Chapter 3

DNA Extraction of Ancient Animal Hard Tissue Samples via Adsorption to Silica Particles

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Abstract

A large number of subfossil and more recent skeletal remains, many of which are stored in museums and private collections, are potentially accessible for DNA sequence analysis. In order to extract the small amount of DNA preserved in these specimens, an efficient DNA release and purification method is required. In this chapter, I describe an efficient and straightforward purification and concentration method that uses DNA adsorption to a solid surface of silica particles. Comparative analysis of extraction methods has shown that this method works reliably for ancient as well as younger, museum-preserved specimens.

Key words: Ancient DNA, DNA extraction, Bones, Teeth, Museum-specimen, Silica, Column

1. Introduction

The most abundant faunal remains are partial skeletons. Bones and teeth are the hardest tissues of vertebrates and can persist for hundreds of thousands of years without fossilization if sediments or permafrost shield them from unstable environmental conditions. When environmental conditions are unfavorable for microbial life that would otherwise metabolize the hard tissue, this can lead to the preservation of DNA molecules within these ancient skeletons. Such conditions are common to permafrost regions, where large numbers of preserved faunal remains have been found. In more moderate climatic ecosystems, well-preserved skeletal remains can be found within sediment deposits in natural shelters such as caves.

Three major obstacles impede DNA analyses of ancient skeletal remains. First, the total amount of DNA preserved in very old bones and teeth is likely to be very small, and often the DNA fragments that do remain are highly damaged (1). The same may be

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true for modern specimens that have been treated with chemical preservatives to prepare them for long-term storage in museums. Second, if DNA is preserved in ancient bones or teeth, it is often contaminated with DNA from bacteria, fungi, or other microbial organisms (2). Third, regardless of the environmental condition from which the sample is excavated, contaminating organic and inorganic compounds, such as humic acid and salts leaking from the surrounding soil, can accumulate in the cavities of these samples over the years. These are often coextracted along with the endogenous DNA of the sample. Therefore, ancient DNA extraction methods need not only to recover DNA molecules preserved in the samples efficiently, but also to remove contaminating compounds that may inhibit subsequent enzymatic reactions.

The solid matrix of bones and teeth promotes their physical preservation and the preservation of biomolecules within them. However, this matrix needs to be disrupted during the extraction process in order to release the DNA molecules into an aqueous solution so that it can be purified. Several DNA purification and concentration methods are used for ancient animal hard tissue samples. The purification method described here is a two-part process, where DNA is first adsorbed to the surface of silica particles and then salts and other contaminating chemicals are removed. The method is identical in concept and very similar in approach to methods employed in various commercially available kits.

In previous comparative analyses, we found DNA purification by adsorption to silica particles in suspension to perform best with respect to amplifiable DNA recovery from ancient bone and tooth samples when guanidinium isothiocyanate (GuSCN) was used as a chaotropic salt to drive the adsorption of DNA (3). GuSCN seems to prevent silica particles from adsorbing potentially inhibiting coextracts that may have accumulated in the samples. One advantage to using a solid phase to pull down the DNA from an aqueous solution is that the particles can be immobilized in an appropriate device. If these device(s) allow parallel processing, salt and other chemicals can easily be washed away and DNA eluted from many samples in parallel. Single column devices are commercially available, and using a vacuum device or a microcentrifuge to remove the buffers in between the steps allows for a moderate throughput for DNA extraction of ancient and historical museum samples (4).

The following protocol is presented using column devices and a vacuum manifold. If no vacuum manifold is accessible, the extraction can be performed using the columns and a microcentrifuge. It is also possible to perform the extraction without the columns by using regular 1.5- or 2.0-mL tubes and resuspension of the silica particles followed by centrifugation, rather than the simpler method (vacuum-mediated washing by flow-through) described below. However, it should be noted that some DNA may be lost as it adheres to the inside surface of the pipette tips during repeated resuspension steps.

The presence of intact cellular structures depends on the degradation state of the sample. A detergent and a reducing agent are recommended for more recent samples (4) and for well-preserved ancient samples such as those from permafrost environments. However, it is usually not necessary to use these when working with ancient specimens (5). Nevertheless, no negative effect has been observed when the detergents and reducing agents used below are included in the extraction buffer, even for very old, nonpermafrost specimens (3). These are therefore included in the extraction buffer described below.

2. Materials

HPLC grade water or water with a similar purity grade is recommended to prepare all solutions and suspensions.

2.1. DNA Release from Bony Specimen

1. Extraction buffer: 0.45 M EDTA (pH 8.0), 1% Triton-X 100, 50 mM DL-Dithiothreitol, 0.25 mg/mL proteinase K (see Note 1).
2. Cutting or drilling tool with exchangeable disposable bits or discs.
3. Mortar and pestle or freezer mill (e.g., SPEX SamplePrep 6750 Freezer/Mill; liquid nitrogen is needed) for grinding sample pieces into fine powder.
4. 15-mL tubes.
5. Rotary mixer, wheel, or similar device to keep samples constantly in motion during incubation steps.

2.2. DNA Purification and Concentration

1. Silicon dioxide (see Note 2).
2. 30% HCl.
3. Binding buffer: 5 M Guanidinium thiocyanate, 0.3 M sodium acetate (pH 5.2). Store in the dark (see Note 3).
4. Washing buffer: 50% Ethanol, 125 mM NaCl, 10 mM Tris–HCl, 1 mM EDTA (pH 8.0) (see Note 4)
5. Elution buffer: 10 mM Tris–HCl, 1 mM EDTA (pH 8.0).
6. 50-mL tubes.
7. 50-mL disposable serological pipettes.
8. Centrifuge capable of holding 15-mL tubes and reaching centrifugal force of 5,000 × *g*.
9. Columns (e.g. MobiCol "Classic," MobiTec, catalog number: M1003).
10. Filter (Filter (large) 10 μm pore size, MobiTec, catalog number: M2210).

11. Filter with 1 μm pore size (e.g., glass microfiber binder free Grade GF/B: 1 μm, Whatman, catalog number: 1821–070).
12. Hole punch with 7 mm diameter.
13. Forceps.
14. Centrifuge capable of holding 2.0-mL tubes and reaching centrifugal force of 16,000 × *g*.
15. Vacuum manifold and vacuum pump.
16. Collection tubes without lids.
17. Disposable VacConnectors (Qiagen, catalog number: 19407).
18. 1.5-mL tubes (see Note 5).

3. Methods

All steps are to be carried out at room temperature.

3.1. Preparing the Silica Suspension

1. Weigh 4.8 g of silicon dioxide into a 50-mL tube, add water to bring the mixture to 40 mL, and vortex extensively.
2. Let large particles settle down for 1 h.
3. Transfer 39 mL from the top of the solution into fresh 50-mL tube and let the solution settle for an additional 4 h.
4. Discard 35 mL from the top of the solution and add 48 μL of 30% HCl to the 4 mL pellet that remains.
5. Vortex, aliquot, and store the silica suspension at room temperature in the dark (see Note 6).

3.2. Preparing the Columns

1. Use forceps to place a large filter with 10 μm pore size in the column. Move the filter to the bottom of the column using the filter insertion tool provided with the filter.
2. Use a hole punch to make a smaller "fine filter" from the filter paper with 1 μm pore size.
3. Using the forceps, place the "fine filter" in the columns, and move it on top of the larger filter using the insertion tool.

3.3. Sample Preparation and DNA Release

1. After removing the surface of the sample with a fresh drilling bit at slow speed, drill into the densest part of the bone or the tooth root. Collect the powder. If a cutting tool is used instead of a drill, remove a compact part of the bone or the tooth root (again after removing the sample surface with a single-use cutting disc or blade). Grind the pieces of sample to as a fine powder as possible using mortar and pestle or a freezer mill. Collect approximately 250 mg of powder per sample into separate 15-mL tubes (see Note 7).

2. Add 5 mL of extraction buffer to each sample. Seal the tubes and incubate them for 16–24 h under constant agitation in the dark (see Note 8).

3.4. DNA Adsorption to Silica, Washing Steps, and Elution

1. Centrifuge the samples for 2 min at 5,000 × *g* and transfer as much of the liquid as possible into new 15-mL tubes.
2. Add 2.5 mL of binding buffer and 100 μL of well-mixed silica suspension to the extraction buffer in each tube. Incubate for 3 h in the dark under constant agitation (see Notes 9–11).
3. Place a disposable VacConnector onto the luer adapter of the vacuum manifold, then place the assembled column onto the VacConnector (depending on the manifold used, up to 24 columns can be handled in parallel).
4. Centrifuge the sample for 2 min at 5,000 × *g*, discard the supernatant, and resuspend the silica pellet in 400 μL of binding buffer. Transfer the suspension to the column and apply the vacuum (see Notes 12–14).
5. Place the column in a collection tube and centrifuge for 30 s at 16,000 × *g* (see Note 15).
6. Place the column back onto the VacConnector of the vacuum manifold. Add 450 μL of washing buffer to the column and apply the vacuum (see Note 16).
7. Repeat the washing step at least once while the column remains on the vacuum manifold (see Note 17).
8. Insert the column into a collection tube and centrifuge for 30 s at 16,000 × *g* (see Note 18).
9. Insert the column into a new, labeled 1.5-mL tube and allow the silica to air-dry by incubating the columns with open lids for about 3 min (see Notes 5 and 18).
10. Add 50 μL of elution buffer onto the center of the silica pellet and incubate the columns for 10 min with closed lids (see Notes 19 and 20).
11. Centrifuge for 1 min at 16,000 × *g* (see Note 21).

4. Notes

1. Always prepare the extraction buffer immediately before beginning the extraction, as proteinase K loses activity rapidly.
2. Recommended silicon dioxide: Sigma-Aldrich, catalog number: S5631.
3. The binding buffer is stable for at least 1 month. This buffer should be stored in the dark.

4. The washing buffer is stable for several months.
5. Low retention or siliconized tubes are recommended, which reduce DNA loss due to tube wall effects.
6. The silica suspension is stable for at least 1 month.
7. Do not exceed 250 mg/5 mL extraction buffer. It is possible to proportionally scale the extraction up or down when more or less sample material is used; use 1 mL/50 mg. It is crucial to adjust the binding buffer volume accordingly (see Notes 10 and 11).
8. Incubation can also be performed at 37°C, where proteinase K is more active than it is at room temperature. This may increase the DNA quantity, especially for younger samples with intact cell structures. Although increasing incubation time was not seen to have an influence in our test series of ancient samples (3), incubation time can be extended in order to completely digest the material; this may also increase the quantity of extracted DNA (6).
9. Silica must be extensively vortexed before adding to the extraction and binding buffer as particles quickly settle down.
10. If more or less extraction buffer was used, adjust the volume of binding buffer accordingly so that the ratio of extraction to binding buffer is 2:1.
11. The volume of silica suspension should also be adjusted proportionally when different extraction buffer volumes are used. The volume of silica suspension should be at least 50 μL, as too few silica particles may result in a loss of DNA molecules. If a very large volume of extraction buffer is used, do not exceed 200 μL of silica suspension per extraction/column, as adding more per column may result in incomplete washing and elution performance; instead, concentrate the extraction buffer prior to the adsorption step using appropriate filter systems (e.g., (2)), or distribute the silica over several columns when more than 200 μL of silica is used. However, the latter will result in higher elution volumes of less concentrated extract.
12. It is recommended that you keep the supernatant until the positive control gives satisfying results. If the extract of the positive control does not contain any DNA, you may repeat the adsorption and purification steps by adding freshly made silica suspension and proceed from the 3-h incubation step onwards.
13. If no columns are used, transfer the silica suspension into a 1.5- or 2.0-mL tube and perform the washing steps by resuspending the silica with washing buffer by pipetting. Then centrifuge for 30 s at 16,000 × *g* to pelletize the silica, and discard washing buffer by pipetting it off. Dry the silica for at least 10 min and resuspend the silica in 50 μL elution buffer by pipetting.

After final incubation for 10 min, centrifuge for 1 min at 16,000 × *g* and pipette off the extract into a fresh-labeled tube.

14. If you are not using a vacuum manifold, this step and all following washing steps can be performed using a microcentrifuge and collection tubes. For an even distribution of the silica particles over the filter and subsequently efficient washing performance, short, slow-speed centrifugation is recommended, followed by a 180° rotation of the column and another short, slow-speed centrifugation step after the silica is applied to the columns.
15. This is a crucial step to remove remaining salts and other chemicals, as remaining GuSCN can lead to incomplete elution of the DNA from the silica and/or inhibit subsequent enzymatic reactions.
16. Fresh VacConnectors are recommended.
17. If the silica is still deeply colored after two washing steps, it is possible to wash the silica with 450 μL binding buffer, followed by centrifugation and at least two washing steps with washing buffer. Washing with binding buffer seems to reduce the amount of colored and potentially inhibiting coextracted contaminants.
18. This is a crucial step to remove any salt and ethanol remains, which may lead to incomplete elution and/or inhibition of enzymatic reactions that follow.
19. If the silica particles are not evenly distributed on the filters, add the elution buffer on top of the thickest part of silica particles.
20. If more than 100 μL silica was used for adsorption, proportional increase of the elution buffer volume is recommended.
21. The elution step may be repeated. However, this increases the volume of extract, but also reduces the concentration of DNA in the extract.

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References

1. Pääbo S, Poinar H, Serre D, Jaenicke-Despres V, Hebler J, Rohland N, Kuch M, Krause J, Vigilant L, Hofreiter M (2004) Genetic analyses from ancient DNA. Annu Rev Genet 38:645–679
2. Noonan JP, Hofreiter M, Smith D, Priest JR, Rohland N, Rabeder G, Krause J, Detter JC, Pääbo S, Rubin EM (2005) Genomic sequencing of Pleistocene cave bears. Science 309: 597–599

3. Rohland N, Hofreiter M (2007) Comparison and optimization of ancient DNA extraction. Biotechniques 42:343–352
4. Rohland N, Siedel H, Hofreiter M (2010) A rapid column-based ancient DNA extraction method for increased sample throughput. Mol Ecol Resour 10:677–683
5. Rohland N, Hofreiter M (2007) Ancient DNA extraction from bones and teeth. Nat Protoc 2:1756–1762
6. Loreille OM, Diegoli TM, Irwin JA, Coble MD, Parson TJ (2007) High efficiency DNA extraction from bone by total demineralization. Forensic Sci Int Genet 1:191–195