

Isolation of DNA from small amounts of elephant ivory

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This protocol describes a method for the extraction of DNA from elephant ivory. These techniques are being used to assign geographic origin to poached ivory by comparing the ivory genotype to a geographic-based gene frequency map, developed separately. The method has three components: ivory pulverization, decalcification and DNA extraction. Pulverization occurs in a freezer mill while the sample is deep frozen in liquid nitrogen, preventing degradation of DNA during the process. Decalcification involves repeated agitation of the sample in 0.5 M ethylenediaminetetraacetic acid over a 4-d period. Extraction follows a modified Qiagen protocol for the extraction of DNA from animal tissue. This method can be used on all forms of ivory. However, DNA recovery is highest when the outermost layer of the tusk, the cementum, is used. When applied to extract DNA from 11 samples, in duplicate, the entire protocol can be completed in 6 d, although much of this time consists of pause points that do not require effort. The protocol provides $0.8 \pm 0.11 \text{ ng } \mu\text{l}^{-1}$ (mean \pm s.e., $n = 48$) of DNA per sample.

INTRODUCTION

The importance of monitoring the origin of elephant ivory poached for the escalating illegal ivory trade has created a need to extract DNA from whole tusks, as well as from ivory carvings and signature seals. In theory, all forms of DNA can be extracted from ivory of any elephant species or subspecies using this method. However, our current applications employ PCR amplification of microsatellite and mitochondrial DNA for assignment of geographic origin of African elephant ivory^{1,2}. Extraction of DNA from ivory may also have applications for evolutionary biology, as DNA can be preserved in ivory for long time periods under a wide variety of environmental conditions³.

DNA can be extracted from any part of the tusk. However, the highest concentration of amplifiable DNA is typically found at the base, where the tusk connects to the skull³. DNA is also more concentrated at the outer most cementum layer of the tusk¹.

There are three major components to extracting DNA from ivory: pulverization, decalcification and DNA extraction. Although

the decalcification step adds several days to the extraction protocol, it increases DNA recoveries from all ivory extractions and is vital for ivory samples taken from the mineral-rich core of the tusk, such as is the case for signature seals and some carvings. The decalcification procedure described in the protocol below was modified from a Qiagen User Developed Protocol intended for the decalcification of bone material (see ref. 4). The protocol was optimized by altering the amount of starting material, reagents, increased centrifugation speed and vortexing.

As DNA is not evenly distributed throughout the ivory, we typically pulverize multiple small pieces of ivory simultaneously to help ensure that DNA is present in the sample. We also routinely extract DNA from 2 to 3 subsamples from each sample, as DNA recovery can vary markedly across extracts. The DNA extraction procedure described in this protocol is modified from the Qiagen DNeasy Tissue Protocol: March 2004 (see ref. 5). We increased the starting material significantly (from 25 to 200 mg), lyse time (to 24 h) and elution time (from 1 to 10 min).

MATERIALS

REAGENTS

- 0.5% (vol/vol) bleach
- Sterile water
- Liquid nitrogen **! CAUTION** Colorless gas. Contents are under pressure. Rapidly expanding gases or liquids can cause frostbite. Use in well-ventilated and cool area; keep container tightly closed when not in use.
- Ethylenediaminetetraacetic acid (EDTA, anhydrous Sigma grade, ~99% titration; Sigma-Aldrich Inc., cat. no. EDS-500G) **! CAUTION** Harmful if swallowed. Irritating to eyes, respiratory system and skin. Wear suitable protective clothing.
- Hydrochloric acid (HCl; Fisher Scientific, cat. no. A144-500) **! CAUTION** Poison. Causes severe burns. May be fatal if swallowed or inhaled. Causes damage to lungs, eyes and skin. Keep the container tightly closed. Loosen closure cautiously. Use under adequate ventilation. Wash thoroughly after handling.
- Sodium hydroxide (NaOH, NF grade, 95.0–100.5%; VWR International Inc., cat. no. EM-SX0588-1) **! CAUTION** Poison. Corrosive. May be fatal if swallowed. Harmful if inhaled. Causes burns to any area of contact. Reacts with water and acids. Use vent hood; store and use in a cool, dry and ventilated area.
- Deionized water

- 100% ethanol (AAPER Alcohol, cat. no. 0010160) **! CAUTION** Inhalation may cause headache, drowsiness, loss of appetite and irritation of throat. Ingestion can cause depression of the central nervous system, nausea, vomiting and diarrhea. Irritant to skin and eyes. Keep away from heat, use under adequate ventilation, avoid breathing vapor, avoid contact with eyes, skin or clothing and wash thoroughly after handling.
- DNeasy tissue kit (Qiagen, cat. no. 69504) **! CAUTION** See <http://www.qiagen.com/ts/msds.asp> for Material Safety Data Sheets (MSDSs) on their products.
- Buffer ATL (supplied in DNeasy tissue kit)
- Buffer AL (supplied in DNeasy tissue kit) **! CAUTION** Harmful and irritant. Harmful if swallowed. Irritating to skin and eyes. Keep away from food and drink.
- Buffer AW1 (concentrate) (supplied in DNeasy tissue kit) **! CAUTION** Harmful and irritant. Harmful if swallowed. Irritating to skin and eyes. Keep away from food and drink.
- Buffer AW2 (concentrate) (supplied in DNeasy tissue kit)
- Buffer AE (supplied in DNeasy tissue kit)
- Proteinase K (supplied in DNeasy tissue kit) **! CAUTION** Sensitizer and irritant. Irritating to eyes, respiratory system and skin.

EQUIPMENT

- Jeweler's saw or liquid nitrogen carrier containing liquid nitrogen



- Clamp or forceps to hold ivory
- Kimwipes
- Thick plastic zip-lock bags (2" × 3")
- Storage bags/tubes
- Eye protection
- Lab coat
- Freezer mill (SPEX CertiPrep 6750)
- Polycarbonate tubes (with stainless steel end plugs and magnet)
- Tube extractor (provided with freezer mill)
- Cryogenic gloves
- Vial rack
- Centrifuge (10,000–13,000 r.p.m. (9,000–15,000g) for 2 ml tubes)
- Rotator/Rotisserie that holds 2 ml tubes and operates at 4 °C
- 4 °C cold room with electrical outlet for rotator
- Stir plate
- 2 ml tubes with screw lids and rubber seals
- Gloves

- Vortexer
- Waterbath (56 and 70 °C capacities) or shaker incubator for 56 °C lyse
- Pipettes (100 and 1,000 µl) and repeat pipettor
- Centrifuge (8,000–13,000 r.p.m. (6,000–15,000g) for 2 ml tubes)
- 1.7 and 2 ml tubes with snap-on lids
- Freezer (–20 °C)

REAGENT SETUP

0.5 M EDTA (pH 7.5) The weight (in grams) of EDTA to be added to 1 liter of deionized water can be determined using the following formula: weight (in grams) of EDTA = (FW/mol)(0.5 mol/liter). FW is the formula (or molecular) weight found on the EDTA bottle. Slowly add EDTA to water in a graduated cylinder on a stir plate. EDTA is soluble in water at pH 3–3.5. To achieve this pH, add NaOH just until all EDTA goes into solution (you should not see any white specks). To raise the pH to 7.5, add HCl. The pH will rise very quickly, so add HCl slowly and check pH often to avoid exceeding the desired pH. **! CAUTION** EDTA, NaOH and HCl are dangerous reagents and must be handled with care. See handling and reagent information under the REAGENTS section.

PROCEDURE

Pulverization ● TIMING ~ 40 min per piece

- 1| Using a jeweler's saw, cut off a piece of ivory ~ 2 cm × 2 cm in size. Cut from the base of the tusk if available; otherwise, use the tip of the tusk. If neither end is available, use any portion of the sample.
 - 2| Prepare starting material using one of the following options.
 - (A) **Preparation of starting material by freezing and hammering**
 - (i) Submerge the cut piece into a small container of liquid nitrogen for 3–5 min.
 - (ii) Remove the piece using large forceps, immediately place in a thick, sterile, zip-loc plastic bag and then double bag it. **! CAUTION** Wear protective clothing (lab coat, eye protection and gloves).
 - (iii) Place the ivory on a hard surface and, using a hammer with the contact end wiped clean with 5% bleach, smash the ivory several times into numerous small pieces.
 - ▲ **CRITICAL STEP** This step must be done cautiously as the sharp ivory pieces can easily tear the bag.
 - (B) **Preparation of starting material using a jeweler's saw**
 - (i) Use a fine-toothed jeweler's saw to cut the ivory into 1 cm × 1 cm pieces.
 - 3| Sterilize the ivory pieces with 0.5% bleach and then rinse thoroughly with sterile water.
 - ▲ **CRITICAL STEP** It is vital to sterilize the cut ivory pieces with bleach to avoid possible sample contamination from the jeweler's saw. The fine teeth of the saw are optimal for cutting ivory but can easily retain ivory powder. Ensure that the bleached sample is rinsed very well; there should be no bleach residue on the sample.
 - **PAUSE POINT** Protocol can be stopped here indefinitely.
 - 4| Combine 1–2 g of the ivory pieces for pulverization.
 - 5| Dry the pieces with a kimwipe and place in a labeled bag until being transferred to the freezer mill tube for pulverization.
 - 6| Place a stainless steel plug in one end of the polycarbonate tube, add 1–2 g of the ivory pieces into the tube along with the magnet and plug the open end with the second stainless steel plug. The pulverization tube and accessories are provided as a component of the freezer mill.
 - 7| Shake the tube by hand to make sure the magnet moves freely.
 - ▲ **CRITICAL STEP** If the magnet does not move freely back and forth, the ivory pieces are too big. Do not attempt to pulverize. To avoid jamming the magnet, cut the ivory pieces into smaller units before pulverizing.
- ? TROUBLESHOOTING**
- 8| Fill the well of the freezer mill with liquid nitrogen up to the fill line.
 - ! CAUTION** Pour the liquid nitrogen very slowly to avoid spillage. Do not fill past the fill line to avoid overflow: this is dangerous and detrimental to the freezer mill parts.
 - 9| Close the freezer mill lid for ~ 3 min allowing the chamber to cool. The liquid nitrogen evaporates quickly until the freezer mill cools to a temperature of –196 °C (boiling/condensation point), requiring the well to be refilled one or more times before beginning pulverization.
 - ▲ **CRITICAL STEP** Close the lid slowly and beware of overflow. Continue to monitor the liquid nitrogen level throughout the entire pulverization process; failure to do so may incur technical problems.



PROTOCOL

10| Place the tube to be pulverized in the main chamber of the freezer mill. Up to two additional tubes can simultaneously be pre-cooled by placing them in the pre-cooling chambers immediately above the main chamber.

11| Submerge the tube holder into the well of the freezer mill by closing the lid and then lock in place.

12| Set the freezer mill to perform two cycles, each comprising three successive time settings. Cycle 1: time 1 (2 min) is the pre-cool time, time 2 (2 min) is the pulverization time, time 3 (5 min) is the post-pulverization cool time; cycle 2: time 1 (2 min), time 2 (2 min), time 3 (1 min). Press run to start the cycle.

▲ **CRITICAL STEP** The freezer mill will shift the magnetic field back and forth at the preset rate of 10 times per second. With each shift, the magnet smashes the frozen cold ivory against opposite stainless steel plugs, pulverizing the brittle frozen ivory while maintaining a temperature of $-196\text{ }^{\circ}\text{C}$ (boiling/condensation point) that prevents DNA denaturation; heat generated by conventional grinding degrades the DNA during pulverization.

? TROUBLESHOOTING

13| Remove the tube and replace with the next tube in line.

! **CAUTION** Be very careful when opening the lid. Hold the base of the freezer mill and open slowly. Check the liquid nitrogen level and refill as needed.

14| Wait for a few minutes to remove the pulverized ivory from the tube. Remove the vial end plug using the extractor, remove the magnet with sterile tweezers and pour sample into the storage tube.

■ **PAUSE POINT** Protocol can be stopped here indefinitely.

? TROUBLESHOOTING

Decalcification ● TIMING 4 d

15| Place 200 mg of pulverized ivory into a 2 ml rubber sealed, screw-top tube.

16| Add 1.0 ml 0.5 M EDTA (pH 7.5).

17| Vortex the sealed tube until ivory is completely in solution (~ 2 min).

18| Agitate tubes on a rotator at $4\text{ }^{\circ}\text{C}$ for 24 h.

? TROUBLESHOOTING

19| Centrifuge samples at $9,000g$ for 10 min at room temperature ($21\text{ }^{\circ}\text{C}/72\text{ }^{\circ}\text{F}$).

20| Decant and discard the supernatant.

▲ **CRITICAL STEP** It is faster to decant the supernatant, but if the ivory does not stay at the bottom of the tube, then pipette out the supernatant instead.

21| Repeat Steps 16–20 two additional times.

22| Add 1.0 ml sterile deionized water.

23| Vortex until in solution.

24| Centrifuge samples at $9,000g$ for 10 min at room temperature.

25| Decant and discard the supernatant.

26| Repeat Steps 22–25 two additional times for a total of three times.

27| Centrifuge samples at $15,000g$ for 2 min at room temperature.

28| Pipette out the remaining supernatant and discard, leaving approximately 200 mg of decalcified ivory for extraction.

▲ **CRITICAL STEP** The pelleted and decalcified ivory sample will be moist but should not be saturated.

■ **PAUSE POINT** Decalcified ivory can be stored at $-4\text{ }^{\circ}\text{C}$ for a few days, but we recommend immediate extraction.

DNA extraction ● TIMING 2 d

29| Add 360 μl of ATL lyse buffer and 40 μl of proteinase K to the 200 mg decalcified samples from Step 28.

30| Vortex until ivory powder is in solution.

31| Place samples in waterbath at $56\text{ }^{\circ}\text{C}$ to lyse the cells for 24 h inverting periodically (or use a shaking incubator).

? TROUBLESHOOTING

- 32| Vortex tubes for 15 s.
- 33| Add 400 μ l of AL buffer to each sample.
▲ CRITICAL STEP It is important to keep a 1:1:1 ratio of ATL lyse buffer plus proteinase K/AL buffer/EtOH.
- 34| Vortex tubes for 10 s.
- 35| Incubate samples in waterbath at 70 °C for 10 min.
- 36| Invert samples.
- 37| Add 400 μ l of 100% EtOH to each sample.
- 38| Vortex tubes for 10 s.
- 39| Pipette 675 μ l of each sample onto a DNeasy Minispin column placed in a 2 ml collection tube.
- 40| Centrifuge at 6,000g for 1 min at room temperature.
- 41| Move the spin column to a new collection tube.
- 42| Pipette rest of the sample onto the spin column.
- 43| Repeat Steps 40 and 41.
- 44| Add 500 μ l of AW1 buffer onto the spin column.
- 45| Repeat Steps 40 and 41.
- 46| Add 500 μ l of AW2 buffer onto the spin column.
- 47| Centrifuge at 15,000g for 3 min at room temperature.
- 48| Place the spin column into a 1.7 ml tube with a snap-on lid.
- 49| Add 100 μ l of AE buffer directly to the DNeasy Minispin column membrane.
- 50| Incubate at room temperature for 10 min.
- 51| Centrifuge at 9,000g for 1 min at room temperature.
- 52| Repeat Steps 49–51 once more for a total of 200 μ l eluate.
- 53| Samples are now ready for PCR amplification.
■ PAUSE POINT Sample should be stored at –20 °C until used.

● **TIMING**

Total work time: 3 h, 38 min
 Total wait time: 4 d
 Steps 1 and 2: 20 min
 Steps 3–14: 20 min
 Steps 15–17: 5 min
 Step 18: 24 h pause point
 Steps 19 and 20: 13 min
 Step 21: 2 d and 36 min
 Steps 22–28: 1 h
 Steps 29 and 30: 4 min
 Step 31: 24 h pause point
 Steps 32–53: 1 h

? **TROUBLESHOOTING**

Troubleshooting advice can be found in **Table 1**.



TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason(s)	Solution
7 and 12	Jammed magnet	Ivory pieces are too large	Remove magnet and ivory chunk. Cut/crush ivory into smaller pieces
12	Polycarbonate tube broke	Tube is exposed to EtOH	Do not expose the tube to EtOH. To clean, use bleach and water only
	Freezer mill error	Lid is not closed properly There is not enough liquid nitrogen in the chamber	Open and reclose the lid Open the lid, check the level and refill as needed
14	Chunks in pulverized ivory	Pulverizing time is insufficient	Adjust settings to include another cycle and/or longer pulverizing time
	Cannot remove the plug from the tube with the extractor	Unit is still frozen from liquid nitrogen	Wait 5–10 min to unfreeze
18 and 31	Supernatant loss during decalcification/extraction incubation	There is evaporation via tube lid; wrong type of tube is used	Use a rubber sealed screw on lid tubes. Ensure the tube is well sealed
31	Tissue not completely lysed	Sample is not agitated during lysis Sample consistency is not fine enough There is too much sample to lyse Decalcification is incomplete	Invert more often during the 24 h incubation period or increase the shaking level Increase pulverization time Decrease the amount of sample to lyse in one tube Add another day of decalcification and/or wash once more
	No data available for analysis following subsequent PCR amplification from extracted DNA	There is little or no DNA in the sample	Select another piece from the sample and repeat the procedure

ANTICIPATED RESULTS

The amount of DNA recovered from an ivory sample is likely to vary markedly between extracts. Some of this variation is predictable based on the part of the tusk being extracted; DNA is most concentrated at the base of the tusk, followed by the tip of the tusk and then the middle portion of the tusk³. The outermost surface of the tusk appears to have higher concentrations of DNA than does the central core of the solid portion of the tusk¹. The high mineral concentration in the core of the tusk also interferes with DNA amplification. Decalcification helps address this latter problem, improving DNA amplification success of all extracts. That being said, DNA is unevenly distributed in all parts of the tusk, causing unpredictable variation across extracts. For this reason, we always start with 2–3 extracts from each sample to address the seemingly random distribution of DNA in ivory. This is similar to our approach for extracting DNA from scat, which also tends to be unevenly distributed⁶.

Using multiple extracts enables one to determine if the ivory has sufficient DNA to warrant further processing. If all three extracts fail to yield a product following subsequent PCR amplification of the extracted DNA, it is probably not cost-effective to pursue the sample further unless it has considerable biological uniqueness. If one DNA extract from the sample yields a good PCR amplification product, while another extract from the same sample consistently fails, it is worth replacing the poor extract with a new extract to assure that enough material exists to amplify sufficient loci for statistical analysis. These latter precautions also help maintain the cost-effectiveness of the overall method.

COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

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