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## [7] Isolation and Analysis of DNA from Archaeological, Clinical, and Natural History Specimens

By CONNIE J. MULLIGAN

### Abstract

The use of ancient DNA (aDNA) in the reconstruction of population origins and evolution is becoming increasingly common. Novel methods exist for the isolation, purification, and analysis of aDNA because these DNA templates are likely to be damaged, fragmented and/or associated with non-nucleic acid material. However, contamination of ancient specimens and DNA extracts with modern DNA is more widespread than is generally acknowledged and remains a significant problem in aDNA analysis. Studies of human aDNA are uniquely sensitive to contamination due to the continual presence of potential contamination sources. Meticulous authentication of results and careful selection of polymorphic markers capable of distinguishing between aDNA and probable DNA contaminants are critical to a successful aDNA study.

### Introduction

Ancient DNA (aDNA) technology has emerged within the past decade as one of the breakthroughs borne of the revolution in molecular biology that began with the polymerase chain reaction (PCR). The enormous

capacity of PCR has stimulated the study of large numbers of individuals that is fundamental to population genetics and has permitted the investigation of DNA samples that are too degraded or damaged for analysis by traditional cloning methods. "Ancient" samples are generally those that were not collected for the purpose of immediate DNA analysis and include archaeological, clinical, and natural history specimens. Because these specimens were not originally collected or preserved for nucleic acid analysis, endogenous DNA is often damaged to an extent that enzymatic amplification can be quite difficult, if not impossible, to achieve. The types of DNA damage that are primarily encountered include modifications of pyrimidine and sugar residues, baseless sites, intermolecular crosslinks, and fragmented DNA (Paabo, 1989).

Damage of aDNA increases the potential for another characteristic of aDNA PCR: that of contamination. Because PCR analysis involves the exponential generation of new, synthetic DNA products from a few molecules, contamination with exogenous DNA in one of the initial PCR cycles can result in exclusive amplification of the contaminating DNA. This possibility is increased in aDNA analysis in which the contaminant is likely to be undamaged DNA that will be amplified preferentially over the damaged endogenous DNA. The growing number of published aDNA studies and number of assayed samples and polymorphic sites may give the impression that all technological hurdles associated with aDNA technology have been overcome. However, identification of contamination and authentication of results remain the most critical issues in aDNA methodology. Early spectacular claims of successful DNA extraction from extremely old specimens, such as 17–20-million-year-old *Magnolia* leaf fossils (Golenberg *et al.*, 1990), 25–135-million-year-old specimens preserved in amber (Cano *et al.*, 1993; DeSalle *et al.*, 1992), and 80-million-year-old dinosaur bones (Woodward *et al.*, 1994), have generally been disproved or cast into serious doubt (Austin *et al.*, 1997; DeSalle *et al.*, 1993; Hedges and Schweitzer 1995; Sidow *et al.*, 1991). Table I lists the controls essential for an aDNA study and suggests additional experiments in the case of questionable or controversial results.

Human specimens are uniquely sensitive to contamination simply because every person involved in the study represents a potential source of contaminating DNA. For instance, analysis of DNA extracted from the Neanderthal type specimen (Krings *et al.*, 1997) revealed two distinct sets of mitochondrial DNA (mtDNA) sequences: one significantly different from modern humans and proposed to be Neanderthal in origin and one identical to the human reference sequence (Anderson *et al.*, 1981) and presumed to reflect modern human contamination. Regardless of the difficulties associated with aDNA analysis, in the past year we have seen a

TABLE I  
CONTAMINATION CONTROLS FOR ANCIENT DNA STUDIES

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Essential controls

- No studies of modern specimens/physically isolated ancient DNA lab
- Disposable labware wherever possible
- Disposable lab coats, gloves, breathing masks, and head coverings
- Dedicated pipetmen
- Filtered pipet tips
- Purchase of molecular biology-grade water and solutions
- Frequent treatment of bench and equipment with 20% bleach solution (autoclaving will destroy living organisms but will not destroy DNA; UV irradiation of solid objects is maximally effective only at a perpendicular angle)
- Multiple DNA extractions for each specimen (from the same and multiple samples, if possible)
- No positive PCR controls
- UV irradiation (254 nm for 20 min) of PCRs before DNA + enzyme addition
- Multiple blank extract and negative PCR controls
- Multiple PCRs of each locus for each specimen
- Appropriate molecular behavior of aDNA, i.e., PCR success is inversely related to amplification fragment size
- Cloning and DNA sequence analysis
- Appropriate phylogenetic sense of results

Additional controls when results are questionable or controversial

- Replication of results in an independent lab
  - Analysis of associated remains to demonstrate feasibility of DNA isolation
  - DNA quantitation or amino acid analysis/racemization analysis of extract to demonstrate organic preservation consistent with DNA survival
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*Note:* aDNA, ancient DNA; PCR, polymerase chain reaction; UV, ultraviolet.

range of aDNA success stories, including (1) a population study of mitochondrial and nuclear markers (single-copy nuclear markers are more difficult to amplify than multicopy mtDNA) in which partial genealogies were reconstructed in a human population about 2000 years old (Keyser-Tracqui *et al.*, 2003); (2) sequence analysis of two Cro-Magnon humans that confirmed the independence of modern human and Neanderthal genetic lineages (Caramelli *et al.*, 2003); and (3) identification of extreme sexual dimorphism in a single species of extinct New Zealand moa that was previously thought to be three distinct species based on overall size differences (Bunce *et al.*, 2003).

#### Methods: Isolation of DNA

DNA can be isolated from any organism. The types of tissue typically available for DNA extraction can be classified as hard (e.g., bone or teeth)

or soft (e.g., muscle or skin). In general, hard tissues are preferred over soft tissues for DNA isolation. Hard tissues typically have not been subjected to any preservation methods. In contrast, soft tissues have often been air-dried (museum skins, mummies, insects in amber) or fixed in formalin (insects, fish, organs) and these preservation methods often increase post-mortem DNA damage (De Giorgi *et al.*, 1994). Both types of tissue are subject to introduction of contaminants whether from bacterial growth on dry remains or groundwater permeation of bones or human handling of museum specimens. Different DNA isolation protocols have been developed for each type of tissue. Hard tissues must be decalcified through use of ethylenediaminetetraacetic acid (EDTA) to release the bound nucleic acid. Preserved soft tissues generally require greatly increased concentrations of and prolonged incubation times with proteinase K (PK) to digest the tissue and release the nucleic acid. A special purification protocol has been developed to facilitate extraction of DNA from formalin-fixed soft tissues (C. Mulligan and N. Tuross, unpublished results). Methods for isolation of DNA from hard and soft tissues are presented in this chapter, with possible modifications listed. In general, a minimalist approach to DNA extraction and purification is recommended to reduce the number of steps in which contamination may be introduced.

*Method 1: Isolation of Nucleic Acid from Hard Tissues (Bone or Teeth)*

1. *Sample preparation.* Brush or wipe obvious dirt from chosen specimens. Break up bone into pieces sufficiently small to fit into a 15- or 50-ml conical tube. If bone can be broken by hand, specimen preservation is likely to be poor. A hammer can be used to fragment well-preserved bone (after placing specimen in plastic bags of sufficient thickness to withstand hammering). Alternatively, bone can be cut using a bone saw or Dremel tool or material can be powdered using a mortar and pestle or Spex mill (Spex Industries, Los Angeles, CA), being careful to control for scatter of dust, which could serve as a contamination source.

2. *Decontamination.* Place fragments or powder in a 15- or 50-ml conical tube so the tissue accounts for only 1/5 of the volume in the tube. Add 20% (v/v) Clorox bleach solution (1.2% sodium hypochlorite) until all material is covered. Gently rock tube in your hand. Friable or powdered material should be exposed to the bleach solution for 45–60 s, whereas well-preserved dense fragments can be exposed for 2–10 min. Pour off bleach solution and fill tube with purified water. Shake tube intermittently for a total wash time of 2–4 min. Repeat water wash two more times. Kemp and Smith (2003) have found that treatment with 33% bleach for

10–15 min or use of “DNA Away” (E&K Scientific, Campbell, CA) is also effective at reducing contamination.

3. *Decalcification*. Place fragments/powder in a fresh 15-ml conical tube. Add sufficient 0.5 M EDTA to cover all material. Ensure that tubes are securely capped and will not leak. Place tubes on a rocking platform (e.g., Nutator [Becton Dickinson, Sparks, MD], and secure tubes with tape so they will not move. Leave tubes at room temperature for 2–4 days (well-preserved material requires more time for complete decalcification). If material has been excavated and stored at cold temperatures, such as excavated from permafrost sediments, DNA isolation should be performed at 4°.

4. *N-phenacylthiazolium bromide (PTB)/PK digestion*. After 2–4 days, add PTB (Trace Genetics, Davis, CA) to a final concentration of 20 mM and add PK (Sigma, St. Louis, MO) to a final concentration of 1 µg/ml. Place tubes on a rocking platform at 65° for an overnight incubation. Wearing gloves, check bone/tooth material for status of decalcification. If there are hard pieces remaining in the tissue and complete decalcification is desired, continue DNA isolation at 65° until all hard calcified bits are gone.

5. *Final step for well-preserved tissue*. If tissue was not well preserved, skip to step 6. If tissue was well preserved, further purification may be unnecessary. Because minimal purification is optimal, the DNA extract may be ready to test for DNA polymerase inhibition at this point. All EDTA must be removed because it will inhibit amplification reactions. First, centrifuge tubes to pellet solid material, and then transfer liquid to dialysis tubing and place tubing in a container with 4 liters of water. The number of water changes required to dilute the EDTA to negligible levels can be calculated based on the volume of the DNA extract(s) and the volume of water used in the dialysis. Typically, two to three changes in 4 liters of water will be sufficient for a 2–4 ml sample. Dialysis against water will increase the volume of the sample by as much as an order of magnitude (e.g., a 3-ml sample will increase to 30 ml, so use sufficient dialysis tubing to accommodate the increase in sample volume). After dialysis, samples must be concentrated to reduce volumes. Samples can be concentrated by partial lyophilization or by filtration through Centricon filters (Millipore, Billerica, MA). Lyophilization is preferable if the necessary equipment is available because the opportunity for introduction of contaminants is reduced and because large volumes are dealt with more easily. The DNA extract is now complete and ready to be tested for the presence of DNA polymerase inhibitors (see below).

6. *Further purification of poorly preserved specimens: Organic extractions*. Add an equal volume of 25:24:1 phenol:chloroform:isoamyl alcohol

(PCI) to the EDTA/PTB/PK solution (be sure to use polypropylene tubes because phenol will destroy polystyrene). Rock tubes for 5–15 min, ensuring that an emulsion forms. Centrifuge the mixture for 15 min at 3000 rpm to separate the aqueous and organic layers (higher speed spins are possible if smaller tubes are used). If the two layers are not well separated, centrifuge again for a longer time. Use a pipette to transfer the top aqueous layer to a fresh tube. Leave some of the aqueous layer behind to ensure that the interface between the two layers, where the denatured proteins collect, is not disturbed. Repeat PCI extraction until no protein is visible in the interface (typically two PCI extractions is sufficient). Add an equal volume of 24:1 chloroform:isoamyl alcohol to the aqueous layer (a final extraction with chloroform removes any lingering traces of phenol from the DNA extract). Rock for 5–20 min, ensuring that an emulsion forms. Centrifuge the mixture for 15 min at 3000 rpm. Use a pipette to transfer the top aqueous layer to a fresh tube.

7. *Ethanol precipitation and washes.* If total sample volume is greater than 500  $\mu\text{l}$ , divide the sample into separate Microfuge tubes with equal volumes of 500  $\mu\text{l}$  or less. Add 1/10 volume 3 M sodium acetate and mix solution well. Add 2 volumes of ice-cold 100% ethanol (do not use <100% ethanol) and mix solution well. Store the solution at  $-20^{\circ}$  overnight to allow a precipitate of DNA to form. Recover the DNA by centrifugation at maximum speed in a Microfuge tube for 10 min at  $4^{\circ}$ . Remove the ethanol supernatant taking care not to disturb the DNA pellet (which may be invisible if you are lucky and have very clean DNA). Add 300  $\mu\text{l}$  of 70% ethanol and recentrifuge at maximum speed in a Microfuge tube for 2 min at  $4^{\circ}$ . Carefully remove the supernatant taking care not to disturb the DNA pellet. Repeat ethanol wash. Store the open tube on the bench at room temperature until all traces of fluid have evaporated. Dissolve the DNA pellet in the desired volume (usually 50–100  $\mu\text{l}$ ) of low TE buffer (10 mM Tris base, 0.1 mM EDTA). The DNA extract is now ready to be tested for the presence of DNA polymerase inhibitors (see below).

*Additional Notes on DNA Isolation and Purification from Hard Tissues.*

a. *Choice of bone or tooth specimens.* Bone or tooth specimens should be chosen that are as complete and as well preserved as possible. In general, bones are preferred over teeth because of the larger quantity of material available in bones. Teeth are often assumed to be sealed against the surrounding environment and, therefore, resistant to contamination, but this is not true as groundwater and associated organisms can permeate the external surface of teeth. Large robust bones, such as femur or tibia, are preferable over small light bones, such as clavicle or rib, because of the

increased density and increased amounts of material in larger bones. When possible, specimens should be chosen that are light colored, have intact epiphyses or edges, have good “heft” to them (assuming the specimens are not fossilized, in which case they will be heavy but lacking in organic material), and have a general appearance of good preservation. If a specimen is small, discolored, or friable, the chances of obtaining amplifiable DNA are lower, and larger amounts of material should be used in the extraction procedure to increase the probability of successful DNA extraction.

b. *Modification of isolation protocol.* Many DNA isolation protocols using skeletal or dental material have been reported in the literature (see Table II for examples of different protocols). The protocol presented here has been used successfully with bovid and human bones and teeth for extraction of DNA representing a total range from 300 to 2500 years before present (YBP) (Kolman, 1999; Kolman and Tuross, 2000; Kolman *et al.*, 1999; M. Ascunce and C. Mulligan, unpublished results). Many modifications to the extraction protocol are possible and some are listed here.

- i. If the material to be extracted is in good condition and endogenous DNA is presumed to be present, smaller amounts of bone or teeth may be used and the extraction protocol can be performed in Microfuge tubes for added convenience.
- ii. It is possible that different amounts of DNA and inhibitors extract at different times during EDTA decalcification (Kolman and Tuross, 2000; Kolman *et al.*, 1999). During the decalcification step, the EDTA can be changed after a few days, replaced with fresh EDTA, and both EDTA extracts processed as separate DNA isolates.
- iii. PTB is a relatively new chemical in the aDNA literature and was first used in the extraction of DNA from late Pleistocene coprolites (Poinar *et al.*, 1998) and from the third published Neanderthal specimen (Krings *et al.*, 2000). PTB is thought to improve DNA yields by cleaving sugar-derived protein cross-links that may entrap DNA. Regardless of the exact mechanism of PTB, PTB does not appear to harm DNA isolations and can be easily incorporated into the extraction protocol at the PK step. Initially, PTB concentrations of 1 mM were tested, although concentrations as high as 200 mM have been used successfully with teeth (Gilbert *et al.*, 2003). It has not been reported in the literature, but PTB may improve DNA amplification if added directly to the PCR.
- iv. Silica-GuSCN is also frequently used in the initial steps of DNA isolation in combination with low levels of EDTA (instead

TABLE II  
SUMMARY OF DNA ISOLATION METHODS USED IN SUCCESSFUL ANCIENT DNA STUDIES

Material extracted	EDTA extraction buffer	Complete decalcification	Silica-GuSCN buffer	Proteinase K digestion	<i>N</i> -phenacyl thiazolium bromide	Organic extraction	Silica purification	Centricon purification/ concentration	EtOH precipitation	References
<b>Hard tissue</b>										
Human bone (300–1000 YBP)	X	X		X						Kolman, 1999; Kolman and Tuross, 2000; Kolman <i>et al.</i> , 1999
Human bone (300–9000 YBP)	X			X		X		X		Kaestle and Smith, 2001
Human bone (~700 YBP)			X	X		X		X		Stone and Stoneking, 1993, 1998, 1999
Late Pleistocene animal remains, Neanderthal (~40,000 YBP)	X			X		X	X	X	X	Greenwood <i>et al.</i> , 1999; Hoss and Paabo, 1993; Krings <i>et al.</i> , 1997
Bear bone (14,000–42,000 YBP)	X			X		X		X		Leonard <i>et al.</i> , 2000
<b>Soft tissue</b>										
Coprolites from extinct ground sloth (~20,000 YBP)				X	X	X	X	X		Poinar <i>et al.</i> , 1998
Frozen sediment cores (600–400,000 YBP)				X		X	X			Willerslev <i>et al.</i> , 2003

relying solely on high concentrations of EDTA) (Hoss and Paabo, 1993). Many DNA purification kits are also based on a silica-GuSCN protocol, such as the Wizard Plus Megaprep DNA Purification System (Promega, Madison, WI). In these protocols, GuSCN facilitates the binding of DNA to the silica so unbound impurities can be washed away. Silica-based methods should be used with caution, however, because aDNA may be bound to protein and may not interact properly with DNA binding resins, resulting in a loss of DNA (Kolman and Tuross, 2000).

- v. Complete decalcification of well-preserved bones may require several weeks if DNA isolation is performed at 4° (Kolman and Tuross, 2000). More typically, complete decalcification requires 3–4 days when performed at room temperature. The level of decalcification can be monitored by feeling the material in EDTA solution (while wearing gloves) and checking for hard decalcified pieces among the soft decalcified material. Complete decalcification of skeletal material may be necessary only for older specimens, as Fisher *et al.* (1993) reported that no decalcification was necessary for bones up to 125 years old.

c. *Purification of DNA extracts.* Minimal purification of DNA extracts will yield maximal recovery of DNA because all purification methods result in the loss of some nucleic acid. Therefore, the number and type of purifications to be performed after initial decalcification should be considered carefully. Although extensive purification seems to be the norm in aDNA studies, I have found that the DNA extracts often can be used immediately after decalcification (and dialysis and sample concentration) (Kolman and Tuross, 2000; Kolman *et al.*, 1999; M. Ascunce and C. Mulligan, unpublished results). If additional purification is deemed necessary, organic extractions most often yield amplifiable DNA extracts and are included in the purification method in the aforementioned protocol. However, phenol extractions can result in losses of up to 50% of total DNA, so organic extractions should be performed only until the organic–aqueous interface is clear of proteinaceous material. Filtration through Centricon filters should not result in appreciable loss of nucleic acid, but filters should be batch-checked for human DNA contamination if human aDNA is being analyzed. Ethanol precipitation of DNA can result in significant loss of low-molecular-weight DNA, such as typical aDNA. DNA binding resin-based methods of purification, such as Glass Milk/GeneClean (Qbiogene, Inc., Carlsbad, CA) and Wizard Megaprep DNA Purification System (Promega, Madison, WI), have been cited extensively in the aDNA literature (Hoss and Paabo, 1993) but should be used cautiously. Kolman and Tuross (2000) reported that silica resin purification

resulted in exclusive recovery of contaminating DNA from extracts containing both endogenous and contaminating DNA. The high probability that aDNA is crosslinked to proteins and may not interact as expected with DNA binding resins provides a cautionary note for purification protocols based on these resins.

*Method 2: Isolation of Nucleic Acid from Soft Tissues (Formalin-Fixed Tissues, Museum Skins, Mummies)*

For the initial steps of DNA isolation from soft tissues, a standard sodium dodecyl sulfate (SDS)/PK digestion is used with two modifications: 1000 times the typical quantity of PK and extended PK incubations are used. Standard phenol:chloroform:isoamyl alcohol (PCI) extraction typically fails to remove all PCR inhibitors from DNA extracts based on formalin-fixed tissues. Therefore, a novel PCI extraction, described by White and Densmore (1992), has been further modified and results in a significant reduction of PCR inhibition (C. Mulligan and N. Tuross, unpublished results).

1. *Sample preparation.* Using a fresh scalpel, dissect a portion of tissue from the chosen specimen that can fit easily into a 2.0-ml tube with a screw cap, about 200–400 mg.

2. *Cell lysis.* Add sufficient lysis buffer (100 mM Tris, pH 8.0, 10 mM EDTA, 100 mM NaCl, 0.1% (w/v) SDS, 50 mM DTT, 0.5 mg/ml PK) to completely cover tissue, typically about 500  $\mu$ l. Incubate at 50° until tissue is completely digested. End-over-end rotation of tubes during incubation will facilitate digestion. If more than 1 day is required for complete digestion, add an aliquot of PK for each extra day.

3. *Frozen PCI extraction.* After digestion, centrifuge tubes to precipitate particulate matter. Transfer supernatant to a clean 15-ml Corex tube (tubes should be muffled at 400° for 15 min or treated with a 20% bleach solution to destroy contaminating DNA). Add 3.5 volumes of SDS:urea buffer (1%/0% [w/v] SDS, 10 M urea, 240 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 6.8, 1 mM EDTA) to the supernatant. Incubate tubes at room temperature for 15–30 min with occasional vortex mixing. Add an equal volume of PCI and incubate at room temperature for 5–10 min with gentle occasional vortex mixing. Spin tubes in a precooled centrifuge at 15,000 rpm for 15 min at –13°. A solid crystallized urea interface must form to obtain maximum extraction of proteins. Tubes should be removed immediately after centrifugation so the organic and aqueous layers do not freeze. If the urea does not crystallize and form a solid interface, a higher concentration of urea can be used or the tubes can be precooled immediately before centrifugation. Remove the aqueous layer and dialyze against two to three

changes of low TE buffer (10 mM Tris, pH 7.5, 0.1 mM EDTA) at 4°. DNA extracts are now ready to test for the presence of DNA polymerase inhibitors (see later discussion).

*Coextraction of DNA Polymerase Inhibitors during DNA Isolation.* During DNA isolation, compounds may co-purify that inhibit the DNA polymerase used in amplification reactions. These compounds may derive from the soil in buried bones or from formaldehyde in fixed soft tissues. Generally, researchers do not attempt to identify the exact nature and source of an inhibitor but focus on eliminating the inhibitor(s). One strategy is to dilute the DNA extracts until the inhibitor is no longer present at inhibitory concentrations. However, excessive dilution may also dilute the DNA to nonamplifiable concentrations. A second strategy is to purify the DNA extracts in an attempt to eliminate the inhibitor without also eliminating the DNA. In practice, both methods are typically used.

Ideally, one should test for presence of inhibition throughout the DNA isolation and purification procedure so the minimum number of purification steps is performed to maximize DNA yield. Presence of inhibition is assayed by amplifying an unrelated DNA template in the presence of varying amounts of the aDNA extract. Dilutions of the aDNA extract are tested to monitor the inhibition (typical dilutions are 1:10, 1:100, and 1:1000). By determining which dilutions are required to eliminate inhibition, one can determine the most effective purification methods and determine when the DNA extract is free of inhibiting compounds. In theory, DNA from any organism that is not studied in the laboratory can serve as a template in the inhibition assay (to avoid introducing contaminating DNA). In the past, manufacturers included control DNA template and primers in commercial PCR kits that could be used as an inhibition assay, but this is no longer true. Currently, my lab uses an inhibition assay with Sendai virus DNA and primers for the P protein (SM464mod = 5'-TGCAGCTGAGAGCAGTCCCC-3' and SM324 = 5'-GATGCCTCACCCGGGATCTAGTTG-3'; PCR profile consists of 10 in at 94°, followed by 28 cycles of 30 s at 94°, 30 s at 55°, 30 s at 72°, and a final 5 min extension step at 72°; S. Moyer and S. Smallwood, personal communication). Magnitude of inhibition is determined by visual comparison on an agarose gel of a control PCR, lacking aDNA extract to a series of PCRs with control DNA plus varying amounts of aDNA extract. To achieve successful amplification of the locus of interest, additional dilution may be required beyond the level indicated in the inhibition assay, for example, in a study of African bovid bone samples approximately 1000 years old, the inhibition assay indicated absence of inhibitors at a 1:10 dilution, but successful amplification was achieved only with a 1:100 dilution (M. Ascunce and C. Mulligan, unpublished results).

*Methods to Improve PCR Success.* Certain chemicals have been found to increase the likelihood of successful DNA amplification. These reagents may be used by themselves or in combination. In some cases, the exact concentration of reagent must be determined empirically. Changes to the PCR profile may also enhance amplification. If initial PCRs do not result in successful amplification of the target DNA, it is recommended to try several or all of the following PCR modifications. Modifications marked by an asterisk are those that appear most frequently in the aDNA literature, suggesting that they most often result in successful DNA amplification.

a. \* $\uparrow$  *Taq* polymerase,  $\uparrow$  # of amplification cycles: Increased amounts of *Taq* polymerase and increased number of amplification cycles are used when amplifying aDNA. Typical amounts are 1–2 units of polymerase and 40–50 cycles. Reamplification of a PCR aliquot or a purified amplified DNA band is not advisable because of the increased likelihood of introducing DNA contamination.

b. \*Hot-start PCR: Hot-start PCR (withholding an essential component of the PCR until the reaction has reached a temperature that inhibits nonspecific priming and primer oligomerization) is used to optimize the yield of desired PCR product and suppress nonspecific amplification. Hot-start PCR can be achieved by either using polymerase–antibody complexes in which the enzyme is inactive until it has been heated at 95° for 5–10 min (e.g., *Amplitaq Gold*; Applied Biosystems, Foster City, CA) or by using wax beads in which the embedded  $Mg^{2+}$  is released only upon prolonged heating (e.g., *HotWax Beads*; Invitrogen, Carlsbad, CA).

c. \*Spermidine: Low levels of spermidine may facilitate amplification of certain DNA samples (Wan and Wilkins, 1993). However, excess spermidine can inhibit amplification, so samples should be tested first for amplification in the absence of spermidine. In two studies, optimal concentration of spermidine was determined to be 400–800  $\mu M$  (Kolman and Tuross, 2000; Kolman *et al.*, 1999).

d. \*Bovine serum albumin (BSA), glycerol, dimethylsulfoxide (DMSO), formamide: BSA and glycerol are used to stabilize protein–DNA interactions, and reagents such as DMSO and formamide facilitate DNA strand separation by disrupting base pairing. However, the mechanism of these interactions is not known and the best additive must be determined by trial and error. Typical additive concentrations are 10–100  $\mu g/ml$  BSA, 10% glycerol, 5–10% DMSO, and 2.5–10.0% formamide (Fisher *et al.*, 1993). BSA and DMSO are the most widely used additives, most likely because they most often result in successful amplification.

e. \*Nested PCR: Nested PCR is the amplification of a single PCR product through the use of two primer pairs in sequential amplification

reactions so the second PCR target is completely contained within the first PCR product. This strategy allows a weak (or invisible) PCR product to be reamplified but increases the specificity of the reaction through the use of two primer pairs.

f. Betaine: It has been suggested that betaine may help stabilize proteins against thermal denaturation and facilitate DNA strand separation by isostabilization of the DNA. Betaine at concentrations of 0.5–2.2 M, in the presence or absence of DMSO, has been found to increase DNA amplification and improve consistency of amplification (Baskaran *et al.*, 1996; Pergams *et al.*, 2003). Betaine has been found to inhibit some DNA polymerases, so addition of betaine may require further modification of the PCR protocol (Baskaran *et al.*, 1996).

g. PTB: The use of PTB to improve aDNA isolation and amplification is still in its infancy. The rationale of using PTB to cleave protein crosslinks and free entrapped DNA may also be reasonable in the context of an amplification reaction.

h.  $\uparrow \text{Mg}^{2+}$ : If residual EDTA in the DNA extract is left over from the decalcification step, the EDTA may reduce the  $\text{Mg}^{2+}$  concentration in the PCR to suboptimal levels.

i. Touchdown PCR: Touchdown PCR (reducing the annealing temperature by 5–10° during the first 10 PCR cycles) is a method used when the annealing temperature of the PCR primers with the DNA template is not known with certainty. Although there should be no uncertainty in this regard with aDNA, this strategy may facilitate the amplification of a complex DNA extract characterized by fragmented or complexed DNA and presence of unknown, possibly inhibitory, chemicals.

#### Postmortem Damage to DNA

Since the advent of aDNA analysis, it has been known that DNA isolated from ancient remains frequently shows evidence of molecular damage (Paabo *et al.*, 1988). Postmortem damage to DNA can take the form of modification of pyrimidine or sugar residues, baseless sites, intermolecular crosslinks, and an average low molecular weight of aDNA due to strand breakage (Hofreiter *et al.*, 2001b). The effects of postmortem damage include (1) fragmented DNA that limits the possible length of PCR product, (2) low to undetectable levels of aDNA that allow preferential amplification of contaminating DNA, (3) random insertion of a nucleotide by the DNA polymerase at a damaged baseless site, and (4) PCR “jumping” in which discontinuous DNA fragments are joined through the amplification reaction. The average lifetime for DNA under physiological conditions and a temperature of 15° has been estimated to be approximately 100,000 years

(Hofreiter *et al.*, 2001b), although low temperatures and low humidity may improve preservation and extend this time limit. Research into postmortem damage has focused on the specific types of lesions that are most prevalent and the distribution patterns of postmortem damage. The most common chemical damage to DNA is deamination of cytosine to uracil and deamination of adenine to hypoxanthine, meaning that transitions ( $\leftrightarrow$  T and G  $\leftrightarrow$  A) are much more common than transversions (Gilbert *et al.*, 2003; Hofreiter *et al.*, 2001a). Comparison of DNA sequences derived directly from PCRs compared to sequences derived from cloned PCR products should identify DNA damage sites (and will detect multiple templates in the DNA extract or PCR indicating contamination). Gilbert *et al.* (2003) identified specific sites in the human mitochondrial control region I (HVRI) that are particularly prone to postmortem damage, and the authors summarize probable misidentification of human haplogroups based on their reported distribution of postmortem damage.

#### Contamination Control and Authentication of aDNA Results

Despite more than a decade of aDNA research, contamination by modern DNA remains a problem because the many sources and modes of contamination are still not known or understood and, therefore, cannot be completely controlled or eliminated. Identification of contamination and authentication of results in aDNA studies rely on the ability to discriminate between aDNA and contaminating DNA. Experiments must be designed with a goal of identifying all DNA contaminants to distinguish convincingly between endogenous and contaminating DNA. Careful selection of polymorphic markers capable of discriminating between aDNA and probable DNA contaminants is critical. Table I lists the controls essential for an aDNA study and presents additional experiments in the case of questionable or controversial results. Sequence analysis of cloned PCR products has emerged as a particularly useful control because it allows one to directly evaluate individual amplification products for evidence of multiple templates in the reaction (indicating contamination) or DNA damage sites (characteristic of aDNA).

Studies of human populations are uniquely prone to contamination because every researcher, archaeologist, or curator represents a potential source of contamination. Richards *et al.* (1995) reported that approximately 50% of animal bones from a Holocene site in England exhibited contamination with human sequences. Human bones should be assumed similarly contaminated. Analysis of human populations in which genetic markers exist that distinguish between the ancient population and probable contaminants should focus on those discriminatory markers to ensure that

contamination is identified quickly. aDNA studies wherein the prehistoric population and likely sources of contamination are genetically similar will be much more difficult to conduct.

Only in nonhuman studies is there control over the presence or absence of contamination sources. However, this control exists only if the studies are performed in a lab in which modern specimens of the organism of interest have never been studied. This restriction is a bit paradoxical because the researchers studying modern populations of an organism are those most likely to be interested in ancient populations of that organism. Typically, this requirement is met by the physical separation of ancient and modern laboratories, although ideally completely different labs would work on modern and ancient specimens. Furthermore, positive PCR controls must never be used because use of modern undamaged DNA represents the purposeful introduction of a potential DNA contaminant. Addition of control DNA to PCRs or storage of control DNA in a separate lab is unlikely to eliminate contamination because it is typically the same researcher handling both modern and aDNA samples. All experiments involving modern DNA, including primer design and PCR optimization, should be conducted in an independent laboratory if possible. These conditions are most easily met by a collaborative project in which a researcher experienced in the analysis of modern populations of the organism teams up with a researcher experienced in aDNA analysis.

### Concluding Remarks

The incredible amount of time and resources necessary for aDNA studies mandate that only questions that cannot be addressed with modern samples should be undertaken. Each aDNA research project must be custom designed with the study populations in mind. Polymorphisms that differentiate between DNA from ancient specimens and any potential sources of contamination must be identified and analyzed. The impressive comparative database of DNA data available for a wide variety of organisms assures the merit of a project assaying those markers in ancient specimens, but only if extensive precautions have been taken to ensure the accuracy and reliability of the data.

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