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Protocols for Ancient DNA Typing

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Summary

Molecular analysis of fossil and archaeological remains has been established as a powerful tool in providing new insight in phylogenetic investigations. The overlapping set of molecular modifications and degradation that forensic samples share with archaeological specimen suggests the application of similar technical approaches to the respective biological material. Polymerase chain reaction is the molecular technique of choice for the retrieval of specimen deoxyribonucleic acid (DNA) molecules. Because of intrinsic sensitivity, potential contaminations from exogenous DNA sources must be monitored through the entire process by the introduction of multiple blank controls. Cloning and sequencing of polymerase chain reaction products often is the only way to discriminate between contaminations and endogenous sequences as well as to identify variable positions from nucleotide modifications/DNA polymerase errors. Phylogenetic analysis and investigations of the pattern of substitutions are an additional and necessary step to validate the retrieved sequence. Comparison with available related samples (modern or extinct) is critical to correctly validate the results and to avoid artifactual data.

Key Words: Ancient DNA, cloning of PCR products, phylogenetic analysis, degradation, contamination, molecular archaeology.

1. Introduction

The investigation of evolutionary relationships among contemporary and extinct species for a long time has been conducted using classical morphological and functional comparisons. Both are still valuable and important methods, and it is somewhat regrettable that there are fewer scientists involved in these "classical" methods. However, the development of molecular techniques has provided new tools for species comparisons that is now bringing new and unexpected results ***(1)***. The pattern of difficulties faced by molecular analysis of fossil remains is similar to the one routinely encountered in genetic analysis of forensic specimens ***(2,3)***. The invention of polymerase chain reaction

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(PCR) was the technical breakthrough in both application fields. This made feasible the retrieval of a minute amount of genetic material, but at the same time it raised issues regarding authenticity of results ***(4,5)***. DNA modifications and contamination are the main issues in result validation ***(6,7)***. Despite these limits, the scientific community has testified a large interest in archaeological molecular analysis, and this has contributed to the creation of a brand new discipline, "molecular archaeology" ***(8–13)***.

PCR allows the retrieval of deoxyribonucleic (DNA) sequences of ancient and minute, often degraded remains (it was by the way the method that revolutionized the forensic analysis of biological stains; ref. ***14***), but its critical sensitivity to low-amount DNA target also is a weak point, making the relevant analyses very prone to molecular contamination. Because of this, one should always seek to avoid contaminations and carefully spot its sources of it during the amplification procedure. Throughout the entire PCR procedure, negative controls must be introduced. The careful control of any possible labware used in the process also is mandatory to prevent cross contamination across various sections of the same laboratory. Disposable plastics, coats, and breathing masks are necessary. An ad hoc workspace with routine ultraviolet (UV) irradiation is necessary to avoid risks of contamination ***(4,5)***, to a point that many laboratories involved in daily ancient DNA (aDNA) analysis are being quickly relocated into rooms with positive pressure.

The protocol we describe should be reproduced in compliance with as many of these precautions as possible. This protocol has been adopted, with minor variants, in a number of aDNA studies ***(15–20)***. We start by a classical phenol–chloroform extraction followed by a silica purification step ***(21,22)***. The organic extraction removes most of the protein molecules present in the sample. Often, especially in the case of bone extraction, several PCR inhibitors might be present as molecular constituents of the samples to test. The silica step is essential to bind the DNA and to get rid of inhibitors that tend to remain in the aqueous phase. The PCR folliws a hot-start approach to avoid mispriming and primer-dimers during the initial denaturing ramping phase.

2. Materials

1. 10% Sodium hypochlorite ("bleach").
2. Double-distilled (dd) water (in alternative autoclaved water exposed to UV light and filtered with centricon 30 can be used).
3. 0.5 *M* ethylene-diamine tetraacetic acid (EDTA), pH 8.0.
4. 5% Sarkosyl.
5. Proteinase K 10 µg/µL.
6. Phenol.
7. Chloroform:Isoamylalcohol 24:1.

8. Amicon Centricon 30 centrifugal filter devices (Millipore, Billerica, MA).
9. Silica (Sigma).
10. HCl.
11. 5 *M* guanidiniumisothiocyanate.
12. 0.1 *M* Tris-HCl, pH 7.4.
13. Ethanol 70%.
14. Acetone.
15. TE, pH 8.0.
16. dNTPs.
17. PCR buffer (supplied with the enzyme).
18. *Taq* polymerase.
19. Agarose reagents and equipment for electrophoresis.
20. Primers of choice ,10 μ*M* each.
21. *Sma*I (e.g. New England Biolabs).
22. *Sma*I-specific buffer (New England Biolabs).
23. T4 DNA polymerase (e.g. New England Biolabs).
24. 10X T4 DNA polymerase buffer (New England Biolabs).
25. 3 *M* Na-Acetate, pH 5.2.
26. Glycogen 1 mg/mL.
27. Ethanol 100%.
28. *Sma*I-cut pUC18 vector, 25 ng/μL, (Pharmacia).
29. T4 DNA ligase (New England Biolabs).
30. ATP 500 μ*M*.
31. Transformation and cell growth reagents.
32. Sequencing reagents.

3. Methods

Several kits are currently available, based on, inspired by, or just competing with to the protocols we describe here. For example, a number of commercially available kits for DNA extraction from archaeological remains have been introduced, and different cloning systems, combined with various sequencing protocols, have been proposed. Our protocols are meant to just exemplify a basic approach, not necessarily the only one.

Most protocols have been conceived and or implemented by Matthias Hoss, Oliva Handt, and Matthias Krings at the Evolutionary Laboratory, Zoological Institute, University of Munich, Germany. They have been published in several articles ***(15–20)***, as such or with alterations.

3.1. Extraction

Most samples in aDNA studies are bone remains. Therefore, our protocol specifically addresses this evidentiary sample. It is, of course, possible to any time modify or readapt the protocol to suit other materials. For example, the

initial digestion step could be modulated to extract from samples with different characteristics, and the silica protocol could be easily introduced at some stage to remove inhibitors.

3.1.1. Sample Preparation

1. Compact, long bone is often better preserved. Cut out a bone section by a sterile saw blade, then rinse it with dd water, repeatedly.
2. Remove the surface of the bone by a scalpel or drill the bone and take the powder material from the inner part. It might be advisable to wash the surface of the piece with 10% sodium hypochlorite for 10 s, or less, and extensively rinse it with dd water. All of this is done to remove possible contaminants (from previous handling) from the sample surface.
3. Grind the bone fragment into fine powder. This is usually the most difficult part of this initial step. Commercially available freeze mills are the best option but are quite expensive. Any mechanical grinding is in principle an equally good option.
4. Weigh the ground bone and transfer 0.4 g to a 2-mL tube. Add 1 mL of 0.5 *M* EDTA, pH 8.0, 5% sarkosyl. Seal with plastic film and mount on a rotary wheel at room temperature (RT) for 48 h, or longer if need. You might want to refill with fresh EDTA after 2 d incubation. In that case, pellet by centrifugation, dispose of the saturated solution, and replace with fresh EDTA (1 mL).
5. After the EDTA treatment, add 10 µL of proteinase K (10 µg/µL) and incubate for another 48 h. More proteinase can be added if required and the incubation time prolonged accordingly.
6. Sample material is then pelleted by centrifugation for 1 min at maximum speed. We suggest keeping the pellet material for eventual subsequent re-extraction.

3.1.2. Organic Extraction

Extract the supernatant with a classical phenol:chlorophorm approach as follows:

1. Add 1 mL of phenol, equilibrated at pH 8.0, vortex, and then centrifuge 3 min at maximum speed. Transfer the aqueous phase to a clean tube—keep in mind that phase reversal caused by high salt concentration in the EDTA solution might occur so the phenol could built the upper layer.
2. Add 1 mL of phenol:chloroform/isoamylalcohol (24:1), vortex, and centrifuge. Move the upper aqueous phase to a clean tube. Note that the phenol and the chloroform:isoamylalcohol (24:1) are in a 1:1 ratio. Prepare and store at 4°C.
3. Add 1 mL of chloroform:isoamylalcohol (24:1), vortex, and centrifuge.
4. Move aqueous phase to a clean tube for the subsequent centricon 30 concentration step, following the supplier's recommendations for centrifugal speed. Wet the membrane for 15 min with 50 µL of dd water, add the aqueous phase from chloroform/isoamylalcohol step to the filter, and spin at recommended rpm.

(Note: the filter is sensitive to chlorophorm, so be careful not to carry it over from the previous steps. Avoid filter dehydration by overcentrifugation. After all the supernatant has been passed through the filter, wash twice with a corresponding volume of dd water.)

Finally, invert the filter to recover the DNA solution by a short centrifugation (2 min). Transfer the DNA solution to a 1.5-mL tube. The recovered volume should be approx 100 µL.

3.1.3. Silica Purification

Because inhibitors of PCR are often present, it is advised to purify the sample. We used a Silica purification process as described previously ***(22)***.

3.1.3.1. Preparation of Reagents for Silica Extraction

3.1.3.1.1. Silica

1. Dilute 4.8 g of SiO_2 (Sigma) in 40 mL of dd water (total volume) using a 50-mL tube, vortex, and sediment by leaving it on the lab bench for 24 h at RT.
2. Remove 35 mL of the supernatant and fill up to 40 mL with dd water. Resuspend silica pellet by vortexing and sediment for additional 5 h at room temperature.
3. Remove 36 mL of the supernatant and add 48 µL of HCl (32%, weight/volume, pH 2.0). Aliquot 0.5 mL in different tubes. The silica is stable for at least 6 mo when stored at RT in the dark.

3.1.3.1.2. Buffer L2

1. Dissolve 24 g of guanidinium thiocyanate in 20 mL of 0.1 *M* Tris-HCl, pH 7.4, in a 50-mL tube. To facilitate dissolution the tube with the solution can be heated a bit, but do not boil it!
2. Add 1 mL of silica. This will bind all the eventual contaminant DNA.
3. Spin down and transfer the supernatant in 50-mL tubes in aliquots of approx 20 mL each. Buffer L2 is stable at least 3 wk at RT in the dark.

3.1.3.2. Silica Purification

1. Add 40 µL of the silica solution plus 1 mL of L2 buffer to the organically extracted solution. Vortex briefly and rotate for at least 15 min (as long as 1 h is feasible).
2. Centrifuge to pellet the silica.
3. Wash twice with 1 mL of ethanol 70% and once with acetone. In all steps, vortex, centrifuge to pellet again and remove the liquid. Avoid touching the pellet with the tip.
4. Dry for a short period at 56°C.
5. Elute the DNA twice, adding 65 µL of TE pH 8.0, per time by dissolving the pellet carefully with a pipette and incubating each time at 56°C for 15 min. Do not pipet up and down in the tip but dissolve by turning the tip gently in the silica. Pellet the silica by full speed centrifugation and collect the supernatant. The final

volume should range between 120 and 130 µL. Aliquot this in 3 different tubes, leaving the silica behind. Store extracts at –20°C. Usually 5 µL of the extract is used for subsequent PCR.

3.2. PCR Amplification

The use of a hot-start protocol is highly recommended ***(23)***. Until a few years ago the only option was to mechanically separate the *Taq* polymerase enzyme from nucleotides, primers, and DNA by a wax layer that just melted as the initial denaturing step started ***(24)***. Nowadays a number of genetically engineered enzymes or antibody-conjugated enzymes, active only at specific temperatures, are available. This makes PCR setup much easier and less prone to contaminations by reducing the necessary number of manipulations. We suggest using those enzymes as a valid alternative to previously described protocols.

The PCR can be set up in a final volume of 20 µL as follows:

10X PCR buffer	2 µL
Primer A, 10 pmolµL	0.5 µL
Primer B, 10 pmol/µL	0.5 µL
dNTPs, 25 m*M* each	0.1 µL
Taq polymerase, 5 Uµ L	0.2 µL
dd water	11.7 µL
DNA extract	5 µL

3.2.1. PCR Conditions

Forty cycles with 15 s 95°C/1 min primer-specific annealing temperature/ 1 min 72°C. Note that by using hot-start polymerases, an extended initial denaturing step has to be included.

If only faint bands are visible after agarose gel electrophoresis, one might consider reamplifying the PCR products. For this purpose, a modified freeze–squeeze protocol ***(25)*** can be applied to quickly recover the PCR products cut out of the agarose gel.

1. Cut out the desired product band with a razor blade and transfer it to a 1.5-mL microtube filled with 100 µL of dd water.
2. Melt it shortly on a heater (between 65 and 90°C) and snap freeze it in liquid nitrogen (alternatively, shock freeze using a dry ice/ethanol bath).
3. After thawing the solution, centrifuge it for 30 min with maximum rpm and use 0.5–5 µL for reamplification under the conditions described above but with only 25–30 cycles and increasing annealing temperature by 3°C.

3.3. Cloning of PCR products

We usually used the *Sma*I-cut pUC18 screening vector and performed blunt-end cloning of the PCR products. A preliminary requirement is not to have a

*Sma*I recognition site internal of your PCR product and to rule out a GGG, CGGG, or CCGGG at the 5' end of the amplification primers.

Despite our positive experiences with blunt-end cloning, it should be mentioned that another very efficient and easy method for PCR-product cloning is at hand nowadays. The so called "TA cloning method" takes advantage of the terminal transferase activity of *Taq* polymerase. The enzyme adds a single, 3'-A overhang to each end of the PCR product. This makes it possible to clone this PCR product directly into a linearized cloning vector with single, 3'-T overhangs. Note that DNA polymerases with proofreading activity, such as *Pfu* polymerase, cannot be used because they provide blunt-ended PCR products. TA cloning kits are available from different manufacturers.

To maximize your cloning rate, it is preferable to have a significant amount of PCR product. If not, a possibility is to try to reamplify the amplicon (refer to **Subheading 3.2.**).

The PCR should be optimized to avoid the amplification of unspecific bands. If this is the case, extract the band of interest from the agarose gel and reamplify it using the method described above.

3.3.1. T4 DNA Polymerase Reaction (Fill-In)

This reaction is necessary to fill-in eventual gaps left at the 3' end of the amplicon by *Taq* polymerase. Mix the following reagents in a 1.5-mL tube: 10 µL of PCR products (from the first amplification); 2 µL of 10X T4 DNA polymerase buffer (New England Biolabs); 1 µL of T4 DNA polymerase (3 units/µL) (New England Biolabs); and 7 µL of dd water. Leave the tube for 20 min at 12°C and then move it to 75°C for 10 min.

It is also possible to T4-treat the PCR band excised from an agarose gel (if the amount is enough):

Bring the purified band up in 10 µL of dd water (purification by, e.g., freeze–squeeze) and add 2 µL of 10X T4 DNA polymerase buffer (New England Biolabs); 1 µL of T4 DNA polymerase (3 units/µl) (New England Biolabs); 5 µL of dd water; and 2 µL of dNTPs (1 m*M* each). Leave the tube for 20 min at 12°C and then move it to 75°C for 10 min.

(Note: When using the PCR product directly from the PCR tube, nucleotides are in excess and are sufficient for the T4 treatment. Using the excised band, the addition of nucleotides is required.)

After the 75°C incubation, a precipitation step follows:

1. Bring the final volume to 100 µL, then add 10 µL of 3 *M* Na-acetate, pH 5.2; 2 µL glycogen (1 mg/mL); and 300 µL of ice-cold ethanol 100%.
2. Spin at 4°C at maximum speed for 40 min.
3. Wash pellet with 500 µL of 70% ethanol.

4. Discard the ethanol being careful to not dislodge the pellet and dry in a speed vac for 3 min.
5. Resuspend in 5 µL of dd water.

3.3.2. Ligation Reaction

The T4-treated, resuspended amplicons are then added to 1 µL of the pUC vector, 25 ng/µL, *Sma*I cut, not dephosphorilated; 1 µL of *Sma*I-specific buffer; 1 µL of *Sma*I, 10 units (New England Biolabs); 1 µL T4 DNA ligase (400 U/µL, New England Biolabs); and 1 µL of ATP (500 µ*M*). Leave at RT (16–25°C) overnight (approx 16 h). After incubation, precipitate and bring up in 20 µL dd water.

3.3.3. Transformation

After ligation and precipitation, competent cells require to be transformed with the ligated plasmid. We routinely used 40 µL of a suspension of readily competent *E. coli* SURE™ cells and transformed by electroporation:

1. To the 40 µL of *E. coli* suspension, add 10 µL of the ligation and transfer to a cooled cuvette for electroporation.
2. Electroporate at 1.6 to 1.8 kV, 200 Ohm, and 25 µFd.
3. Transfer the suspension to 1 mL of regular SOC medium for 20-25 minutes (at maximum to avoid sister clones). Gently shake at 37°C.
4. Plate 20–200 µL of the cell suspension on a selective medium afterward. In our experiments we used standard LB-agar plates with IPTG/X-gal ***(26)***. Incubate plates overnight at 37°C.

Note: Our regular transformation efficiency was around 4×10^8 transformants/µg circular plasmid; generally, we plated 50 µL of 1000 µL. The used plasmid contains the *E. coli* gene LacZ. The LacZ gene codes for the production of an enzyme called β-galactosidase. β-Galactosidase converts substrates such as X-Gal into a product of blue color. In order for the gene to be actively transcribed from the DNA and for the enzyme to be produced, the activator called IPTG is added.

Within the LacZ gene, there are multiple cloning sites (including the used *Sma*I site) where the plasmid may be cut and DNA may be added. Because of its location within the enzyme-coding sequence, the foreign DNA disrupts activity and function of the enzyme. The disrupted enzyme activity is observed as a white bacterial colony (if the enzyme is fully functioning meaning no insertion has taken place, each colony is of bright blue color). Very small inserts of foreign DNA may lead to light blue colonies.

3.3.4. Colony PCR Screening

The blue–white selection is a preliminary screening method to maximize the retrieval of cloned PCR products. The white colonies then have to be additionally screened by colony PCR to avoid sequencing of false-positive clones. Moreover, colony PCR generates a sufficient amount of the desired PCR product for sequencing. This protocol follows the one described ***(27)***.

1. Per PCR add:

dd water	9.4 µL
BSA, 10 µg/µL	1.25 µL
10X *Taq* buffer	1.25 µL
Plasmid universal primers, 10 µ*M*	0.25 µL
	0.25 µL
dNTPs, 25 m*M* each	0.05 µL
Taq polymerase, 5 µ/µL	0.04 µL

2. Distribute the PCR master mix into reaction tubes.
3. Touch the selected colonies with a micropipet tip and pipet then, one by one, into the PCR tubes. To create a back-up plate, touch first an LB plate and then pipette in the PCR tube. PCR cycling should be performed as follows: an initial denaturation step of 90°C for 5 min then 30 cycles; 95°C for 30 s; (X°)C for 1 min (annealing temperature must be adjusted according to the used primers); 72°C for 1 min; and a final hold of 4°C until tubes are recovered from the PCR machine.
4. PCR products are loaded on a 2% agarose gel (3 µL of the 12.5 µL reaction volume). The colonies containing the insert should show a length equal to 144 base pair (bp) plus the insert length (if the pUC18 vector is used). Colonies of interest can be selected for the subsequent sequencing step.

3.4. Sequencing

Sequencing strategies are quickly evolving from four-lane-radioisotopes-X-ray films procedures to single-lane multifluorescent multichannel processing. Each laboratory has its own sequencing technology with optimized ad hoc protocols. We used 1.5 µL of the colony PCR to perform the sequencing reaction, without any prior purification step. In the case of a single lane multichannel detection system, a prior purification step removing unincorporated nucleotides and primers is necessary for subsequent sequencing steps. Critical information can be recovered by individual suppliers.

We also note that direct sequencing of the PCR product, avoiding a cloning step, is sometimes used in aDNA analysis. In principle, when dealing with animal remains, the probability of a contamination is very low so a direct sequencing approach can be a possibility. Additionally, polymerase-induced errors should be easily identified as overlapping peaks in the electrophero-

grams. Sequencing of different amplification products should permit discrimination between those and endogenous variable positions.

3.5. Phylogenetic Analysis

When an ancient sequence is obtained, the phylogenetic plausibility of the finding should be checked. The crucial importance of this step is easily stressed by retelling the now classical example of the so-called dinosaur bone analysis *(8,9)*. The clamorous announcement was quickly called in doubt by the mere fact that the relevant sequence was clearly too closer to humans than to birds or reptiles as one would have expected. The whole case is a formidable caveat about the absolute need of result authentication. Consulting a database with contemporary species is part of the protocol when the consistency of the final consensus sequence to the original expectations is needed. At this stage, all apparent mutations should be retrieved in evolutionarily neighboring species. A large excess of unseen variants should be regarded as suspicious.

There is plenty of phylogenetic software to use for reconstructing relationships between "ancient" sequences and the rest of known sequences—some are available free of charge. Softaware programs differ one another with respect to the tree reconstructing method. PAUP (http://paup.csit.fsu.edu/index.html) (http://evolution.genetics.washington.edu/phylip/getme.html), Phylip, and tree-puzzle (http://www.tree-puzzle.del) are among the most used (see the attached web addresses for download information; ref. *28,29*).

4. Notes

aDNA is an extremely difficult field. Not only might it be painstaking to successfully establish a laboratory in the field, but ensuring authenticity of the results is every time challenging. Since the beginning, aDNA analysis has lived on a flimsy equilibrium between the "hall of fame" of sensational results (with the invariable footage of the daily press) and forceful retractions. In the past, sequences of extinct organisms (including lithified samples and amber entombed insects), millions of years old, have been described *(10,30)*, and they have triggered the hunt to older and even more spectacular preys. Some of these publications are clearly unlikely, but they were never retracted and the results are occasionally found in citation lists and textbooks *(9,11,31)*. Some of these results (the claims of DNA retrieval out of amber-entombed insects or bones of a dinosaur) have invariably ended up to inspire the media and the movie industry.

Another example for illusions inspired by aDNA reports are the claims of cloning a mammoth, which cannot work neither technically nor theoretically but of course raised dreams and hope by a broad spectrum of people *(32,33)*. Today we know that there is a theoretical upper limit for DNA preservation *(34)*, even if our methods are more sophisticated than earlier.

The recent dispute over DNA sequences obtained from 24,000-yr-old modern human (Cro-Magnon) has also suggested that the problem of authenticity is always open even if a research group acts in full conformity with the proposed criteria of authentication ***(4,5,35–37)***.

How can one come to terms with the whole issue is hard to say. A newcomer in the field has better start working with animal samples before talking humans. The example of aDNA research on mammoth shows that there are many very well preserved specimen available, even proven to contain nuclear DNA ***(38)***. These samples are less prone to contamination than human samples. The following rules should be adopted anyway.

1. In general it is decisive to control for possible contaminations throughout all steps of aDNA analysis, from extraction to PCR amplification. Several controls have to be added at each step. A blank control for the extraction has to be performed each time using the same extraction materials without adding samples. This control has to pass over all the extraction steps and must be included in subsequent amplification reactions. Additionally, two PCR controls, with no DNA added but containing the same reagents, have to be included to monitor eventual contaminants present in the reagents or occurring during amplification set-up. Similarly, if a reamplification is performed, a reamplification PCR control must be included.
2. Another laboratory's assistance should be always sought in the authentication process, if only to ensure that the whole sequence production process has been started afresh, with no contamination risks from the same source.
3. The samples we deal with are precious and unique and should therefore be treated with respect. There should always be a reason to believe that DNA might have survived in the material. Whether the often proposed determination of amino acid racemization by high-performance liquid chromatography is the method of choice is not clear ***(39)***. The advantage of this method is that only minute amounts of the sample is needed; however, it is not proven that the racemization of certain amino acids directly permits predictions about DNA survival. Other hints to amplifiable DNA in a specimen could be the surrounding conditions at the place of excavation. A very good hint would also be successful DNA retrieval from other samples of the same layer at the excavation site. It might therefore be an option trying to extract and analyze DNA from less valuable bone fragments from the same site.

   Also because of its cellular abundance, mitochondrial (mt)DNA has been the target of choice in most ancient DNA analyses. Despite this, mtDNA bears the problem that there are translocated pieces in the nuclear genome of many species that might be mistaken for authentic organellar mtDNA sequences ***(40)***. In order to avoid this, it was suggested to take explicit measures to authenticate mtDNA sequences in newly studied taxa or when any suspicious sequence is retrieved.

aDNA and molecular archaeology/evolution are fascinating fields that recruits enthusiastic young scientists, and we have no doubts about the fact that most problems affecting the field will be solved in the future. In the meantime, no chance to verify and critically question results should be missed.

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