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Technical Note: Improved DNA Extraction From Ancient Bones Using Silica-Based Spin Columns

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ABSTRACT We describe a simple method for extracting polymerase chain reaction-amplifiable DNA from ancient bones without the use of organic solvents. Bone powders are digested with proteinase K, and the DNA is purified directly using silica-based spin columns (QIAquick™, QIAGEN). The efficiency of this protocol is demonstrated using human bone samples ranging in age from 15 to 5,000 years old. Am J Phys Anthropol 105:539–543, 1998. © 1998 Wiley-Liss, Inc.

The advent of the polymerase chain reaction (PCR) (Saiki et al., 1985) has made it possible to amplify and analyze DNA isolated from ancient bones, mummified tissue, and even fossilized material. The studies of DNA provide great potential for research in anthropology, archaeology, forensic science, biology, and the evolutionary sciences (Herrmann and Hummel, 1994). There are, however, factors that limit the extent to which ancient DNA can be analyzed. In particular, one must cope with intrinsic problems such as low template quantity, poor template quality, and the presence of PCR inhibitors. To maximize the potential for success, DNA extraction protocols should be capable of purifying trace amounts of DNA while at the same time removing potential inhibitors of PCR. In addition, extraction protocols should be designed to minimize the potential for contamination with modern DNA (genomic or PCR-amplified DNA).

Currently, there are two widely used extraction protocols for ancient DNA studies. The first method, designated the Centricon™ approach (Hagelberg and Clegg, 1991), involves proteinase K digestion to solubilize the DNA. The DNA is then separated from the cellular debris through a series of phenol-chloroform extractions and concentrated by passage through a Centricon™ microconcentrator. The Centricon™ contains an anisotropic membrane that retains macrosolutes (including DNA) while allowing low-molecular-weight solutes to pass through. As the volume of the solute is reduced, the concentration of the retained DNA increases. Unfortunately, this method also concentrates any potential inhibitors of PCR that happen to fall above the molecular weight cutoff of the Centricon™ membrane.

The second method for purification involves the use of silica particles which have a high binding capacity for DNA molecules (Höss and Pääbo, 1993). DNA extracts are treated with silica powders under conditions which allow the DNA molecules to become immobilized on the silica particles. The silica particles are then pelleted by centrifugation, washed, and treated to elute the purified

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DNA. This method has the advantage of being more specific for DNA and less likely to co-purify PCR inhibitors. However, silica particles are powerful PCR inhibitors, and care must be taken to ensure that the final extract is free of residual silica particles.

In this report, we describe a simple extraction protocol that combines features of both the Centricon™ and silica-based approaches. The method involves the use of silica-based spin columns that simultaneously concentrate and purify DNA. Bone powders are digested with proteinase K and loaded directly onto QIAquick™ spin columns, eliminating all extraction steps involving organic solvents (phenol-chloroform). We demonstrate that this method is highly effective for purifying PCR-amplifiable DNA from ancient human bones.

MATERIALS AND METHODS

Bone samples

Five human bone samples ranging in age from 15 to 5,000 years old were used in this study. The oldest samples are a 2,000-year-old rib bone and a 5,000-year-old mandible from two archaeological sites in China. The other samples are a 15-year-old humerus that had been stored in a laboratory, a 200-year-old humerus that had been buried in a pine coffin, and a 300-year-old femur that had been buried directly in the ground. Morphologically, all samples are generally in a good state of preservation.

Extraction protocol

Throughout the extraction procedure, care was taken to minimize contamination with modern DNA. Bone powders were prepared in a facility dedicated to the analysis of archaeological specimens, using separate equipment and reagents. Non-disposable equipment (e.g., drill bits) was decontaminated between samples, and latex gloves and protective clothing were worn when handling the bones.

The DNA extraction protocol described here utilizes a commercially available QIAquick™ PCR Purification Kit. QIAquick™ spin columns are originally designed to trap PCR products that are larger than 100 bp and smaller than 10 kb, while at the same time excluding nucleotides, proteins, and salts. These columns are ideally suited for ancient DNA samples since the DNA templates are highly degraded and the target regions for amplification generally are quite small (e.g., 150 to 250 bp); DNA molecules that are larger than 10 kb associated with ancient remains are more likely to be postmortem and/or modern bacterial or fungal DNA instead of authentic ancient DNA.

Bone powders were generated by drilling the bone surface that previously had been polished with sandpaper. Depending on the age and state of preservation, from 0.5- to 5-g samples were taken from each bone. Larger bone samples were intentionally used in this study to obtain sufficient amounts of DNA for multiple comparisons of different protocols. Bone powders were dissolved in 8 mL extraction buffer (0.5 M EDTA pH 8.0, 0.5% sodium dodecyl sulfate, and 100 µg/mL proteinase K) and incubated in a shaking waterbath at 55°C overnight and then at 37°C for 24 hours.

The extraction solution was centrifuged at 2,000*g* for 5 minutes, and 1.75-mL aliquots of the supernatant were transferred to 2.0-mL centrifuge tubes and spun in a microcentrifuge (12,800*g*) for 5 minutes. The supernatant was then transferred to a 10-mL tube and mixed with 5 volumes of QIAquick™ PB buffer. Using a sterile disposable pipette, 750 µL was loaded directly onto a QIAquick™ column and centrifuged at 12,800*g* for 1 minute. The flowthrough was discarded and the process was repeated until all of the extract had been passed through the column. The DNA was washed by adding 750 µL of QIAquick™ PE buffer and centrifuging for 1 minute. The flowthrough was discarded and the DNA was then eluted from the column by loading 100 µL TE buffer and centrifuging for 1 minute.

Centricon™ 30 microconcentrators (Amicon Division, Danvers MA) were used to concentrate the DNA solution after phenol-chloroform extraction (Hagelberg and Clegg, 1991). Centricon™ 30 microconcentrators were also employed to concentrate the digested bone solution to reduce the number of loadings required to pass all of the extract through the QIAquick™ column. This was accomplished by loading the extract onto a

Centricon™ 30 microconcentrator and spinning at 2,000*g* until the retentate was reduced to the desired volume. The retentate was then collected, mixed with 5 volumes of QIAquick™ PB buffer, and loaded directly onto the QIAquick™ column in a single loading.

PCR amplification of repetitive α-satellite DNA (D17Z1)

The overall success of the extraction protocols was assessed based on the ability to amplify a 211-bp target sequence using PCR. The target sequence was derived from a highly repetitive α-satellite sequence specific for human chromosome 17 (locus D17Z1) (Waye and Willard, 1986). The D17Z1 repeat sequence has a copy number of 500–1,000 and therefore can be detected with a high degree of sensitivity using PCR (Warburton et al., 1991). The following primers were used for PCR amplification: 5′-CAA ATC CCC GAG TTG AAC TT-3′ and 5′-AAA ACT GCG CTC TCA AAA GG-3′. PCR amplification was carried out using the GeneAmp™ Thermocycler Model 2400 (Perkin-Elmer, Norwalk CT) in a 50-μL reaction volume containing 50 mM KCl and 10 mM Tris-HCl, 2 mM $MgCl_2$, 0.2 mM dNTP, 1.5 mg/mL bovine serum albumin (BSA), 0.25 U Taq polymerase, 100 pmoles of each primer, and 5 μL of DNA template. PCR was run for 32 cycles of 94°C for 40 seconds, 53°C for 20 seconds, and 72°C for 20 seconds. Five microliters of PCR product was separated by electrophoresis on a 2% NuSeive/2% agarose gel cast and run in 1 × TBE (90 mM Tris Borate, 2 mM EDTA). The 100-bp ladder (Life Technologies, Gaithersburg, MD) was used as a size marker. After electrophoretic separation, the gel was stained with ethidium bromide and photographed under UV illumination.

RESULTS

Several different procedures were used to extract DNA from the proteinase K–digested bone solutions. For comparative purposes, four protocols have been identified in this study as follows:

A. Phenol-chloroform extraction followed by Centricon™ concentration.
B. Phenol-chloroform extraction followed by Centricon™ concentration and QIAquick™ purification.
C. Direct purification from proteinase K digests (without any phenol-chloroform extractions) using Centricon™ concentration followed by QIAquick™ purification.
D. Direct purification from proteinase K digests (without any phenol-chloroform extractions) using only the QIAquick™ column.

The PCR results obtained for the five DNA samples extracted using the four protocols are shown in Figure 1. With ***protocol A*** (phenol-chloroform extraction and Centricon™ concentration), only the samples from the 200- and 15-year-old bones (lanes 5 and 6, respectively) showed detectable amplification. In contrast, all of the samples extracted using ***protocol B*** (phenol-chloroform extraction, Centricon™ concentration, QIAquick™ purification) showed strong amplification. A probable explanation for this difference is the presence of PCR inhibitors in DNA samples extracted using ***protocol A.*** This notion is supported by the observation that several of the DNA samples extracted using ***protocol A*** were pigmented, with the sample from the 300-year-old bone (lane 4) appearing dark brown and the samples from the 5,000- and 2,000-year-old bones (lanes 2 and 3, respectively) appearing light brown. In comparison, all five samples prepared using ***protocol B*** were free of pigmentation and readily amplified.

It was of interest to determine if the QIAquick™ columns could be applied directly to the proteinase K–digested bone solutions, without the need for phenol-chloroform extractions. As shown in Figure 1, the QIAquick™ columns yield PCR-amplifiable DNA from proteinase K digests that were concentrated using the Centricon™ columns (***protocol C***). Moreover, the QIAquick™ purification method can be applied directly to the proteinase K digests (***protocol D***).

To monitor potential contamination, extraction blanks containing everything except bone powders were carried through the entire process. For all four protocols, the extraction blanks were negative for D17Z1 amplification (Fig. 1, lane 1). This provides

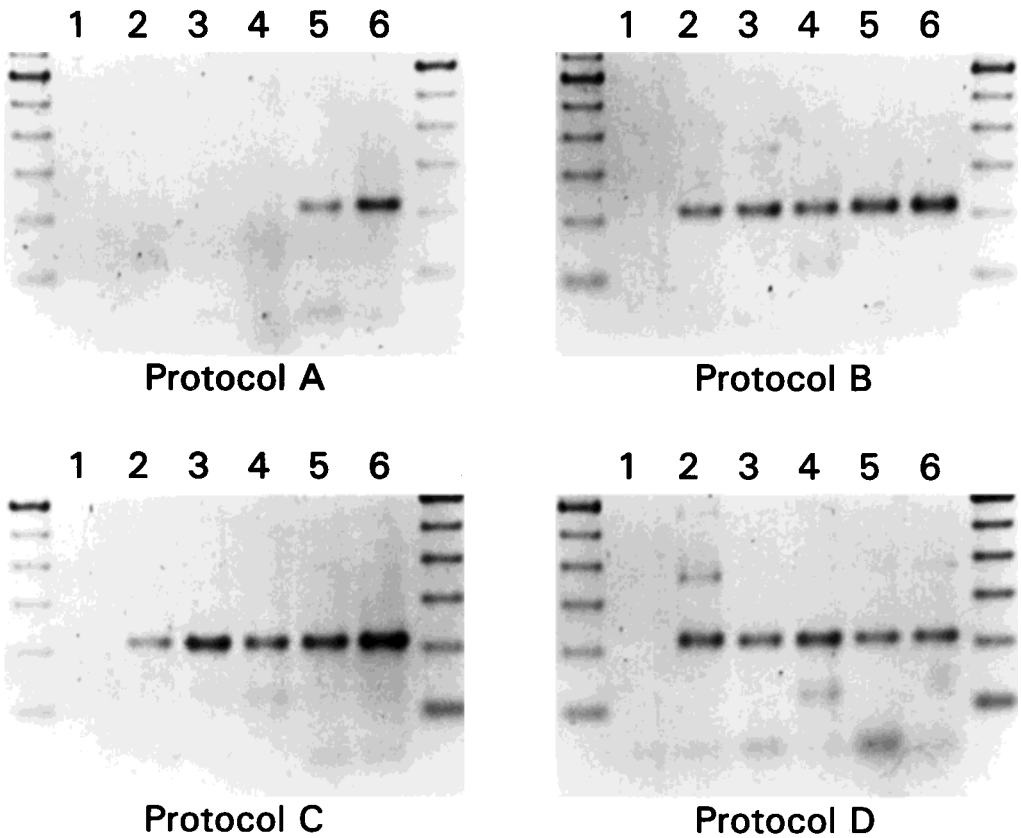

Fig. 1. Reverse image of ethidium bromide–stained gel of PCR products of DNA isolated from ancient bones. **Lanes 1:** extraction blank (no DNA); **lanes 2:** 5,000-year-old bone; **lanes 3:** 2,000-year-old bone; **lanes 4:** 300-year-old bone; **lanes 5:** 200-year-old bone; **lanes 6:** 15-year-old bone.

indirect evidence that the positive D17Z1 results obtained for the various bone samples were not due to systemic contamination introduced during DNA extraction or PCR amplification.

DISCUSSION

Protocols for ancient DNA extraction should optimize the recovery of DNA and minimize the impact of PCR inhibitors. The number of steps in the procedure should be minimized to lessen the possibility for contamination. Ideally, commercially available reagent kits should be employed to ensure consistency and reduce the technical requirements of the facility.

Our results demonstrate that QIAquick™ columns may be superior to Centricon™ microconcentrators, particularly with respect to the recovery of PCR-amplifiable DNA. This was most evident for the bone samples in lanes 2, 3, and 4 (Fig. 1). ***Protocol A*** yielded pigmented DNA preparations that could not be amplified. Subsequent purification of this sample using the QIAquick™ column (***protocol B***) removed all traces of pigmentation and allowed the sample to be amplified. Similarly, ***protocols C*** and ***D*** yielded clear DNA solutions that were readily amplified. Based on these observations, it is evident that the QIAquick™ columns are effective in removing pigments that often inhibit PCR (Yang et al., 1997).

Protocol D offers several advantages over other methods. In a single step, the DNA is concentrated and separated from non-DNA substances that could inhibit PCR. A potential limitation of ***protocol D*** is that the capacity of the QIAquick™ column is only 750 µL (125 µL proteinase K digest plus 625

µL PB buffer). If the starting volume of the proteinase K digest is greater than 125 µL, multiple loadings of the QIAquick™ column are required. In our experience, as many as four or five loadings are practical. For larger volumes, we recommend decreasing the volume first using Centricon™ concentration (***protocol C***).

Protocols C and ***D*** are particularly well suited for ancient DNA research because they can be applied directly to proteinase K digests, without the need for phenol-chloroform extractions. Not only does this eliminate the need for a vented fume hood, but it reduces the number of manipulations and the potential for contamination. Moreover, the commercial availability of the QIAquick™ kits makes it possible to carry out DNA extractions in conventional museum, anthropology, and archaeology laboratories.

Protocols for isolating and analyzing DNA from ancient bones must safeguard against contamination with modern DNA. In the present study, the issue of authenticity was not formally investigated because the sole purpose of the study was to compare the relative efficacies of the protocols for isolating amplifiable human genomic DNA from ancient bones. The important issue is whether the positive PCR amplification results were due to human genomic DNA isolated from the bones and not from contaminating DNA templates introduced during the extraction protocol or PCR setup. The fact that the reagent blanks were negative for all four protocols indicates that systemic contamination was not a problem (Fig. 1, lane 1).

On a final note, we now have considerable experience using the QIAquick™ extraction protocols described in this report. These protocols have been used in two separate studies; one involving bones and teeth from a pioneer cemetery that is 126–174 years old (Dudar, unpublished results), and another involving Roman bones approximately 2,000 years old (Yang, 1997). In both studies, the QIAquick™ protocols have yielded amplifiable DNA, the authenticity of which has been verified by typing of short tandem repeat markers or by mitochondrial sequencing.

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