Chapter 2

A Phenol–Chloroform Protocol for Extracting DNA from Ancient Samples

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Abstract

The myriad downstream applications of ancient DNA (aDNA) analysis all ultimately require that sequence data are generated from extracts of ancient material. DNA extraction from tissues known to contain preserved biomolecules (e.g. teeth, hair, tissue, bone) relies on subtle modifications of a basic technique that has been in use for nearly two decades. Multiple DNA extraction protocols have been introduced, with varying levels of success depending on tissue type and the long-term preservation environment to which the ancient tissue was exposed. Here, we describe the phenol–chloroform method for extracting aDNA from any tissue type. This commonly employed method allows for the recovery of total nucleic acid content with minimal loss of low molecular weight double-stranded DNA.

Key words: Ancient DNA, Extraction, Bone, Teeth, Hair, Tissue, Phenol, Chloroform

1. Introduction

Over the past quarter century, there has been an enormous increase in the use of genetic data extracted from the preserved remains of plants and animals in questions of evolution, taxonomy, and population genetics. To extract DNA from ancient tissues, most of these studies have relied on simple modifications of a DNA extraction technique that was used in the late 1980s to generate the first ancient genetic data (1, 2). This technique, which has been known as the phenol–chloroform technique, has stayed in favour due to its ease of application and ability to harvest complete nucleic acid fractions. Like all DNA extraction techniques using aDNA, working in a sterile environment and taking careful measures to prevent contamination of the ancient samples and extracts throughout the protocol are crucial in the successful extraction of endogenous genetic material (3, 4).

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The phenol–chloroform method relies on an initial digestion step to break down crystalline minerals, proteins, and complex lipids present in the sample. Further processing sequesters the nucleic acid fraction from other components by repeated separation into hydrophobic and aqueous phases. Final passing through a membrane filter concentrates the DNA in the sample. The final solution of total DNA can be used for PCR, next-generation sequencing, and other applications.

2. Materials

All reagents, consumables, and equipment used throughout this protocol should be sterilised, either through purchase or through processing with UV irradiation or cleaning with bleach solution and ethanol (5–7). Plasticware should be sterile, single-use, and preferably designed to prevent cross-contamination (e.g. pipette tips with aerosol barrier). All solutions should be prepared using ultrapure water (18.2 MΩ at 25°C).

2.1. Sample Preparation

1. Hand-held drill with disposable abrasive discs.
2. Sodium hypochlorite (bleach, 10–20% solution).
3. Ethanol (95–100%).
4. Freezer mill, shaker mill, or other device for grinding samples into powder (see Note 1).
5. Aluminium foil.
6. *N* prepared sterile plastic tubes (15 mL) (see Note 2).

2.2. Chelation

1. EDTA chelation buffer: Ethylenediaminetetraacetic acid (EDTA) 1 M solution pH 8.0 (see Note 3).
2. Rotary mixer, wheel or similar device to keep samples constantly in motion during incubation steps, suitable for use with 15-mL tubes.
3. Centrifuge suitable for use with 15-mL tubes.

2.3. Digestion

1. 1× Buffer:15 mM Tris–HCl (pH 8.0), 2.5 mM *N*-phenacylthiazone bromide (see Note 4).
2. 10×: Sodium dodecyl sulphate (Fisher) 10%w/v (see Note 5).
3. 10×: 25 mg mL^{-1} Proteinase K (see Note 6).
4. 10×: 500 mM Dithiothreitol (DTT) (see Note 7).
5. Laboratory incubator large enough to accommodate rotator.
6. Rotary mixer, wheel or similar device to keep samples constantly in motion during incubation steps, suitable for use with 15-mL tubes.

2.4. Phase Separation

1. 2 *N* pre-prepared 15-mL phase separating tubes (light gel see Note 8) containing 6 mL of saturated pH 6.6 Phenol (see Note 9).
2. *N* pre-prepared 15-mL sterile plastic tubes containing 6 mL of chloroform (see Note 10).
3. Rotary mixer, wheel or similar device to keep samples constantly in motion during incubation steps, suitable for use with 15-mL tubes.
4. Centrifuge suitable for use with 15-mL tubes.

2.5. Concentration

1. *N* labelled micro-concentrators with a nominal molecular weight limit of 30 kDa and able to process 6 mL of solution (see Note 11).
2. Centrifuge suitable for use with 15-mL tubes.
3. *N* labelled sterile plastic tubes (1.5 mL).

3. Methods

As the method described below attempts to extract degraded, damaged DNA from samples that may be anywhere up to several hundred thousands of years old, it is necessary to work in an isolated aDNA workspace, ideally one that is isolated from normal molecular biology (especially PCR) work (4).

3.1. Sample Preparation

1. Prepare the work area by sterilising surfaces. First wash all surfaces with bleach and then rinse with ethanol. Wait until the surfaces dry completely before proceeding. A fume hood with integrated extraction fans is an ideal location for this protocol to be performed. Prepare several layers of aluminium foil to collect powdered sample as it is produced.
2. Thoroughly abrade the external surface of the bone/tooth sample using a hand-held drill with disposable cutting discs or equivalent (see Note 12). Discard the resulting powder, for example by collecting it in the upper layer of aluminium foil and discarding the foil and powder.
3. Reduce the bone/tooth section to powder using a shaker mill, freezer mill or similar device (see Note 13). The speed and other conditions of the powdering device should be adjusted to suit the mineralisation state of the sample. Generate as fine a powder as possible to maximise the surface area of the sample that will eventually contact the chelation solution (6, 8).
4. Collect the powder and transfer it to a labelled, sterile tube (15 mL).

3.2. Chelation

1. Add 15 mL of 1× chelation buffer to the powdered sample (see Note 14).
2. Add 15 mL of 1× chelation buffer to a labelled tube that does not contain any powder. This will be the negative extraction control.
3. Be sure that the powder and chelation buffer are well mixed. Place all 15-mL tubes on the rotary mixer and rotate overnight at room temperature.
4. Concentrate the samples and negative control by centrifugation at 4,000 × *g* for 10 min or until all organic content has pelleted at the bottom of the tube.
5. Remove eluate and retain pellet (see Note 15).

3.3. Digestion

1. Prepare 4.2 mL of the 1× digestion buffer for each sample and add to this each tube containing an organic pellet using.
2. Add 0.6 mL of the 10× solution of SDS to each sample.
3. Add 0.6 mL of the 10× solution of proteinase K to each sample.
4. Add 0.6 mL of the 10× solution of DTT to each sample.
5. Place all 15-mL tubes on the rotary mixer. Place the rotator in the oven and rotate overnight at 55°C (see Note 16).

3.4. Phase Separation

1. Decant each digested sample into a corresponding pre-prepared tube containing phenol.
2. Place all 15-mL tubes on the rotary mixer and rotate at room temperature for 10 min
3. Centrifuge at 8,000 × *g* for 10 min. The two phases will separate. If phase-lock or phase-divider tubes are used, the gel should have formed a barrier between the aqueous and hydrophobic layers (see Note 17). Decant the aqueous layer into the second, pre-prepared tube containing phenol.
4. Place all 15-mL tubes on the rotary mixer and rotate at room temperature for 10 min.
5. Centrifuge at 8,000 × *g* for 10 min. As before, the two phases will separate. Decant the aqueous layer into a pre-prepared chloroform tube.
6. Place all 15-mL tubes on the rotary mixer and rotate at room temperature for 5 min.
7. Centrifuge at 8,000 × *g* for 5 min. The two phases will separate.

3.5. Concentration

1. Carefully transfer the aqueous layer by pipette to a micro-concentrator (30 kDa membrane) (see Note 18).
2. Centrifuge at 8,000 × *g* until the sample has completely passed through the membrane. Discard the filtrate.

3. Add 5 mL of ultrapure water to each sample and centrifuge again at 8,000 × *g* until resolution into a final solution of 100–200 μL (see Note 19). The eluate will now contain your DNA.
4. Transfer the remaining solution by pipette to a sterile storage tube (e.g. 1.5 mL, see Note 20).
5. Store the DNA extract at –20°C (see Note 21).

4. Notes

1. Shaker mills, freezer mills, and other similar devices use friction to reduce samples to powder. The increase in surface area that results from powdering the sample allows for more efficient digestion.
2. Prior to beginning the protocol, prepare N sufficiently labelled tubes, where N is the number of samples plus negative controls (i.e. if 7 samples and one negative control are to be extracted, prepare $N = 8$ labelled tubes, $2N = 16$ phenol tubes, etc.).
3. EDTA is a strong chelator that is able to bind metallic ions such as Ca^{2+} and Mg^{2+} that are released during digestion.
4. Some experiments have shown that *N*-phenacylthiazone bromide may be useful in freeing DNA that has been chemically cross-linked to other biomolecules through diagenetic processes (9, 10).
5. SDS is a detergent that allows the solubilisation of lipids present in biological samples and denatures proteins. 10× SDS solution should be stored in the refrigerator. At low temperature, SDS can precipitate out of solution. If this occurs, place it in a warm oven for 5 min until the detergent has resolubilised.
6. Proteinase K is a protease that cleaves proteins, reducing them to their constituent amino acids. Proteinase K should be stored in the freezer where it will remain stable for several months.
7. DTT is a reducing agent that can cleave cystine–cystine bridges and disrupt the tertiary structure of some proteins, prior to digestion. DTT should be stored in the freezer where it will remain stable for several months.
8. Phase-lock or phase-dividing gels are useful in this capacity as they allow easy decanting of the aqueous phase. The inert gel can be added to tubes, and upon centrifugation, forms a barrier between the two phases. In the absence of gel, the aqueous phase can be transferred by careful manual pipetting.
9. Phenol is dangerous. Extreme caution must be exercised when aliquotting and transferring phenolic samples. Familiarise yourself with the appropriate safety information and correct disposal

methods before use. Polyethylene glycol should be kept to hand wherever phenol is used and those performing the technique should acquaint themselves with emergency procedures in case of phenol spills.

10. Chloroform is hazardous and should be handled with caution.
11. Concentrators use a membrane barrier of pre-determined pore size to prevent the passage of molecules larger than a certain molecular weight. A pore size with a molecular weight cut-off of 30,000 Da should prevent the loss of any nucleic acid larger than about 50 bp in length, but will allow removal of almost all other digested biomolecules.
12. This step removes any preservative coatings and potentially adsorbed environmental contaminants. Recent work suggests that the speed setting of the drill and the amount of friction produced can have a negative effect on DNA recovery (11). Generally, use the lowest speed setting possible for abrasion and cutting of bone and tooth.
13. Some of the sample should be retained for further extractions. Replication, either internal or external, may be necessary for ancient samples.
14. To adapt the protocol for tissue/hair/nail, the overnight chelation stage can be omitted. Begin at the digestion stage and add 0.5 mL of 1× Chelation buffer to the digestion buffer, SDS, proteinase K, and DTT (12). Follow the remainder of the protocol as described.
15. The eluate is also likely to contain DNA and can be retained for processing, either in parallel or at a later date (8).
16. The temperature setting for digestion can be modified depending on the sample. Recent work suggests that lower temperatures may have a beneficial effect on DNA recovery. If the temperature is lowered, increase the length of time the samples are left to rotate until complete digestion is achieved (8).
17. Very occasionally, the phase-lock tubes may not separate properly between aqueous and hydrophobic phases. In this case, care should be taken when manually removing the aqueous phase by pipette.
18. Adding a small volume of ultrapure water to the filters prior to adding the extract may aid in absorption of DNA to the membrane. Depending on the volume of extract to be processed, this step may have to be repeated multiple times until the entire sample has passed through the membrane.
19. Flushing water through the membrane after the entire sample has been passed through may help to further remove any potential inhibitors from the final extract.
20. After extraction, DNA can be roughly quantified by measurement on a spectrophotometric platform. Note that this does

not give an indication of how much of the DNA in the extract is derived from the sample vs. from co-extracted environmental contaminants.

21. It may be useful to subdivide the final extracts into aliquots of 20–50 μL and to use these as necessary. DNA is susceptible to damage from repeat freeze–thaw cycles (13) and should be defrosted as infrequently as possible.

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