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The Extraction and Analysis of Ancient DNA From Bone and Teeth: a Survey of Current Methodologies

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The last decade has seen a deluge in publications reporting the extraction of DNA from ancient sources. It is beyond the remit of this paper to discuss the extensive accomplishments in this field although, suffice to say, a current literature search unearths hundreds of papers. Methodology from this area of study has not only affected archaeology, but has impacted upon population genetics, conservation biology, forensic medicine, pre-natal diagnosis and medicine. In this paper, we discuss the extraction and analysis of ancient DNA from bone and teeth samples. We cover basic 'cleanroom' procedures and the precautions required to extract authentic ancient DNA. We also review recent extraction procedures, appropriate commercial products and provide techniques and advice to improve polymerase chain reaction (PCR) efficiency. Where possible, we provide internet contacts for the suppliers of consumables and equipment. In addition, we describe a cheap and efficient direct sequencing method that we have found extremely useful for examination of ancient DNA PCR products.

Keywords: Ancient DNA, Archaeology, Contamination, DNA extraction, DNA sequencing, Polymerase chain reaction

INTRODUCTION

Studies of ancient DNA (aDNA) have always attracted a great deal of interest, both from scientists and the general public. Unfortunately, during the early to mid-1990s, a number of sensational and subsequently discredited reports describing the retrieval of DNA from fossilised plants, amber-entombed insects and dinosaur bones cast a shadow over the credibility of the field (Golenberg *et al.*, 1990; DeSalle *et al.*, 1992; Cano *et al.*, 1993; Woodward *et al.*, 1994). As a consequence of these debacles, scientists have concentrated their efforts on more realistic goals and in recent years they have been rewarded with truly exciting results such as the extraction and analysis of mitochondrial DNA (mtDNA) from the original Neandertal type specimen (Krings *et al.*, 1997).

Perhaps because of the healthy scepticism surrounding the field, many workers have invested a great deal of energy and time in developing and perfecting more appropriate methods and experimental strategies for the retrieval and analysis of genuine aDNA sequences. The purpose of this article is to take stock of these developments and to assess current methods and techniques for the extraction, purification and analysis of aDNA from archaeological material and museum specimens.

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The favoured methods for aDNA extraction are the phenol/chloroform method (Hagelberg and Clegg, 1991) and the silica method (Boom *et al.*, 1990; Höss and Pääbo, 1993). Subsequent experimenters have combined these two methods (Richards *et al.*, 1995). Recent comparisons made by Yang and co-workers (1998) have suggested that, in many cases the phenol/chloroform stage of the extraction process is unnecessary.

The minuscule amounts of aDNA recovered using these extraction methods is usually amplified using the polymerase chain reaction (PCR) and the resulting products are then directly sequenced. Most ancient DNA studies concentrate on short mtDNA sequences. Through a fortuitous set of circumstances, the mtDNA molecule is ideally suited for archaeological genetic studies. The mitochondrion organelle and its associated small circular genome (approximately 16 kb in mammals) is present at a very high copy number in most cells (usually 1000–10,000 copies). This implies that the survival of mtDNA fragments during archaeological diagenesis is much more likely than that of single-copy nuclear DNA. Another important factor which favours mtDNA for aDNA studies is that, due to its rapid rate of evolution, lack of recombination and exclusively maternal mode of inheritance, it has become the target of choice for studies of evolutionary relationships and genetic diversity. GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) therefore contains a huge repository of mtDNA sequences characterised from a wide range of species – particularly those from the most rapidly evolving control region segment.

Criteria for Sample Selection

The limiting factor for any aDNA study is the quality of the starting material. If a sample is selected which contains no amplifiable aDNA, then even the most efficient extraction procedure will not be successful. Careful sample choice is therefore a priority. Another major consideration is whether the sample has been processed on site. Generally speaking, human material which has been handled without appropriate precautions during excavation may not be suitable for aDNA extraction.

Although skeletal material is more likely to be devoid of amplifiable DNA (Hagelberg and Clegg, 1991), those bones that do contain endogenous DNA will tend to be more suitable for the amplification of larger products than samples of ancient soft tissues (e.g. Richards *et al.*, 1995). A plausible explanation for this observation may be that the durable exterior surface of bone can provide protection and a sterile barrier against bacterial and enzymatic decay. In addition, DNA binds to an inorganic component of bone called calcium hydroxyapatite, and it has been suggested that this adsorption results in a two-fold decrease in depurination therefore making the DNA more stable (Lindahl, 1993).

Compact (cortical) bone extracts are preferred over spongy (cancellous) bone, as this dense bone is more likely to retain structural integrity with minimal bacterial and fungal contamination. It has also been suggested that teeth should be used where possible as they are normally the most well preserved skeletal remains (Ginther *et al.*, 1992; Merriwether *et al.*, 1994). The chance of contamination *in situ*, or during excavation/curation, is much lower with teeth compared to bone as extraction samples can be taken from the very centre of the tooth if the appropriate equipment is used.

Conditions Considered Favourable for the Preservation of Ancient DNA

Reliable large-scale sampling of genetic information from skeletal material is highly dependent upon a better understanding of the depositional conditions that are conducive to DNA preservation. The degree of DNA preservation is not always closely linked to the gross morphological/macroscopic condition of the sample. This is

especially true of sites that are, or once were, waterlogged. Wet sites, such as peat bogs, often yield material with good general preservation, but the likelihood of retrieval of DNA is dependent on the pH (neutral pH is desirable: Thomas and Pääbo, 1993). However, the general consensus is that cold and dry depositional conditions are best (e.g. Hauswirth *et al.*, 1994). Thorough dehydration will provide greater protection for DNA against water-requiring autolytic cellular enzymes. Temperature is also a crucial factor; in our experience, although samples may have been excavated in an extremely arid environment, unless the ambient temperature is relatively low, DNA is unlikely to survive. We have recently completed a large-scale survey of cattle archaeological material from Anatolia, the Middle East and northern Europe. Very few of the samples from the first two locations gave any mtDNA amplification whatsoever and if they did, it tended to be sporadic and difficult to reproduce. Contrary to this, many samples from various archaeological contexts in northern Europe contained sufficient quantities of aDNA for reproducible PCR amplification and direct sequence determination (MacHugh *et al.*, 1999; MacHugh, unpublished data).

Although bone should provide some protection against water introgression, over long periods of time the porous nature of bone (even when fossilised) encourages the degradation and removal of DNA through water seepage. For example, Anglo-Saxon and Romano-British specimens, subject to a fluctuating water table, have been found to render little or no amplifiable DNA, and any results that are obtained are almost impossible to reproduce (Richards *et al.*, 1995). The bones sampled from the Tudor warship, the *Mary Rose*, which was sunk in the English Channel in 1545 (Richards *et al.*, 1995), were preserved in constant anoxic conditions under silt so aiding DNA retrieval. This suggests that environmental stability, rather than simply presence or absence of water, is the most important factor in DNA survival. A recent comparison of the effect of environmental factors upon DNA preservation identified that cool cave conditions, even with the presence of water, gave the highest yield of DNA (Burger *et al.*, 1999). Based on their comparative studies, the same authors have compiled a list of factors which they consider most important for the survival of archaeological DNA (Table I).

The recovery of aDNA from bone is not exclusively dependent on the age of the sample, but rather it is thought that survival is related to the preservation state which can be affected by ethnological practices influencing burial conditions (Hagelberg *et al.*, 1991; Colson *et al.*, 1997). Successful extraction is dependent on the treatment of the body and its eventual resting place once dead. Bones from the same excavation site can give different results and different degrees of DNA preservation can even be observed in replicate samples of the same bone (Hagelberg and Clegg, 1991).

Methods for Assessing Sample Preservation

Without objective criteria with which to assess DNA survivability, it is quite likely that the investigator will spend long hours working with material that may never yield reproducible aDNA. A good starting point is to choose bones with excellent macroscopic preservation. Previous studies have found a good correlation between relatively subjective measures of bone preservation and the presence of amplifiable endogenous DNA (Richards *et al.*, 1995). Histological preservation and estimation of the survival of the organic component of the bone were also found to be good indicators of DNA durability (Colson *et al.*, 1997).

The most scientifically rigorous method for assessing whether ancient tissue samples contain endogenous DNA is considered to be the procedure described by Poinar and colleagues (Poinar *et al.*, 1996). This method involves the use of high performance liquid chromatography (HPLC) to estimate the extent of racemization of various

TABLE I Favourable conditions leading to aDNA preservation in archaeological material

Factor	Comments
Absence of microorganisms	Microorganisms and their metabolites can destroy DNA completely.
Absence of UV radiation and radioisotopes	UV irradiation only affects the surface of a sample.
Aridity	Under dry conditions, hydrolytic and oxidative damage is reduced.
DNA adsorption to mineral surfaces such as hydroxyapatite	Mineral surfaces stabilise DNA molecules.
Rapid inhumation after death	The formation of gas and the breakdown of soft-tissue structures accelerates infestation by microorganisms.
Hard and dry tissue samples	Hard and dry tissues prevent physical and chemical reactions. Bones and teeth protect organic residues against chemical reactions and microbial infestation.
Low temperature	Low temperatures retard most chemical reactions and inhibit the growth of microorganisms.
Neutral or slightly alkaline pH value	As environmental pH decreases, both DNA and its surrounding material (bone or teeth) will be degraded or destroyed.
Presence of chelating humic and fulvic acids	Phenolic geopolymers prevent aerobic activity.
Storage at low temperature	Samples should be stored at least as cold as the ambient temperature where they were excavated. For longer term storage, the use of a −20 °C freezer is highly recommended.

Reproduced with modifications from Burger *et al.* (1999).

amino acids (aspartic acid, alanine and leucine). In other words, the equilibration between the D-enantiomer of these amino acids and the biological L-enantiomer, which takes place once metabolism ceases, is used as a surrogate indicator of DNA degradation. Poinar and co-workers found that for aspartic acid a D:L ratio higher than 0.08 from a particular sample was generally incompatible with the survival of amplifiable mtDNA. Although this method can provide a very useful indicator of DNA survival, its use in the wider archaeogenetics community has been limited by a lack of both expertise and the appropriate equipment.

Precautions for Avoiding Contamination

Although contamination will always be the bane of ancient DNA studies, with due consideration, care and an appreciation of the potential sources of contamination, it should be possible to minimise the occurrence of contamination episodes in the laboratory. The schematic shown in Fig. 1 outlines the likely sources of contamination for a standard extraction and purification method used to obtain aDNA from archaeological material.

In many respects, those studies which focus on human archaeological material are the most challenging, and will inevitably produce the most contamination-related artefacts as compared to non-human samples. The most likely source of modern exogenous DNA entering a bone or tooth sample will be from the archaeologists and other workers who handle the material during excavation and subsequent morphological analysis. This point is illustrated by standard archaeological procedure for bone cleaning. A widely used method is to wash excavated bones by hand in warm water – an efficient route to wholesale contamination of any sample (Brothwell, 1981; Bass, 1987). It is therefore recommended that all specimens (in particular human samples) intended for aDNA work are always handled using disposable gloves.

It is necessary to protect the samples from contamination at every stage of the aDNA extraction procedure. Ideally, even when using gloves, samples should be taken directly from the excavation site with the minimum amount of handling. When working on human samples, or samples of great antiquity, protective clothing should be worn when sampling, extracting and setting up PCR experiments. This clothing can include the following disposable items: coverall with hoods, shoe covers, and facemasks (Merck Ltd.: www.merck-ltd.co.uk). Two pairs of sterile gloves can be worn together, with the sleeves of the overall taped to the inner pair of gloves to prevent exposure of the wrist skin.

The preparation of most samples begins with cleaning and powdering. Ideally, these should take place in a separate room where PCRs are never carried out. The external layer of the sample should be cleaned vigorously with an industrial grit- or sand-blaster (Guyson Corporation: www.guyson.com), fine sandpaper or a scalpel blade. However, abrasive techniques to remove surface contamination may not remove all extraneous material and are only useful for hard material of a reasonable size. If possible, sample cleaning should include UV irradiation of the external surface of the tooth or bone using a standard UV crosslinker (Ultra-Violet Products Inc.: www.uvp.com; Stratagene: www.stratagene.com). Samples are sometimes soaked in bleach for a short period of time; teeth are particularly amenable to this treatment due to the hard protective enamel coat. For obvious reasons, soft porous material should not be soaked in bleach solutions.

Under ideal circumstances, extractions should be performed in a positive pressure room and PCRs set up in a class II containment hood (ICN Flow) within this cleanroom. Failing this, the room for extraction should at least be physically separated from downstream work areas. If cloning or second-round PCR is required, this should be set up in a lab where no modern work studying the same genomic regions has been conducted. Sequencing, however, can be performed in the lab alongside modern work, since contamination is no longer a problem at this stage. Any containment hoods for PCR set-up or sample preparation should be equipped with shortwave (254 nm) UV sources for surface DNA decontamination. These UV sources should be left switched on when the containment hoods are not being used. *CAUTION: shortwave UV is mutagenic and can damage eyesight very rapidly. Always use containment hoods with UV-protective shielding and personal UV face-covers.*

All general equipment must be dedicated for pre-PCR work and apparatus from a post-PCR laboratory should never be taken into the clean-

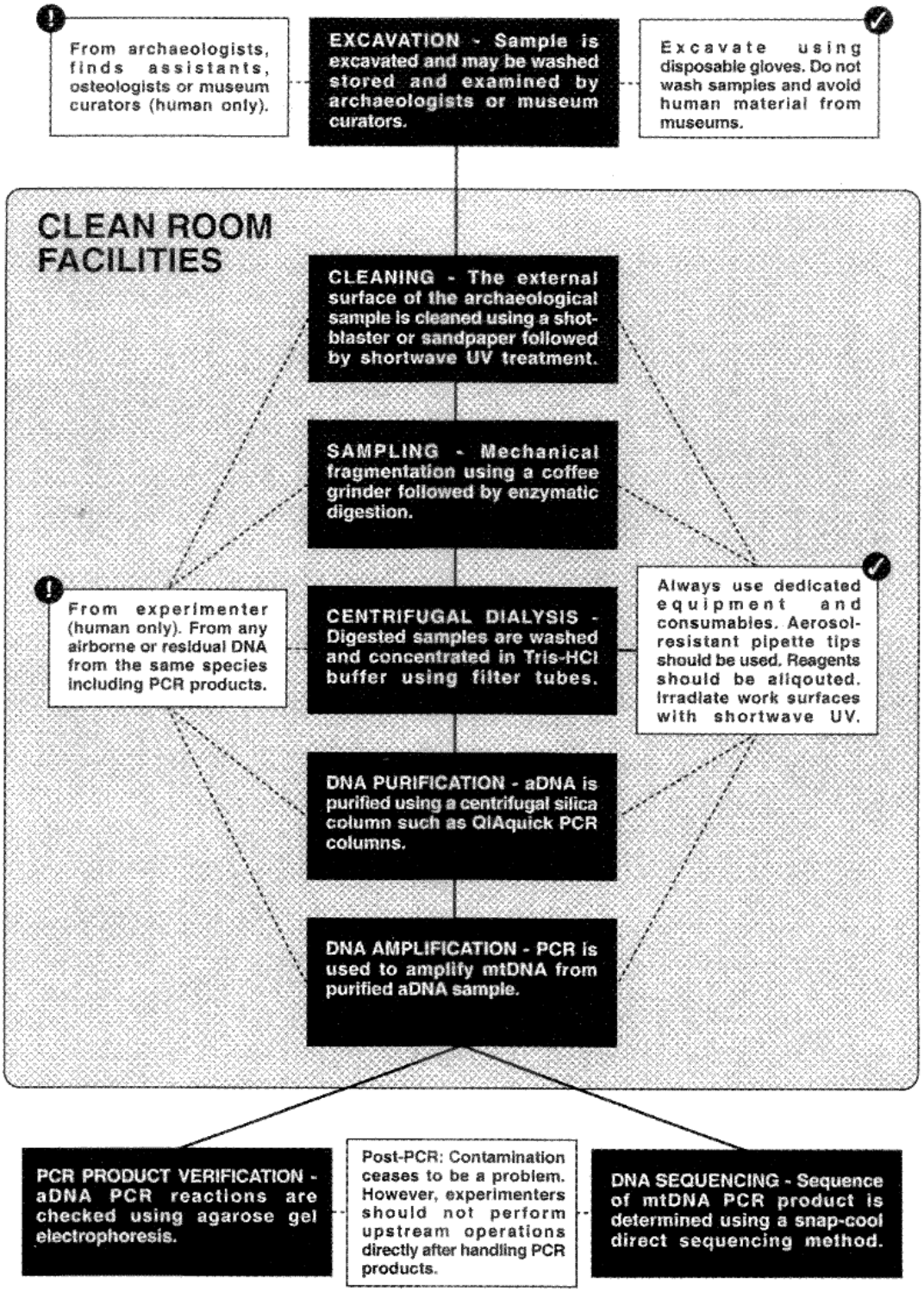

FIGURE 1 Schematic showing a typical aDNA workflow and potential sources of contamination

room facility. Access to the cleanroom should be controlled. If the work involves the retrieval of ancient human DNA, then the DNA sequences for the various genetic loci examined by a group should also be known for all cleanroom workers. In order to prevent contamination by aerosol particles of the aDNA extractions and PCR set-ups, a one-way working procedure should be maintained. If the experimenter has worked on post-PCR stages, such as running an agarose gel or sequencing, then he/she should not set up a PCR reaction subsequently on the same day. Useful reviews of general PCR laboratory set-up and UV decontamination procedures are given by Dieffenbach *et al.* (1995a) and Cone and Fairfax (1995).

Reproducibility and Duplicate Extraction of Ancient DNA

Due to the considerable problems with extraneous contamination involving modern DNA, caution must be exercised over aDNA results and most workers in the field have placed a heavy emphasis on the need for stringent precautions (e.g. Sykes, 1991). This is especially important where the aDNA study involves humans or related species, or in the case of non-hominoid material, if the scientists involved are also working on modern samples from the same taxa. It is recommended that at least two extractions from each sample should always be undertaken. These should yield identical results and any discrepancy should be noted on publication. If at all possible, and especially with very important or contentious work, inter-lab duplication should be attempted, as carried out, for example, with the Ice Man and Neanderthal specimens (Handt *et al.*, 1994b; Krings *et al.*, 1997). Reproducibility between labs has been demonstrated on numerous occasions; recent cases being analyses of *Mammuthus primigenius* - the woolly mammoth (Hagelberg *et al.*, 1994; Höss *et al.*, 1994), and the extinct ground sloth – *Mylodon darwinii* (Höss *et al.*, 1996; Taylor, 1996). However, this is usually time-consuming, impractical and difficult to arrange for less high profile studies. Therefore, in our opinion, as long as duplicate samples are extracted and analysed, this type of reproducibility should not be insisted upon. This is particularly relevant if the sample material is of limited quantity, unique or extremely valuable (Handt *et al.*, 1994a).

EXTRACTION METHODS

A range of aDNA extraction methods have been developed specifically for bone and tooth material. These procedures are required to separate and purify the DNA from proteins and other organic components of bone or teeth which may inhibit DNA amplification via PCR. A selection of these methods are discussed here including the original silica-based method (Höss and Pääbo, 1993; adapted from Boom *et al.*, 1990), the phenol-chloroform/silica column method (Richards *et al.*, 1995), the *'Geneclean For Ancient DNA'* method, the collagenase/dispase/lysozyme method of Pusch and Scholz (1997), and the simplified silica-column DNA extraction method recommended in a recent assessment of various procedures (Yang *et al.*, 1998).

Amplification of aDNA samples using *Taq* polymerase can be inhibited by many naturally-occurring compounds found in soil and groundwater (Höss and Pääbo, 1993). Atoms and molecules such as iron, cobalt and fulvic acids, which can become incorporated into archaeological material, will severely affect the performance of most DNA polymerases. The removal of these PCR inhibitors is the most important function of the DNA extraction and purification step in aDNA work. Amplifiable aDNA is usually fragmented into segments between 100–1000 bp which means that kits originally intended for the clean-up of PCR products (which are usually in this size range) can provide a rapid, highly efficient purification step. Most recent methods incorporate one of these column-based kits and it is important to be aware of the range of such products available (Table II).

TABLE II A selection of commercial kits suitable for the purification of aDNA extracts

Product name	Company website	Principle of action	Comments
Qiagen Qiaquick PCR purification kit	www.qiagen.com	Silica matrix	Widely used in aDNA extraction procedures
Hybaid Recovery DNA purification kit II.	www.hybaid.com	Silica matrix	Fast but does not isolate fragments under 200 bp
Roche High Pure PCR product purification kit.	biotech.roche.com	Glass wool matrix	Designed to isolate PCR products larger than 100 bp
Sigma GenElute PCR DNA purification Kit	www.sigma-aldrich.com	Silica matrix	90–95% recovery of DNA fragments 300 bp to 10 kb
Camgen Selex PCR purification kit	www.cmtech.co.uk	Size exclusion resin	May not provide adequate purification for aDNA
Mo Bio Labs Inc. UltraClean PCR clean-up kit	www.mobio.com	Silica filter matrix	Over 100 bp fragment size
Stratagene StrataPrep PCR purification kits	www.stratagene.com	Silica-based fibre matrix	Very clean DNA ($O.D._{260/280}$ ratios between 1.8–2.0)
Promega Wizard PCR Preps DNA purification system	www.promega.com	Resin-based	Suitable for aDNA extraction but cumbersome
Life Technologies (Gibco-BRL) Concert rapid PCR purification system	www.lifetech.com	Silica membrane	Not suitable for use in aDNA protocols because column has no lid

The Original Silica-Based Method (Höss and Pääbo, 1993)

The efficiency of PCR amplification can be greatly increased if a chaotropic salt bridge method is used to remove potential inhibitors that co-extract with aDNA. This method is a 'simple, rapid and reliable protocol for small-scale purification' (Boom *et al.*, 1990) of many forms of DNA. It exhibits reasonably high extraction efficiency and does not require any specialised equipment.

In brief, the cleaned bone sample is ground to a fine powder under liquid nitrogen in a freezer mill (Spex Industries, ISA: www.instrumentssa.com). Approximately 0.5 g of this powder is added to 1000 µl of extraction buffer consisting of 5 M guanidium thiocyanate, 0.1 M Tris-HCl pH 7.4, 0.02 M EDTA and 1.3% Triton X-100 (Sigma: www.sigma-aldrich.com). This mixture is then incubated overnight at room temperature. Bone powder is then pelleted by centrifugation and the supernatant is added to 40 µl silica suspension (Boom *et al.*, 1990). This is incubated at room temperature for 10 min and after washing is eluted at 56°C in two aliquots of 65 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). *CAUTION: care must be taken with the preparation of any buffers containing guanidium thiocyanate as it produces a toxic gas (hydrogen cyanide) when in contact with acid.*

It is advisable to microcentrifuge the extract before removing aliquots for PCR or further cleaning steps, as even minimal amounts of silica

can inhibit the enzymatic reaction. The DNA can be further purified with an electroelution step (Allaby *et al.*, 1999) that removes Maillard products (carbohydrate/protein complexes) and humic and fulvic acids, followed by ethanol precipitation.

The silica extraction method was developed specifically to remove unknown inhibitors that co-extract with the aDNA. However, this method can be problematic as any traces of silica remaining in the buffers can cause inhibition of downstream enzymatic reactions including PCR. Also, due to the extremely efficient DNA-binding properties of silica, the buffers are prone to contamination, making it very time-consuming to ensure contamination artefacts are minimised. It is also thought that the original protocol can shear DNA and leads to a low recovery of highly fragmented aDNA.

The Phenol-Chloroform/Silica Column Method (Richards *et al.*, 1995)

With this method, the exterior of the bone or tooth is first cleaned by sand- or grit-blasting, then mechanically digested using a coffee grinder and chemically digested with Proteinase K. A number of phenol/chloroform extraction steps are performed to remove cellular debris and residual protein. This is then followed by purification using a silica column to remove any impurities that may inhibit subsequent PCR reactions.

Between one and five grams of powder are incubated in 0.5M EDTA pH 8.0 on a rotary shaker at room temperature for at least 24 h to decalcify the material. Next, 0.5% Sarkosyl (BDH: www.bdh.com) and 100 $\mu g\ ml^{-1}$ Proteinase K (Roche Molecular Biochemicals: biochem.roche.com) are added and incubation continued at 37°C. The DNA is then extracted using standard phenol/chloroform procedures (Sambrook *et al.*, 1989). The samples are diluted four-fold in sterile water and the excess EDTA is removed by centrifugal dialysis with Centricon-30 microconcentrators (Amicon: www.millipore.com). The samples are then purified further using guanidium thiocyanate/silica-gel columns (Wizard PCR Preps DNA Purification System, Promega: www.promega.com). This method has been modified for use with smaller amounts of archaeological material (100–300 mg), and addition of bacteriophage lambda DNA (1.35 $\mu g\ ml^{-1}$ of the initial extraction volume) has also been found to improve extraction efficiency (Bailey *et al.*, 1996; Colson *et al.*, 1997).

This protocol has been particularly useful for samples such as bones from the *Mary Rose* that have absorbed a number of potential inhibitors (Richards *et al.*, 1995). The use of a silica column avoids the carry-over problems associated with the original silica method, as described above. However, the original Promega syringe-based columns are awkward to use and more prone to contamination when compared to newer centrifugal-based products. We have found that guanidium thiocyanate glass fibre columns, such as the High Pure PCR product purification columns (Roche Molecular Biochemicals: biochem.roche.com), perform extremely well in this protocol and are much easier to use (MacHugh *et al.*, 1999).

The 'Geneclean For Ancient DNA' Method

'Geneclean For Ancient DNA' is a commercially available kit designed specifically for isolation of DNA from non-viable tissues or samples that have historical or forensic value (BIO101 Inc.: www.bio101.com). The reagents are carefully formulated to prevent contamination with extraneous DNA.

Briefly, the protocol includes the following steps. After incubation of the homogenised sample in 'DNA DeHybernation' solution, the sample is centrifuged to remove debris. 'Ancient DNA Glassmilk' is added to the supernatant in a spin column, and the DNA-matrix complex is washed with three separate wash solutions. Finally, the DNA is eluted in a DNA-free solu-

tion. It is claimed that the resulting sample is readily amplifiable by PCR.

Two different DeHybernation solutions are supplied with the kit. DeHyb A is a guanidinium-based solution, and DeHyb B an aqueous EDTA-based solution. Unfortunately, with the large diversity of samples and preservation conditions, the manufacturers admit that it is not possible to determine which DeHybernation solution will be most efficacious with particular samples and they suggest preliminary work to determine the most suitable buffer. Unfortunately, this is not always possible with samples of limited quantity.

DNA Extraction via Multi-enzymatic Treatment (Pusch and Scholz, 1997)

This protocol originally appeared on the *Elsevier Trends Journals Technical Tips Online* website (tto.biomednet.com). Pusch and colleagues describe an interesting method which uses a combination of collagenase, dispase and glycosidase lysozyme (Roche Molecular Biochemicals: biochem.roche.com). They state that their procedure ensures that high quality DNA can be isolated from small quantities of bone powder. The use of collagenase assumes more importance in the light of a follow-up paper by the same group which indicated that collagen type I is a major inhibitor of DNA amplification in human archaeological samples (Scholz *et al.*, 1998). The method is described in full on the *Technical Tips Online* website; what follows is a brief summary.

A very small quantity of bone powder (50 mg) is incubated with collagenase and dispase in a microcentrifuge tube for 1.5 h at 37°C on a benchtop shaker. The enzymatic reactions are stopped by adding EDTA. A sucrose-containing detergent buffer is added and the mixture is incubated overnight at room temperature. A rapid freeze-thaw step is then used to fragment any remaining bone particles. The sample is then digested with lysozyme (this digestion serves as an indicator of bacterial contamination – see the full protocol). After a boiling and centrifugation step, the supernatant is transferred to another tube and the DNA is precipitated with isopropanol and glycogen. After standard washing steps the precipitate is resuspended in TE buffer.

We have not assessed this method in our laboratory. However if, as the authors seem to have found, collagen type I is a major inhibitor of DNA amplification then it may prove to be a very useful protocol. The authors also suggest that a glass-wool or silica column can be used to ensure the dissolved DNA is completely pure.

The Rapid QIAquick Method (Yang *et al.*, 1998)

Recently, Yang and colleagues (Yang *et al.*, 1998) compared four different methods of extracting DNA from Proteinase K-digested bone solutions. They contrasted results obtained by extraction with phenol-chloroform followed by Centricon microconcentration (Amicon: www.millipore.com), with and without an additional QIAquick PCR purification step (QIAGEN: www.qiagen.com), with those obtained directly from the digested bone solutions followed either by concentration and purification or by purification alone. The protocol using QIAquick purification on its own is recommended by Yang *et al.* because the DNA is concentrated in a single step and separated from contaminating substances that can inhibit the PCR. However, when the starting volume of the bone digest is large, it is suggested that the volume be decreased using an additional Centricon microconcentration step prior to purification.

The recommended protocol is as follows. Samples of 0.5–5.0 g are taken from bone that has been cleaned, in this case with sandpaper, and drilled. Bone powder is dissolved in 8 ml extraction buffer (0.5 M EDTA pH 8.0, 0.5% sodium dodecyl sulphate, 100 μg ml^{-1} Proteinase K) and incubated in a shaking water-bath at 55°C overnight, followed by 24 h at 37°C. The extraction solution is centrifuged at 2000 × *g* for 5 min, and 1.75 ml aliquots of the supernatant are transferred to

2.0 ml centrifuge tubes and spun in a microcentrifuge (12,800 × *g*) for a further 5 min. The supernatant is then transferred to a 10 ml tube and mixed with 5 vols QIAquick PB buffer. Using a sterile disposable pipette, 750 µl is loaded directly onto a QIAquick column and centrifuged at 12,800 × *g* for 1 min. The flow-through is discarded and the process repeated until all of the extract has passed through the column. The DNA is washed by adding 750 µl QIAquick PE buffer and centrifuging for 1 min. The flow-through is discarded and the DNA eluted from the column by addition of 100 µl TE buffer followed by centrifugation for 1 min. The DNA is then ready to be amplified by PCR.

We have assessed this method extensively and recommend it with some important modifications. Even with moderate digest volumes, it is impractical to add 5 vols PB buffer and then do many repetitive centrifugation steps through the QIAquick column. We therefore reduce the digest volume and remove the EDTA by using 4-ml centrifugal filter tubes with a 30 kDa molecular mass cut-off point (Eppendorf: www.eppendorf.com), which are similar to the Centricon tubes but less prone to contamination, followed by a wash with 10 mM Tris-HCl pH 7.5. This dramatically reduced volume requires much less PB buffer and just two initial centrifugation steps in the QIAquick column are needed.

The QIAquick system is only one of the many commercially available column-based PCR product purification methods. We have successfully used a number of other column-based systems for this protocol. Table II lists other products and includes the website details where more information is available. This technology is under constant development so regular reviews of the methods are recommended.

IMPROVING THE EFFICIENCY OF PCR AMPLIFICATION

PCR is the cornerstone of archaeological genetics. The incredible sensitivity and power of the technique has allowed molecular biologists to analyse the very small amounts of archaeological DNA which have survived for hundreds, thousands or even tens of thousands of years. PCR is a deceptively simple technique – relatively easy to understand in principle but often frustratingly difficult to optimise in practice, particularly with material containing highly degraded target DNA in a complex background of fungal and bacterial DNA. There are numerous introductory texts outlining the basic principles underlying the PCR and we feel it is unnecessary to provide a detailed explanation of the PCR process. What we hope to do instead is to provide a guide to PCR optimisation and to concentrate on practical advice which will assist scientists initiating a programme in aDNA research.

PCR Primer Design and Optimisation

The subject of primer design has been an active area of debate since the inception of PCR during the mid-1980s. There are numerous technical discussions in the scientific literature, on the internet and in commercial documentation. However, the researcher needs to be aware that in some cases, different sources contradict each other in relation to aspects of primer design such as, for example, the correct calculation of primer melting temperature (T_m) and the corresponding annealing temperature (T_a). The primer design guide we present here is a distillation of information from a wide variety of sources. Good starting points if further reading is required are Dieffenbach *et al.* (1995b), Beasley *et al.* (1999) and Innis and Gelfand (1999).

Because primer design can greatly affect the yield of amplified product, it is imperative that anyone interested in amplifying aDNA is aware of the key principles involved in the selection of the most appropriate primer pair to amplify a given DNA segment. The main aim of primer design is to achieve a balance between two goals: the specificity and the efficiency of amplification. The issues are as follows:

1. *Primer oligonucleotide length.* The general consensus concerning primer length is that for conventional PCR (as opposed to long-range PCR) the optimum primer length is between 18–24 nucleotides. Statistically, it is highly unlikely that primers of this length will be present more than once in a typical eukaryotic genome. Although most researchers tend to aim for about 20 nucleotides as the standard length of their PCR primers, recent large-scale statistical analysis of the contribution of primer design to PCR success or failure indicates that the primer length should be between 21–26 nucleotides with an optimum of roughly 23 nucleotides (Beasley *et al.*, 1999).
2. *Primer nucleotide content.* The conventional wisdom regarding the GC content of the primers is that a window of 40–60% is acceptable for most PCR applications. However, based on their statistical analysis, Beasley *et al.* (1999) recommended that, if possible, a threshold of 50% GC content should never be exceeded. In particular, their analysis indicated that the GC content near the 3′ end of the primer should be relatively low. A useful rule-of-thumb is that the five nucleotides at the 3′ end should include no more than two G and/or C nucleotides.
3. *Avoid polynucleotide stretches.* It is important to avoid tracts of monotonous single nucleotide repeats in a primer intended for PCR. The complexity of the primer is reduced and mispriming with non-target DNA is more likely. In addition, poly A/T stretches anneal relatively weakly (because of the nature of the chemical interactions between nucleotides that occur during primer annealing) and primers which contain these may be prone to 'breathing', where not all of the primer forms a permanent attachment to the annealing site.
4. *Minimise the potential for inter- and intra-primer interactions.* A golden rule of primer design is that the 3′ ends of the two primers should not be complementary, because if they are then primer-dimer artefacts will be preferentially synthesised and may out-compete amplification of the target DNA, particularly if the latter is present at low copy number. This also applies to palindromic sequences at the 3′ end of a primer (e.g. -CATG or -CG) as these allow the primer to form a dimer with itself. It is also important to reduce the potential for other molecular interactions within and between primers such as the formation of hairpins and other secondary structures.
5. *Sequence composition at the 3′ end.* This aspect of primer design is has been quite contentious. Many workers have recommended the use of a 'GC clamp' at the 3′ end of each primer. This means that the target site for primer annealing is chosen so that the two nucleotides at the 3′ end of the primer are G and/or C, so that a particularly strong association is formed between this part of the primer (the point from which DNA synthesis is initiated) and the target DNA. Taking into account the issues mentioned in point 4, suitable GC clamps would be -CC or -GG, with the same clamp used with both primers. However, more recent discussions of the subject have suggested that to reduce the likelihood of primer-dimer formation, and hence to increase yield, an AA dinucleotide at the 3′ end of each primer is advantageous (Innis and Gelfand, 1999; Zangenberg *et al.*, 1999). We have obtained high product yields after amplifying mitochondrial DNA from archaeological sources using primers which do not contain a GC clamp. If a GC clamp is included in the primer design then consideration of point 2, above, places further restrictions on the choice of annealing site, due to the need to ensure that the overall GC content in the 3′ region of the primer is not too high.
6. *Check candidate primers against the DNA databases.* Primer sequences might inadvertently be chosen from segments which are homologous to non-unique DNA regions such as

repetitive elements or viral insertion sequences. Primer sequences can be checked rapidly using a web-based BLAST server (www.ncbi.nlm.nih.gov/BLAST/) which searches for matches within the existing sequence databases.

7. *Chose primer-pairs with similar melting temperatures*. Under PCR conditions (usually 50 mM salt concentration), the melting temperature (T_m) of a primer can be estimated using the following equation:

$$T_m = 69.3 + (0.41 \times \%GC) - (650/length)$$

The T_m values for two primers in a pair should not differ by more than 5°C. When setting up the first PCR with a new pair of primers an annealing temperature (T_a) that is 3°C lower than the average T_m of the two primers is used. If no products are formed then the annealing temperature is reduced by 2°C increments in subsequent experiments until a product is seen. If non-specific products are formed then the annealing temperature is raised in 2°C increments.

There are a range of commercial and non-commercial primer-design computer programmes available. There are also web-based online primer-design tools which provide similar features to the stand-alone packages. Most of these programmes take into account the criteria described above. A useful compilation of online primer-design sites can be found at www.alkami.com/primers/refdsgn.htm.

'Hot Start' versus Conventional PCR

Primers that anneal to non-target locations on the template may give rise to non-specific amplified products. In addition, as outlined above, some primers can anneal to themselves or to the second primer of the pair to give another type of non-specific product, called a primer-dimer. These products are normally less than 80 bp in length and frequently occur when the template DNA is at a low concentration or completely absent. Primer-dimers are the result of one primer acting as the template sequence for extension of the second primer. Once this primer-dimer extension product has been synthesised it can be further amplified and can take over as the main product of the reaction, leading to no visible amplification of the genuine template DNA if this was at low concentration.

The cheapest way to alleviate the problem of primer-dimers is to set up all reactions on ice, but a much more efficient method is to use 'hot-start' PCR (D'Aquila *et al.*, 1991; Erlich *et al.*, 1991). Hot start PCR is a simple modification of the original PCR process, whereby the amplification reaction is initiated at an elevated temperature. A number of different methods can be used to achieve this, but essentially the objective is to prevent the DNA polymerase from functioning during set-up and the initial heating to the denaturation temperature. In addition to a dramatic reduction in primer-dimer artefacts, hot-start procedures provide other benefits including reagent assembly at room temperature, increased yield and better specificity.

One approach to achieving hot-start PCR is to use wax beads which create a physical barrier between the enzyme and essential reaction components. This wax barrier melts during denaturation and allows the components to mix (Chou *et al.*, 1992). However, the wax bead approach can be laborious and prone to contamination. It has been largely superseded by automated hot-start procedures which use heat-activated enzyme systems. A popular method for automated hot-start PCR uses a chemically modified form of *Taq* DNA polymerase called AmpliTaq Gold (Birch *et al.*, 1996) which is commercially available from PE Biosystems (www.pebio.com). This enzyme is activated for PCR by heating at 95°C for 10 min.

A related method for achieving automated hot-start PCR is to use an antibody-mediated DNA polymerase. There are a range of these systems available from a number of different manufacturers: for example, the Platinum and AdvanTaq enzymes available from Life Technol-

ogies and Clontech respectively (www.lifetech.com; www.clontech.com). These enzymes are *Taq* DNA polymerases that have been complexed with an antibody which inhibits polymerase activity until denatured by heating at 94–95°C for 1–3 min. In addition to providing a hot-start capability, these enzymes generally exhibit a broader range of magnesium tolerance and require less optimisation than conventional *Taq* polymerase systems.

It is important to realise that the enormous commercial potential of the PCR process has encouraged the discovery and development of many innovative new enzyme systems. There are a wide range of products on the market, many of which show great promise for aDNA studies. Researchers should ensure that they stay abreast of these developments using the internet. A recently published collection of reviews provides a good starting point for those interested in the current thinking concerning PCR enzymes and the overall optimisation of the PCR process (Innis *et al.*, 1999).

Addition of Cosolvents to PCR Reactions

Amplification efficiency (yield of product) and specificity (absence of non-specific products) can be improved by the addition of a cosolvent enhancer to the PCR reaction. These additives work in various ways but most of them enhance separation of DNA strands during the denaturation step of PCR, thereby improving the efficiency and specificity of primer annealing. The cosolvents that are most often recommended by researchers include: 1–10% (v/v) dimethyl sulphoxide (DMSO); 5–20% (v/v) glycerol; 10–100 μg ml^{-1} bovine serum albumin (BSA); 1.25–10% (v/v) formamide; non-ionic detergents such as 0.05% (v/v) Tween 20 and 0.01% (v/v) Triton X-100; and 10–100 μM tetramethyl ammonium chloride (TMAC). Although DMSO and glycerol are probably the most popular, there is evidence that BSA increases the efficiency of a PCR more than either of these, possibly because high concentrations of BSA binds to enzyme inhibitors present in the aDNA preparation (e.g. Hagelberg *et al.*, 1989). Different additives might be useful for particular aDNA samples, but no study has been published to quantify their relative values. In our experience with amplification of mitochondrial sequences from aDNA, additives such as DMSO, formamide and TMAC have not proven particularly valuable and, in some cases, additives have had a detrimental effect on PCR yield. A useful website concerning PCR additives can be found at info.med. yale.edu/genetics/ward/tavi/PCR.html.

PCR Contamination

The ability of PCR to use very few, possibly just one, molecule as the template for an amplification reaction is the main reason why this technique has become so important in aDNA studies. From ten starting molecules in a 100 μl reaction (probably typical for an aDNA PCR), it is theoretically possible to generate 1.0×10^9 PCR product molecules after 30 cycles (Cimino *et al.*, 1990). This ability also results in the well-documented contamination problems associated with aDNA and other PCR applications that use small amounts of starting template, such as pre-natal and clinical diagnosis (Kitchin *et al.*, 1990; Porter-Jordan and Garrett, 1990). The problem arises because completed PCR reactions contain such high concentrations of the target sequence (1000–10,000 molecules per nanolitre) that even the smallest aerosol particle generated during or after a PCR contains many more potential template DNA molecules than the number of aDNA molecules added to the reaction.

This type of contamination can best be prevented by complete physical separation of the laboratory where DNA extractions and PCR experiments are set up, from the area where PCR products are handled and analysed. The separation needs to be very rigorous, with isolation and dedicated use of pipettes, plasticware and glassware, reagents, water and all technical devices.

Ventilation systems must not connect labs. Contamination can even occur via clothing of people who go between the two labs. Solutions ideally should be kept in small carefully-labelled aliquots dedicated solely for work with aDNA. Independent multiple extractions should be performed and numerous control (blank) extractions should be carried out in parallel with the aDNA extractions (see below). These blanks will detect contamination sources in extraction reagents and solutions (Pääbo *et al.*, 1989). Negative PCR controls (PCRs set up with water rather than DNA) will detect contaminated PCR reagents.

Carry-over of PCR products from one reaction to another can also be reduced by an enzymatic method (Longo *et al.*, 1990). This approach is based in the use of uracil *N*-glycosylase (UNG, available from a number of suppliers including Roche Molecular Biochemicals: biochem.roche.com). UNG acts on both single and double-stranded DNA that contains the unusual base deoxyuracil (dU), stimulating cleavage of the DNA. The enzyme has no activity on RNA or on DNA that does not contain dU. PCRs are therefore set up with deoxyuridine triphosphate (dUTP), which means that the resulting products contain dU bases. The products can be analysed in the normal way, for example by DNA sequencing. Any products which are carried over to a subsequent PCR are degraded by treatment of the PCR mix, before amplification, with UNG for about 10 min at 20–50°C. The primers and the new template DNA, which do not contain dU, are unaffected, but the carried-over PCR products are broken down. UNG is heat-labile (completely inactivated after about 2 min at 95°C) so it does not function once the new PCR begins and hence does not degrade the new, dU-containing products that are made. The only problem is that dUTP is not incorporated in PCR reactions as easily as the normal nucleotides and it is usually necessary to raise the concentration of dUTP relative to the other nucleotides (600 μM compared to 200 μM). This usually necessitates a concomitant increase in the $MgCl_2$ concentration (usually 2.5–3.0 mM instead of 1.5 mM).

Merriwether *et al.* (1994) suggest that all tubes, pipettes and reagents for extraction and amplification (except any enzymes, including the DNA polymerase, or the primers) should be UV irradiated as this forms crosslinks in contaminating DNA, leaving it unavailable for use in the PCR. All surfaces and shared laboratory equipment (for example, centrifuges) should be cleaned with sodium hypochlorite (bleach) regularly.

PCR Controls to Monitor Contamination

Controls useful during PCR amplification of aDNA samples include extraction blanks to monitor the contamination of extraction reagents and PCR ('water') blanks to monitor the contamination of PCR reagents. Extraction blanks can be performed by running through the entire extraction procedure with no starting material, but it is advisable to use a bone from a species other than the one under investigation (e.g. a cattle bone if human remains are being studied). A bone from a different species which has been excavated from the same archaeological site is particularly useful as this means that the extraction blank not only monitors contamination of reagents but also provides some indication of possible contamination of the material during excavation.

Positive controls, comprising modern DNA, are frequently used to monitor the success of aDNA PCRs. If the positive control works, but the aDNA PCRs give no product, then it can be concluded that the PCRs are working effectively and there is either no aDNA present or the aDNA extractions contain PCR inhibitors. Unfortunately, the positive control is a potential source of contamination, and it is better to generate an 'artificial' positive control DNA. This control DNA is designed to give an amplification product with the primers being used, but one that has a sequence different from the aDNA target sequence, and so can be distinguished from

the aDNA PCR products. For example, when investigating the 12S ribosomal RNA gene of the mtDNA of a putative macaque skeleton from Pompeii, Bailey *et al.* (1999) generated an artificial control DNA that contained 'monkey tails' (the annealing sites for the macaque primers) but had an internal sequence from cattle mtDNA. In this case, the artificial positive control also differed in size from the aDNA target and so could be used in quantification experiments.

Degradation of Ancient DNA and the Use of Short PCR Targets

Lindahl (1993) has persuasively argued that the damage that occurs to DNA molecules over time can severely limit the study of aDNA from very old material. Under normal conditions, DNA will degrade to short fragments over several thousand years and will be damaged by chemical reactions such as depurination. However, it is feasible that DNA can survive for tens of thousands of years, especially if the remains have been consistently at low temperatures. Partial dehydration of the sample may also facilitate DNA survival over time, although 'dry' DNA is more prone to base damage since the helical structure of the DNA is compromised.

Degradation of target DNA is a problem for PCR, because fragmentation sets limits to the size of the products that can be amplified successfully. DNA from archaeological specimens is usually of low average molecular size and low copy number due to various forms of damage. DNA is vulnerable to attack by a variety of chemical and physical processes. These include oxidative processes (which cause chemical modification of sugar residues and pyrimidines, particularly thymine, leading to greatly reduced C and T content (Pääbo *et al.*, 1989), breakage of strands, and formation of cross-links (Sykes, 1991). This damage may prevent useful sequence data being obtained or can give false results. Damage may also cause the DNA polymerase to stall, slowing down initial amplification cycles therefore allowing modern non-specific contaminants or primer-dimers to be preferentially amplified. For these reasons, primers that amplify short sequences are recommended. It is also a great help if, where possible, the primers are designed to amplify species- or genus-specific sequences.

There are mechanisms in the living cell that repair damage to DNA. These are usually effective, and only when they fail are mutations introduced into the genome. DNA polymerases usually have a certain inherent capacity for DNA repair but in the case of damaged positions this can lead to the wrong nucleotide being incorporated. If misincorporation occurs during PCR then some of the products will have the wrong sequence. If this is suspected then cloning of the product, followed by sequencing of individual clones, can identify the true sequence (Handt *et al.*, 1994b).

DIRECT SEQUENCE DETERMINATION FROM aDNA PCR PRODUCTS

During the last ten years, direct sequencing of PCR-amplified DNA has become a routine and relatively straightforward task for most laboratories. There have been a number of reviews outlining the various strategies that can be used to obtain sequence information from PCR products as small as 100 bp in length (e.g. Rao, 1995; Kelley and Quackenbush, 1999). Many methods require asymmetric PCR, biotinylated primers and other complications which may not be ideal for aDNA research. Instead, we present a simple and cheap method which we have found extremely useful for manual sequencing of aDNA PCR products. The protocol described here is a more detailed version of that described by MacHugh *et al.* (1999).

An advantage of this protocol is that each of the original PCR primers can be used as a sequencing primer and that the sequence can be determined from both strands using the same

PCR product. In addition, the method uses standard reagents available in the Sequenase manual sequencing kit (Amersham Pharmacia Biotech: www.apbiotech.com), although these can also be prepared *de novo* from readily available materials. The procedure is based on a method for direct sequence determination from double-stranded DNA using snap- or flash-cooling and a DMSO-based series of reactions. We routinely obtain >400 bp of sequence from PCR primers with products ranging in size from 100 bp to 1.5 kb, which is more than sufficient for typical ancient mtDNA PCR products, which are usually 100–250 bp.

PCR products are amplified normally and purified from excess primers, unincorporated nucleotides and other buffer components using either High Pure or Concert PCR purification columns (Roche Molecular Biochemicals or Life Technologies: biochem.roche.com; www.lifetech.com). After elution and re-suspension in TE buffer to a final concentration of approximately 50–200 ng μl^{-1}, the annealing reaction shown in Table III is assembled in standard 0.5 ml PCR tubes. If the same sequencing primer is to be used for each template, a master-mix can be assembled consisting of everything except the double-stranded amplicon template. This mixture is denatured by heating to 95°C for 3–4 min in a pre-heated PCR machine. The annealed samples are then snap-cooled by immediately plunging into liquid nitrogen. *CAUTION*: *Care must be taken with liquid nitrogen; protective clothing, gloves and eye glasses should always be worn.* While this annealed primer-template mix is left sitting in liquid nitrogen, a labelling master-mix, sufficient for the number of templates, is assembled (Table III).

Each of the four Sequenase termination mixes (G, A, T and C) is added separately to 50% DMSO solution in a 9:1 ratio to give a working concentration of 5% DMSO. The amount of termination mix required depends on the number of samples. A volume of 2.5 µl of each DMSO-containing termination mix is aliquoted into a labelled 60-well Nunclon Microwell (Terasaki) plate (Life Technologies: www.lifetech.com). The annealed template-primer tubes are then removed quickly from the liquid nitrogen and 5 µl of labelling reaction master-mix is added to the side of each tube and the tube flick-spun in a microcentrifuge to simultaneously thaw and mix the reactants. After spinning, further mixing can be performed by gentle pipetting with a micropipette. The labelling reaction is allowed to proceed at room temperature for at least 2 min before 3.5 µl from each tube is added to the appropriate termination mixes in the Terasaki plate. These termination reactions are carried out at room temperature for a further 5 min and then stopped by the addition of 4 µl of Sequenase stop solution. Up to 12 samples can be sequenced simultaneously using this method if pipetting steps and reaction times are carefully monitored using a laboratory timer.

The reactions in the trays can then be stored in a freezer and heated to 90°C before gel fractionation by standard polyacrylamide gel electrophoresis.

EVALUATING THE AUTHENTICITY OF ANCIENT RESULTS

After aDNA has been extracted, amplified and sequenced, the results must be verified to ensure they are authentic. There is a general consensus in the field of aDNA research that if the sequence data obtained is to be accepted scientifically then it must be reproducible. Exactly what this reproducibility means has been debated on numerous occasions at various scientific meetings (e.g. Sykes, 1991, 1995).

TABLE III Direct sequencing of PCR products

Component	*Volume added per reaction (μl)*	*Final amount/concentration*
Annealing reaction		
Purified PCR product	2.0	100–400 ng DNA
10 μM PCR primer	2.0	20 pmoles
Sequenase buffer	2.0	–
50% DMSO	1.0	5%
Ultrapure water	3.0	–
Labelling reaction		
100 mM dithiothreitol	1.50	30 mM
50% DMSO	0.50	5%
Sequenase labelling buffer	0.40	–
Ultrapure water	2.10	–
[α-^{35}S]dATP	0.50	5 μCi
Sequenase enzyme	0.25	3 units

The Sequence Should Make Phylogenetic Sense

The sequence should be compared with those from other related organisms present in the GenBank DNA sequence repository (www.ncbi.nlm.nih.gov). Most recently extinct species will have closely-related sibling species that may have mtDNA sequence data available for comparison. An aDNA sequence is more readily accepted as genuine if it is phylogenetically compatible with a modern relative. The GenBank database is a particular boon for aDNA studies because sequence information from modern samples may not need to be collected in the same laboratory as related ancient samples. Sequences that have been retrieved from samples less than 100,000 years old should fit into a phylogeny of modern relatives, even if the sample is of an extinct species (Cooper *et al.*, 1992). Blind testing, where the experimenters are unaware of the exact identity of samples, has also been advocated for authenticating aDNA sequences (Yang *et al.*, 1997).

Fragmentation of aDNA

Based on studies of DNA degradation and the observation that shorter length fragments are easier to amplify from ancient samples than longer ones, there is a general opinion that most aDNA gradually fragments into short pieces of approximately 100–150 bp over time. Therefore one criteria which is used to assess the authenticity of a particular aDNA-containing sample is whether long PCR products can be amplified. Although this depends on the age of the sample, if long products (say, greater than 300 bp) can be amplified, this would cast doubt on the validity of any short PCR products that were previously obtained.

FUTURE DEVELOPMENTS AND CONCLUSIONS

Perhaps the most exciting current technological innovation which will have a bearing on aDNA research is the emergence of real-time quantitative PCR. Using this technique, the initial copy number of a DNA template can be estimated much more accurately than is possible with the earlier methods, which were based on conventional PCR with internal controls. In outline, the new method involves monitoring the kinetic growth curve of a PCR reaction in real-time

using a CCD camera and appropriate fluorescence-based detection formats. A threshold point during the log-linear phase of the reaction is identified and data from unknown samples can then be calibrated using standards of known template concentration. Accurate quantification of initial copy number of the ancient DNA templates would be a major advance in the field of archaeological genetics. The archaeological context of DNA preservation could be analysed in much greater detail than previously possible and contamination artefacts could be more easily identified. At the present time only two manufacturers provide the appropriate equipment and it is relatively expensive (PE Biosystems and Roche Molecular Biochemicals: www.pebio.com; biochem.roche.com). However, this situation may change as more companies bring out PCR equipment capable of real-time kinetic amplification monitoring.

Another promising development has been the identification of a reagent which may help to release DNA trapped within sugar-derived condensation products (Maillard products), considered to be common components of ancient DNA extracts. This reagent is *N*-phenacylthiazolium bromide (PTB) and it has been found to be extremely effective in purifying DNA from 11,000 year-old coprolites from an extinct ground sloth (Poinar *et al.*, 1998).

On a similar theme, a method to make aDNA extractions more amenable to PCR amplification has been reported by Pusch *et al.* (1998). In this paper they describe a procedure to repair degraded duplex DNA from prehistoric samples using *Escherichia coli* DNA polymerase I and T4 DNA ligase (Stratagene and Roche Molecular Biochemicals: www.stratagene.com; biotech.roche.com). This method holds great promise as it is relatively straightforward and uses standard molecular biology reagents (unlike the PTB method described above).

The exploration of various methods which use other preserved macromolecules as surrogates for DNA survival has been extremely useful. The original work describing the use of HPLC to estimate the extent of racemization of various amino acids (Poinar *et al.*, 1996) has recently been expanded to encompass the use of flash pyrolysis with gas chromatography and mass spectrometry to evaluate protein survival in aDNA extracts (Poinar and Stankiewicz, 1999). These methods, although beyond the means of many researchers, hold out the best hope for assessing DNA survival in particular samples, and also perhaps for determining the most appropriate extraction methods for particular sample types.

Notwithstanding the recent progress in methods for analysing archaeological DNA, the basic advice outlined in this review should still serve as a reasonable guide for future studies of aDNA in both human and non-human material. In our opinion, the most important factors for aDNA success are as follows:

- Only use well-preserved archaeological samples for analysis.
- Work in dedicated labs with strict cleanroom procedures.
- Experimental design should be well thought out for all aspects of aDNA work.
- Perform DNA extractions using the modified Yang *et al.* (1998) procedure.
- Design primers carefully.
- Always use modern automated 'hot-start' PCR DNA polymerases.
- Keep up to date with developments in this rapidly moving field.

Ancient DNA research is an expensive, time-consuming and destructive method and should not be used in place of other archaeological methods if these are equally informative. This said, the study of aDNA has huge potential. Although the field of aDNA was rocked by discredited reports of fossil DNA in the early- to mid-1990s, important discoveries have been published during the last decade. These include the retrieval of Neandertal mtDNA – a fatal body blow to the multiregional theory of human evolution (Krings *et al.*, 1997, 1999); the phyloge-

netic analysis of a range of extinct Pleistocene fauna (Cooper *et al.*, 1992; Janczewski *et al.*, 1992; Hagelberg *et al.*, 1994; Höss *et al.*, 1994, 1996; Krajewski *et al.*, 1997); and the first large-scale genetic surveys of ancient human populations (Stone and Stoneking, 1993, 1998, 1999; Oota *et al.*, 1995, 1999; Stone *et al.*, 1996). Based on the progress made during the last decade, the new century should herald an exciting new era for archaeological genetics.

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