 mM EDTA), DNA's favorite solution. Therefore, when this procedure is completed, the DNA will be dissolved in TE.

A. Materials

1. 3 or 5 ml syringe
2. sterile cotton & sterile forceps (both autoclaved)
3. 15 ml tube
4. parafilm
5. Sephadex G50 equilibrated in TE Buffer (10 mM Tris, 1 mM EDTA, pH 8.0)
6. 1 ml pipette
7. organically-extracted DNA
8. transfer pipette & bulb
9. centrifuges (Jouan or IEC)

B. Procedure

1. With a gloved hand, unwrap the syringe and remove and discard the plunger and the cap on the end where the needle would be inserted.
2. Insert a small piece of cotton into the syringe barrel, pushing it to the end with a 1 ml pipette.
3. Insert the cotton-plugged syringe into a 15 ml tube and insert the tube in a holder of some kind or a test tube rack (vertical).
4. Swirl the Sephadex beads and pour them into the syringe until the bead volume is near the top of the syringe.
5. Cap with parafilm.
6. Centrifuge the tube and syringe for 1 minute at 1500 rpm to remove the excess TE buffer.
7. Uncap, remove the syringe, and shake out the TE from the tube.
8. Reinsert the syringe in the 15 ml tube.
9. Dropwise, add the DNA sample to the bed of the column (do not allow the DNA solution to flow

between the sides of the bed volume and the syringe. It must soak into the sephadex).
10. Cap with parafilm.
11. Centrifuge for 3 minutes at 1500 rpm.
12. Remove and discard the syringe, **NOT the tube! The liquid in the tube is your DNA**.
13. To concentrate the DNA, proceed to microconcentration (8) or lyophilization (9) below.

(8) MICROCONCENTRATION

A. Materials

1. desalted DNA
2. Amicon or Millipore microconcentrator
3. TIME (this step could take up to 3 hours)

NOTE: The microconcentrator you will use excludes molecules > 10,000 MW (so anything less than that will pass through). This will exclude DNAs > 100 bp or DNAs with high GC > 80 bp. Anything smaller will pass through and be discarded. The purpose is to concentrate the DNA sample down to 50-100 ul.

B. Procedure

1. Apply the sample to the microconcentrator that excludes molecules > 10,000.
2. Follow the manufacturer's instructions regarding the appropriate speed (usually 5000 to 8000 rpm). Centrifuge at the recommended speed for 20 minutes.
3. Check the volume that has been collected in the bottom reservoir (mostly TE and very small degraded DNA) or the volume lost from the upper reservoir (DNA). From this information, determine the rate that the volume is decreasing, for example, 1 ml per 20 minutes.
4. From the rate of volume lost per 20 minutes, estimate the remaining time you must centrifuge. For example, if the volume is decreasing 1 ml per 20 minutes, and you need to remove another 2 ml from the upper reservoir, you will need to centrifuge for 40 additional minutes.
5. Centrifuge for the time determined in steps 3 and 4.
6. Check the volume change again. If necessary, centrifuge some more.

NOTE: One can ask "why not centrifuge for 3 hours and come back?" If the upper reservoir is allowed to go completely dry, you may not be able to recover the DNA off the filter.

7. Once the volume is 50-100 ul or so, carefully remove the DNA from the upper chamber. Transfer it to a sterile 0.5 ml microcentrifuge tube.
8. Try to determine the volume as you remove it. This can be done using the micropipettes and adjusting the volume. Another way to determine the volume is to preweigh the microcentrifuge tube, then weigh it after you have transferred the concentrated DNA into that tube.
9. Clearly label the tube and place it in the appropriate box in the refrigerator.
10. Proceed to the DNA yield gel.

(9) LYOPHILIZATION OF DNA

NOTE: This is a suitable alternative to Microconcentration but a second desalting step is required.

A. Materials

1. DNA samples in 15 ml tubes
2. acetone (need not be pure)
3. ice bucket
4. parafilm
5. hypodermic needle (from sterile syringe) -- to puncture parafilm just before freeze-drying

B. Procedure

1. Samples must be frozen in a dry ice-acetone bath.
2. Be sure tubes are clearly labeled near the TOP of the tubes with BLACK SHARPIE INK (all other colors tend to dissolve in acetone vapors).
3. Remove the caps and apply parafilm very snugly (at least two layers, wound around the edge of the tube; if the parafilm is not tight, it will come off as it becomes stiff during freezing). Using a syringe needle, poke several holes in the parafilm.
4. Insert the tubes into the dry ice-acetone bath (once it stops bubbling); be sure that the labels on the tubes are ABOVE the solvent. Allow the tubes to freeze for at least 5 minutes.
5. Insert the tubes into the lyophilization vessel (made of vacuum-resistant glass).
6. Cap the lyophilization vessel, then insert the metal tube into a port of the lyophilization unit.
7. Turn the stopcock until the vacuum is heard pulling from the vessel. Observe the vacuum gauge to be sure that a vacuum is restored.
8. The next day, come back and turn the stopcock to release the vacuum. DO THIS SLOWLY so that the dried solutes, much of which is DNA, are not sucked out of the tube.
9. Cap the parafilm with a fresh piece of intact parafilm.
10. The DNA can be stored indefinitely in this condition, but usually we dissolve the sample in 200 ul of DNA-free H_2O right away.

NOTE: The sample now has the DNA and all solutes concentrated from the original volume of 5 ml or so to 100 ul. This is a > 200-fold concentration. The salts are a problem; they will be > 1 M and will inhibit PCR. Therefore, they must be removed from the sample and equilibrated in TE buffer.

11. Desalt the sample using the protocol in (7) above.

(10) DNA YIELD GEL (rarely done anymore)

A. Materials

1. 1/10 of your DNA sample (for example, 5 or 10 ul) transferred to a 0.5 ml tube or as a dot to a piece of parafilm.
2. sample loading dye (0.05% bromphenol blue, 0.05% xylene cyanol, 50% glycerol, 50 mM Tris-HCl, pH 8.3, 10 mM EDTA)
3. 100 base pair DNA marker
4. TE buffer
5. 0.5x TBE Buffer
6. 1.5% agarose gel in 0.5x TBE, prepared as follows:

-- 0.6 g agarose in 40 ml 0.5x TBE
-- melt, cool, & add 10 ul 5 mg/ml ethidium bromide (WEAR GLOVES)
-- pour gel & allow to harden & cool to room temperature

7. electrophoresis apparatus (gel box, tray, comb, power supply)
8. micropipettes & tips
9. photographic film, camera, etc.

B. Procedure

1. Adjust the DNA sample to 18 ul using TE buffer (if you removed 5 ul, add 13 ul TE).
2. Add 2 ul of sample loading dye.
3. Submerge the gel.
4. Apply the sample to the well of the agarose gel submerged in the gel box containing 0.5x TBE.
5. Also apply 100 bp DNA markers to a lane.
6. Electrophorese for 1 hour at 100 volts.
7. View and photograph the results.
8. What do you see? There will probably be degraded DNA averaging 100 to 200 bp and a high molecular weight bacterial DNA band.
9. Proceed to DNA profiling.

(11) DNA PROFILING (separate protocol)

A. Materials

1. purified DNA
2. PCR reagents
3. primers (Amelogenin, TPOX, FES.FPS, mitochondrial)

B. Procedure

1. Select the procedure based on what you have done previously. Add 2 ul of your purified bone DNA per PCR.
2. Start with amelogenin.
3. If at first you get NO product, try reducing the amount of DNA by half.
4. Try mitochondrial next, then try the STRs.
5. When running STRs, you should run your own DNA. Hopefully any results you get for the bone DNA will not always precisely match your own!

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