DECONTAMINATION TECHNIQUES IN ANCIENT DNA ANALYSIS

By

Kathryn E. Watt
BA (HONS), Simon Fraser University, 2003

THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF ARTS

In the
Department
Of
Archaeology

SIMON FRASER UNIVERSITY

Summer 2005

APPROVAL

NAME: Kathryn Elizabeth Watt

DEGREE: M.A.

TITLE OF THESIS: Decontamination Techniques in Ancient DNA Analysis

EXAMINING COMMITTEE:

Chair: Dr. A.C. D'Andrea
Associate Professor

Dr. D. Yang
Senior Supervisor

Dr. M.F. Skinner
Professor

Dr. D. Hildebrand
Forensic Science Programme, B.C.I.T.
Examiner

Date Approved: August 3, 2005

ABSTRACT

Contamination is one of the most troublesome aspects of ancient DNA analysis. Resulting from the ease with which samples may be contaminated, decontaminating ancient remains has become a necessary step in ancient DNA analysis. Unfortunately, there have been no controlled studies of the efficacy of current decontamination techniques.

This study examined a variety of chemicals to test their effectiveness at removing DNA within solution. Bleach, being the most effective chemical destroyer of DNA, was subsequently tested in a controlled experiment using an artificial DNA fragment for contamination and an ancient animal proxy. Results indicated that submersion in 100% household bleach for 5 to 10 minutes was the most efficient technique for removing contaminant DNA on ancient bone surfaces. However, this treatment may not adequately decontaminate heavily and deeply contaminated bone samples since 100% bleach could not remove contaminant DNA that has been soaked into bone, even after 20 minutes of exposure.

ACKNOWLEDGMENTS

Many thanks go out to my supervisory committee, Dr. Dongya Yang and Dr. Mark Skinner for providing guidance and discussion throughout this project. Special thanks goes to my senior supervisor, Dr. Yang, for teaching me the laboratory skills required for ancient DNA analysis, allowing me the opportunity to work with him and work in the ancient DNA laboratory, and providing much appreciated technical and editorial help over the past two years. This study was supported in part by Yang's grants from the Social Science and Humanities Research Council of Canada and SFU Discovery Park Grant. Gratitude also goes out to Dr. Dean Hildebrand of BCIT, for not only acting as my external reviewer, but also for allowing me to take part in projects in his lab and teaching me different techniques.

Much appreciation goes to Camilla Speller my lab partner and friend, whom helped me in many ways both in the lab and out, without you I would never have made it through.

Credit must also be given to my beloved husband Darren who has stood by me through many, many, years of higher education, supported me and urged me on when I was not sure I could do it.

I must also recognize the efforts of Andrew Barton and Shannon Wood for assistance during sampling and analysis of the elk and whale remains. Many thanks also go to Karl Hillis and Sarah Padilla for their technical assistance in the

SFU ancient DNA laboratory. I am also grateful to all my friends, both at SFU and abroad, for all their encouragement.

Finally yet importantly, I am thankful for the moral support from my parents, Heather and Rob Watt, and my brother Greg and sister, Jennifer who listened to hours of complaints. Thank you all!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

GLOSSARY

Generic terms:

100bp Ladder™ (Invitrogen)	The 100bp DNA Ladder is designed for sizing double-stranded DNA from 100 to 1,500bp, with each band indicating 100bps.
3% H_2O_2	3% Hydrogen peroxide
10% Bleach	Diluted full strength commercially available household bleach to 10%, i.e. 1ml/10ml H_2O
100% Bleach	Full strength commercially available household bleach
Artifact	Something that is not typical of the actual substance, but a result from a process
Base pair	Two nitrogenous (purine or pyrimidine) bases (adenine and thymine or guanine and cytosine) held together by weak hydrogen bonds. Two strands of DNA are held together in the shape of a double helix by the bonds between base pairs. The number of base pairs is often used as a measure of length of a DNA segment, for example 500 bp.
Cross-link	To join by creating covalent bonds (of adjacent chains of a polymer or protein).
DNA Away™ (Molecular Bioproducts)	Commercial product used to eliminate DNA contamination from work surfaces and equipment
ddH_2O	Double-distilled water
dsDNA	Double-stranded DNA
Endogenous	Part of the internal environment of a living organism.
Exogenous DNA	DNA originating outside an organism that has been introduced into the organism.
HCl	Hydrochloric acid

Mass Ladder™ (Invitrogen)	Designed for estimating the mass (quantity) of unknown DNA samples
NaOH	Sodium hydroxide
Normality (N)	A unit of concentration expressed as equivalents per litre.
Polymerase Chain Reaction (PCR)	A powerful method for amplifying specific DNA segments which exploits certain features of DNA replication. For instance, replication requires a primer and specificity is determined by the sequence and size of the primer. The method amplifies specific DNA segments by cycles of template denaturation; primer addition; primer annealing and replication using thermostable DNA polymerase. The degree of amplification achieved is set at a theoretical maximum of 2^N, where N is the number of cycles, e.g. 20 cycles gives a theoretical 1048576-fold amplification. In addition to primers and DNA polymerase, PCR reactions must contain template DNA (the DNA to be amplified) and the DNA "building blocks", deoxynucleotide triphosphates (dNTPs, which include dATP, dTTP, dGTP, and dCTP).
Primer	A short sequence (of RNA or DNA) from which DNA replication initiates and the enzyme adds additional monomeric subunits to (dNTPS).
Ultraviolet radiation (UV)	Electromagnetic radiation in the region of 200 to 400 nm.
v/v	Volume-to-Volume ratio: Describes the concentration of a substance in a mixture or solution. Thus, 2% v/v means that the volume of the substance is 2% of the total volume of the solution or mixture
w/v	Weight to Volume ratio: Describes the concentration of a substance in a mixture or solution.

Thesis Specific Terms:

ArtDNA	Artificially created DNA fragment for contamination and quantitative purposes
Blank Extraction Control	Lysis buffer solution that has no bone powder added. This control is used to indicate contamination in the extraction and purification processes in the laboratory.
Negative PCR Control	PCR reaction in which no DNA template is added. This control is used to indicate contamination of the reagents and materials in use in the PCR setup laboratory.
Target DNA	The DNA sought after, the animal DNA within the bone

CHAPTER 1: INTRODUCTION

Ancient DNA (aDNA) has allowed archaeologists to answer fundamental questions regarding disease (Zink *et al.* 2005, Zink *et al.* 2004, Zink *et al.* 2003, Mays *et al.* 2001, Zink *et al.* 2001, Taylor *et al.* 1996, Faerman *et al.* 2000, Haas *et al.* 2000, Braun *et al.* 1998, Drancourt *et al.* 1998, Raoult *et al.* 2000), human evolution (Krings *et al.* 1997, Ovchinnikov *et al.* 2000, Adcock *et al.* 2001, Cooper *et al.* 2001, Serre *et al.* 2004, Scholz *et al.* 2000), species identification (Yang *et al.* 2004, Barnes *et al.* 2000, Newman *et al.* 2002, Fleischer *et al.* 2000, Barnes *et al.* 1998), and animal and plant domestication (Freitas *et al.* 2003). However, the analysis of ancient DNA is still fraught with technical difficulties revolving around the minute amounts of preserved DNA, its fragmentary condition and the real risk of contamination. Contamination is a threat to any ancient DNA project because modern DNA will overwhelm the degraded ancient DNA during amplification, potentially leading researchers to make false claims about positive results. Ensuing from the likelihood of contaminant DNA being responsible for many results in the early 1990s, calls resounded throughout the field for proving the authenticity of extracted DNA (Pääbo. 1989, Austin *et al.* 1997). Consequently, many authentication criteria were published to prevent contamination from entering a sample within the laboratory setting and to prove the legitimacy of results (Poinar. 2003, Cooper and Poinar. 2000, Pääbo *et al.* 2004). However, even with the vigilant use of these authentication protocols,

samples may still be contaminated prior to entering the lab, and we must rely upon decontamination techniques to remove this exogenous contaminant DNA. However, little discussion has revolved around how to remove contamination present on bone samples effectively.

1.1 Contamination

Contamination can be defined as any molecule of DNA that is similar to the target molecule of DNA (Yang and Watt. 2005). Contamination is the greatest threat to any ancient DNA study due to the hypersensitivity of the Polymerase Chain Reaction (PCR) (Yang *et al.* 2003). This is because PCR is a means by which large amounts of DNA product may be amplified from as little as a single cell (Innis. 1990). When working with ancient DNA, potential modern contaminant DNA often outnumbers the target DNA. Therefore, in order to authenticate the results of an ancient DNA study we must first exclude the possibility of contamination (Poinar. 2003)

Despite the fact that almost all sources of contaminant DNA are theoretically avoidable or controllable, contamination occurs readily (Hummel. 2003). How often and how much contamination arises is dependant upon whether analyses can detect it and the steps taken in the lab to avoid it. However, no matter how rigorous the steps taken to minimize contamination in the laboratory, results may not be authentic if the contamination within a sample cannot be detected or removed.

1.1.1 Contamination Sources

Numerous sources of contamination must be taken into consideration when designing an ancient DNA study. The source of contamination, however, will depend upon the research subject. For example when working on ancient human remains, contamination can come from modern human DNA or other human remains that one may be using as a reference. Yet when working with animal remains as long as the study is carefully designed one need not worry about human contamination.

PCR product carry-over is likely the most detrimental type of contamination. PCR product carry-over results from the introduction of amplification products at a pre-PCR analysis step. PCR products can contain up to $10^{12} - 10^{15}$ amplified DNA molecules in as little as a 50µl reaction (Kwok and Higuchi. 1989). Upon opening of the PCR tube, aerosol droplets can easily escape attaching themselves to any material. These particles are effortlessly transported on clothing, shoes or through ventilation systems, back to the pre-PCR laboratory. When compared to 1g of an ancient sample which may only contain $10^5 - 10^6$ copies of DNA template (Handt *et al.* 1996, Cooper *et al.* 2001) the introduction of even one droplet of aerosol PCR product, which can contain a thousand times more DNA, can quite clearly overwhelm the authentic sample (Willerslev and Cooper. 2005).

Contamination by human DNA is another common type of contamination encountered when working with ancient human remains because human DNA is ever-present in the environment. Human contamination may be introduced into the laboratory in two different manners: through the purchase of contaminated

reagents, tubes and tools used in the laboratory (Hummel. 2003); and through human handling of samples prior to, or during analysis. The latter source can readily occur when archaeologists or researchers who possess DNA markers similar to the target, sweat, breathe on, handle or wash the ancient human samples (Serre *et al.* 2004, Cooper. 1997).

Further contamination can occur during morphological examination of ancient remains prior to DNA analyses, for example, samples that have been a part of a teaching collection, reference collection or stored in a museum. Cross-sample contamination may occur when samples are placed in close proximity, (i.e., stored in the same bag or drawer) but can also occur in the laboratory during any step in the pre-PCR process.

Contamination is not of great concern in work with modern DNA, because modern target DNA is usually plentiful and intact. However, in the study of ancient DNA, contamination is a major difficulty because the recoverable ancient DNA, if any, consists of low copy number, degraded template. Ancient DNA is subject to many taphonomic factors within the burial environment, which causes the molecules to breakdown via hydrolysis and oxidation (Lindahl. 1993), leaving only small fragments of DNA available for PCR amplification. Small fragments of DNA (100 – 500bp) will likely survive for no more than 10,000 years, or perhaps up to a maximum 100,000 years in permafrost areas (Poinar *et al.* 1996, Willerslev and Cooper. 2005). While claims have been made of plant and bacterial DNA recovery from ice core samples of upwards of 100,000 years (Willerslev *et al.* 2004), the authentication of these results has not been

substantiated. Although time is a factor in the preservation of DNA, of greater importance are temperature, humidity and microbial activity (Lindahl. 1993, Hofreiter *et al.* 2001); these such taphonomic influencing processes were often not taken into account in early ancient DNA studies, resulting in unrepeatable experiments and spurious results.

1.1.2 Contamination in Ancient DNA Studies

In the beginning of ancient DNA as a field, hopes were elevated that the theoretical time limits of preserved DNA would be high. The first studies of ancient DNA focused on well-preserved remains from the skin of animals and mummified human tissue (Higuchi *et al.* 1984, Pääbo. 1985, Pääbo. 1989). Unfortunately, difficulties were associated with the molecular cloning of this DNA, and its fragmented, damaged nature did not allow for reproducible results (Pääbo *et al.* 2004). Further, cloning resulted in artifacts and false sequences, and phylogenetic analyses were problematic (Hagelberg. 1993). Although these studies might have demonstrated that DNA was preserved in these samples, most of the recovered DNA originated from microbial sources.

With the invention of PCR in the late 1980's (Mullis. 1990) came the routine amplification of genetic material from even a single molecule. One of the drawbacks of the technique's sensitivity within ancient DNA studies was the potential for contamination from modern DNA sources. Additionally, with amplification came the threat of contamination from previously amplified PCR products. Consequently, false positive results stemming from laboratory contamination became much more likely.

Astounding and highly publicized early studies claiming the preservation of million-year-old DNA are now refuted, including: studies describing the recovery of DNA from dinosaur bones (Woodward *et al.* 1994); fossil plants (Soltis *et al.* 1992, Golenberg. 1991); and amber-entombed insects (Cano *et al.* 1992b, Cano *et al.* 1992a, Poinar *et al.* 1993, DeSalle. 1994). These results were discredited in the early 1990's, some due to human and microbial contamination (Hedges and Schweitzer. 1995, Zischler *et al.* 1995, Gutierrez and Marin. 1998) and others because the results could not be reproduced (Sidow *et al.* 1991, Austin *et al.* 1997, Olsen and Hassanin. 2003). As a result, a cloud of suspicion was cast on the integrity of the field of ancient DNA.

Resulting from this period of scepticism, rampant publishing ensued, with numerous papers discussing at length both contamination controls and criteria for authenticating sequences (Poinar. 2003, Cooper and Poinar. 2000, Pääbo *et al.* 2004, Austin *et al.* 1997, Montiel *et al.* 2001, Pruvost and Geigl. 2004, Spencer and Howe. 2004, Hummel *et al.* 1999, Yang *et al.* 1997b, Richards *et al.* 1995, Hofreiter *et al.* 2001, MacHugh *et al.* 2000, Stoneking. 1995, Beraud-Colomb *et al.* 1995, Pääbo *et al.* 1989, Cano. 1996). These criteria continue to evolve as the science progresses; however, most efforts have focused on the detection of contamination rather than the removal of it.

Due to the vast amount of extant human DNA found in the environment, the problems associated with the authenticity of ancient human DNA are far more serious than those of ancient faunal and plant remains. The results of many studies have been questioned due to the lack of independent replication (Adcock

et al. 2001, Cooper *et al.* 2001, Gill *et al.* 1994, Knight *et al.* 2004) or the evident contamination within samples (Handt *et al.* 1994). Contamination is in fact so prevalent in studies involving human remains, that even those that can claim authentic sequences still encounter contamination on some level (Krings *et al.* 1997, Ovchinnikov *et al.* 2000, Serre *et al.* 2004, Yang *et al.* 2003, Hofreiter *et al.* 2001, Kolman and Tuross. 2000, Garcia-Bour *et al.* 2004, Vernesi *et al.* 2004, Handt *et al.* 1996, Wandeler *et al.* 2003, Alonso *et al.* 2001, Melton and Nelson. 2001, Cipollaro *et al.* 1999, Stone and Stoneking. 1999). Despite the problems in performing ancient DNA analysis using human remains, there are still multitudes of archaeological, paleoanthropological and forensic questions that can only be addressed through this technique, including questions regarding disease, kinship relationships, human evolution, population genetics and forensic questions of identity. Therefore, efforts directed at controlling and removing contamination in ancient DNA studies are exceedingly important.

1.1.3 Contamination Controls and Authentication

Two approaches were taken by members of the ancient DNA community to address concerns of authenticity of ancient DNA sequences: first to outline protocols for the prevention and minimization of contamination and secondly, to list criteria that should be used to authenticate sequences. Criteria such as the use of blank and negative controls, separation of pre and post PCR work, wearing of protective clothing and use of disposable reagents and lab materials are now standard protocols.

Avoidance of PCR carry-over contamination must be undertaken vigilantly and laboratories should be designed with geographically separate pre-PCR and post-PCR work areas. Further, personnel should only be moving on a daily basis from ancient to modern labs or pre-PCR to post-PCR workspaces. Other simple precautions such as equipping the labs with positive air pressure and cleaning regularly with bleach and ultraviolet light (UV) irradiation are also effective if rigorously maintained (Willerslev and Cooper. 2005).

Several protocols have been devised to reduce the risk of contamination from consumables (Hummel. 2003). Included are the use of UV irradiation and DNase on equipment and supplies (Eshleman and Smith. 2001). Other protocols include extensive cleaning of tools for prolonged periods with a variety of techniques including UV for 72 hours and 50% bleach for 48 hours (Willerslev and Cooper. 2005).

In the field during excavation of remains, contamination can be reduced and avoided by wearing, masks, gloves (although it is not always reasonable for field crew to do this in sweltering heat) and by carefully containing the sample in a clean bag, using clean tools (Yang and Watt. 2005). If ancient DNA analysis is a part of a study, development of clean collection protocols are critical to help avoid contamination.

In the lab the possibility of cross-sample contamination can be minimized, by strictly enforcing the separate handling of each sample i.e., changing gloves between samples, cleaning of all tools that come into direct contact with each

sample (saw), not touching the inside of reaction tubes and changing the aerosol-tight pipette tips between samples.

Criteria revolving around DNA sequence analysis contend that results must make phylogenetic sense, behave in the right molecular way, and results must be reproducible on three levels: from the different extracts, different amplifications and independently in a separate laboratory (Poinar. 2003, Cooper and Poinar. 2000). As well, results should be cloned to determine the extent of polymerase miscoding due to damage and as a check on the level of contamination within a source. Unfortunately, the latter criteria, which can elucidate results, are often ignored due to logistical difficulties and worries regarding costs and intra-lab contamination.

Blank extraction and negative PCR controls have been touted as sufficient indicators of all types of contamination (Poinar. 2003, Cooper and Poinar. 2000). However, it has been shown that even those studies in which blank and negative controls are clean are not necessarily contaminant free due to a carrier effect (Kolman and Tuross. 2000, Handt *et al.* 1994). This phenomenon is not well documented or explained in the literature. Handt *et al* (1994) describe the carrier effect as a process by which sugar or microbial DNA binds to a very low level of contaminating DNA releasing it from its adsorbed state on plastic-ware allowing the product to become available as PCR template. Alternatively, Kolman and Tuross (2000) described the carrier effect as a situation in which two types of DNA, (exogenous and endogenous), are needed to increase the DNA concentration to a critical level for PCR amplification. If the DNA concentration

within the sample is high enough, the target and exogenous DNA then begin to compete for reagents during amplification (Kolman and Tuross. 2000). The carrier effect as described by Kolman and Tuross is less often an issue in ancient DNA studies since well-designed primers should preclude the amplification of exogenous DNA (*e.g.,* microbial). Although this phenomenon is not well understood, it is clear that the contamination was derived prior to the bone samples entering the laboratory.

Sample contamination is much more difficult to detect than systematic contamination stemming from the laboratory reagents or personnel, and it is particularly problematic in studies of ancient human remains (Serre *et al.* 2004). Sample contamination does not always invade every sample (i.e., systematic contamination) but has a tendency to affect one sample and not others. Moreover, it is difficult to distinguish between contamination and authentic sequences, as it is possible that bone samples excavated and curated from different sources are likely to produce different sequences (Cooper. 1997). Even more problematic is the likelihood that samples curated in museums have been handled on numerous occasions over time resulting in contaminant DNA behaving like authentic ancient DNA (Willerslev and Cooper. 2005) making the criteria of an inverse relationship between amplification strength and fragment length irrelevant (Handt *et al.* 1994). Further, a heavily contaminated sample is also problematic as repeat amplifications and extractions will likely show the same sequence. To resolve these issues independent replications should be

undertaken using different elements from the same individual i.e. a bone and a tooth.

The evaluation of DNA preservation can also provide useful information about the likelihood of DNA retrievability. The first technique for this evaluation is to look for other biomolecules as predictors for DNA preservation. The use of amino acid racemization (Poinar *et al.* 1996), flash pyrolysis (Poinar and Stankiewicz. 1999) and thermal aging (Smith *et al.* 2003) have been advanced as methods for testing preservation potential. Unfortunately, many of these methods are expensive and not entirely reliable; for example, there is little agreement over the suitable threshold of amino acid racemization (Serre *et al.* 2004, Poinar *et al.* 1996, Schmitz *et al.* 2002) and the Neanderthal type specimen, which contained authentic DNA, fell outside the proposed range for successful DNA recovery (Krings *et al.* 1997). Amplifying DNA from other associated faunal remains can also be used as an alternative means to predict the survival of ancient DNA.

It is clear that contamination within an ancient DNA laboratory has been adequately recognized and some guidelines and protocols have been proposed to avoid and detect the contamination. However, there seems to be a loophole in this process. If the samples have been previously contaminated, they can make these in-lab contamination controls meaningless. Therefore, efforts must be in place to deal with these previously contaminated remains in ancient DNA studies.

1.2 Decontamination

1.2.1 Decontamination Methods

Ancient DNA researchers have to assume that samples are contaminated in some way before they enter the laboratory. For this reason, decontamination techniques are a necessity to increase the likelihood that only authentic ancient DNA is amplified during PCR. Currently there are numerous techniques used in laboratories to decontaminate bone samples. The currently available decontamination methods include the physical removal of bone surfaces, the chemical destruction of contaminant DNA, UV irradiation of surface contaminant DNA and a combination of methods.

From a survey of 106 ancient DNA studies from peer-reviewed journals focusing on recovering DNA from ancient bone or tooth samples, it became clear that the most common technique used to decontaminate samples was through the physical removal of the sample surface, with 79 of 106 studies examined using some type of physical method (Appendix 1). In particular, the use of a dremel, drill or shot-blasting are the most common physical surface removal techniques with 28 studies using these methods The next most common physical techniques were the use of scalpel blades, sandpaper, and brushes, respectively. Chemical methods were the next most prevalent techniques in use with 37 studies using some variety of chemical for decontamination through rinsing or wiping. Of the chemicals used, bleach was the most common with 31 of the 37 studies using this chemical. Twenty-seven studies undertook decontamination with the aid of UV irradiation.

Of the 106 studies surveyed, 56 undertook decontamination using only one technique, 45 studies used a combination of techniques, and five did not use any decontamination method (Figure 1.1).

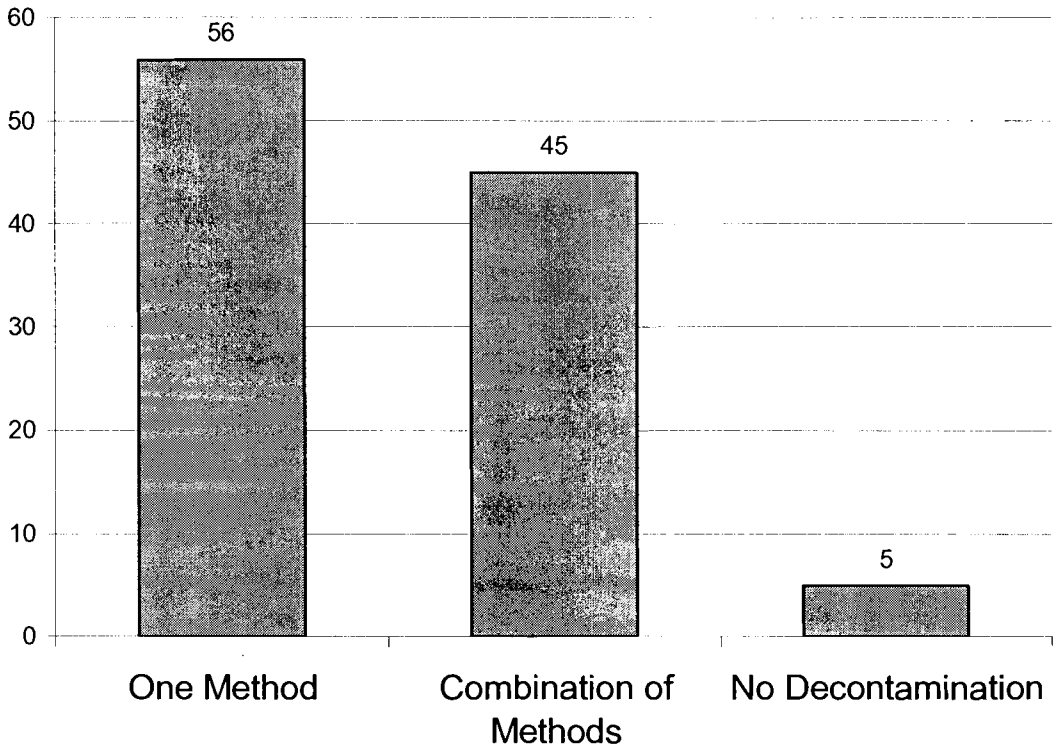

Figure 1.1 Graph representing the decontamination techniques surveyed in 106 published ancient DNA studies (See appendix).

The use of a physical method and bleach, or a physical method and UV were employed in combination in eight studies respectively (Figure 1.2). The use of other decontamination combinations took place in 16 studies, while a physical technique, bleach and UV, were employed in seven studies each.

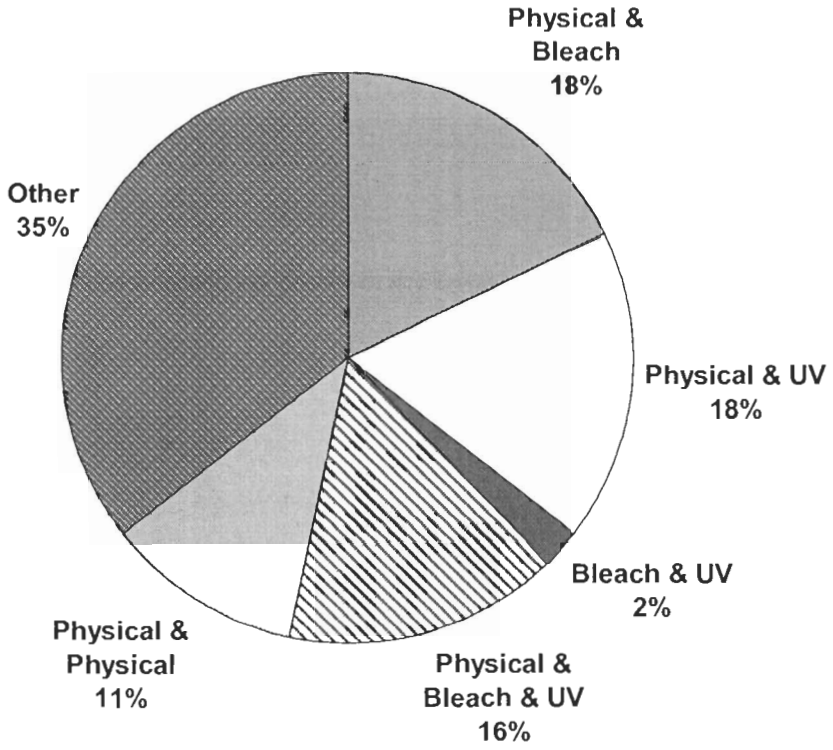

Figure 1.2 Chart displaying the types of decontamination techniques used in combination (See Appendix A for description of 'Other')

As is clear from the survey, there is no consensus in the field regarding which method is most appropriate to remove contamination from ancient bone and teeth samples. As the effectiveness of these techniques has not been systematically evaluated, it is still unclear whether they are capable of removing contamination from bone surfaces, or more importantly, contamination that may have deeply penetrated into bone tissues. Additionally there are concerns about the techniques' destructiveness in regards to ancient target DNA.

1.2.2 Physical Decontamination

The use of physical contaminant removal techniques follow a common-sense approach, as it is logical that the removal of outer surface bone will leave cleaner bone underneath. Physical removal can be accomplished in a variety of ways: with a scalpel blade, sandpaper, dremel tool or sandblasting. However, there could potentially be many difficulties associated with the physical removal techniques. It is difficult to determine how much of the surface should be removed in order to eliminate all contaminant DNA, simply because samples vary in size and shape and therefore vary in the amount of surface bone that can be removed. Out of necessity, decisions concerning bone removal quantities must be made on a case-by-case basis; for example, it is easier to remove a large quantity of surface bone when a large sample is used but you cannot follow the same procedure for a very small sample. There may also be other concerns with physical methods, for example, sandpaper may allow for contaminant DNA present in the bone powder to be pushed into the interior of a bone sample, while sandblasting and dremel tools may produce aerosols that could cause sample cross-contamination.

1.2.3 Chemical Decontamination

Chemical decontamination methods are based on the assumption that some chemicals may break down DNA molecules. For example, hydrochloric acid (HCl) works through the process of hydrolysis, depurinating DNA, rendering the DNA unsuitable for PCR amplification (Savage and Plaut. 1958). As was

noted above the use of bleach is the most common technique to remove contaminant DNA from the bone surface.

Household bleach contains the active chemical ingredient sodium hypochlorite in concentrations of 5.25 – 6%. Hypochlorites (-OCl) have long been shown to be an effective antibacterial agent; they are very microbicidal (Hidalgo *et al.* 2002) causing bacterial cell death within seconds of exposure (Albrich *et al.* 1981). Sodium hypochlorite is a strong oxidizing agent and when introduced to DNA at varying concentrations can cause base modifications and the manufacture of chlorinated base products (Hawkins and Davies. 2002, Hayatsu *et al.* 1971, Ohnishi *et al.* 2002, Prutz. 1998, Whiteman *et al.* 1997). In particular, it has been shown that pyrimidines are more susceptible than purines to oxidative damage by hypochlorite (Whiteman *et al.* 1999, Whiteman *et al.* 2002). It has also been demonstrated that widespread denaturation of double-stranded DNA occurs upon treatment with hypochlorous acid resulting from the loss of hydrogen bonding (Prutz. 1998, Hawkins *et al.* 2003, Prutz. 1996). These lesions produced by oxidation can cause a loss of structure, which can be reduced further into base fragments (Lindahl. 1993). Prince and Andrus (1992) found that treatment of DNA (free-floating in solution) with 2.5% Clorox (v/v) caused "extensive nicking of DNA" indicated by slower mobility of the DNA through the gel. It was also concluded that 10% Clorox eliminated amplification of a DNA fragment as small as 76bp after 5 minutes of treatment. Based on this information, questions of safety to the already degraded ancient DNA housed within skeletal remains are inevitable.

Bleach has been used as a bone-decontaminating agent for many years (Ginther and Issel-Tarver *et al.* 1992, Rosenbaum and Egan *et al.* 1997). However, the concentration and exposure time vary between studies. Prince and Andrus' 1992 study demonstrated that 10% bleach (assuming w/v or ~ 0.6% NaOCl) was quite effective at destroying DNA in solution, even more so than 1.33N HCl. However, Richards *et al*'s (1993) study established that bone submerged in 10% bleach was not as effective as shotblasting the bones surface to remove contamination, stating that "bleach may be useful in treating less well-preserved remains in which contamination may have penetrated the pores of the bone" (295). Later Kolman and Tuross subjected powdered bone for 2 minutes to 20% bleach (w/v) followed by extensive washing with double distilled water. The results of this study illustrated that although this treatment reduced the amount of contaminant DNA amplified it did not completely eliminate it (Kolman and Tuross 2000).

A recent study was published with the intent of determining an efficient method for decontaminating maggot crops intended for DNA analysis (Linville and Wells 2002). Results of this study demonstrated that 20% bleach was more effective than H_20 or DNase treatment, and did not interfere with PCR amplification or analysis of crop content mtDNA.

While these studies provide valuable information regarding the usefulness of bleach as a DNA eradicator, two of the four studies have not addressed the needs of researchers who primarily study ancient DNA. Neither of the studies by Linville and Wells (2002) or Prince and Andrus (1992) looked at bone or tooth

samples, leaving questions of how these concentrations react with bone tissues and the DNA housed within it. The other studies, while focusing on hard tissues, did not test higher concentrations of bleach or lengthy periods due to the understandable fear of destroying the DNA within the bone. Based on the evidence that the contaminant DNA was reduced but not totally removed it could logically be inferred that a stronger concentration or longer exposure time might eliminate the contamination. Clearly, previous studies also indicate that there might be a difference in terms of chemical decontamination reactions in solution and in bone tissues.

1.2.4 UV Irradiation Decontamination

UV radiation can cross-link DNA templates making them unsuitable for PCR amplification. UV is typically split into three intervals based upon wavelengths: UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm) (Ke *et al.* 2005). Ultraviolet light causes excitation of orbital electrons, raising them to higher energy levels (Nias. 1998). UVC rays never reach the earth as oxygen and nitrogen absorb it in the atmosphere creating ozone. Typical UV manufactured bulbs radiate at 254 nm or 300 nm, and fall into the UVC and UVB categories respectively. Studies have shown that intracellular biomolecules strongly absorb UVC light, which induces DNA damage and mutation (Ke *et al.* 2005, Sakar and Sommer. 1990).

Exposure of DNA to UV radiation generates predominately cyclobutane pyrimidine dimers and (6-4) photoproducts (Choi and Pfeifer. 2005, Cadet *et al.* 2005). The dimeric photoproducts are formed by adjacent pyrimidines,

particularly thymine bases, through both UVB and UVC exposure resulting in unusable templates for PCR (Ravanat *et al.* 2001, Ou *et al.* 1991). UV has also been shown to create inter-strand and protein-DNA cross-links (Douki *et al.* 2003, Smith. 1964, Hall and Ballantyne. 2004).

Sakar and Sommer, who exposed varying quantities of DNA to different wavelengths for various amounts of time, undertook one of the first studies of UV as a decontamination technique for laboratory reagents (Sakar and Sommer. 1990). They concluded that UV was most effective at the 254nm wavelength, and would prevent amplification of up to 30ng of human factor 1X complementary DNA. Additionally, it was noted that UV did not affect either the Taq polymerase or the primers (Sakar and Sommer. 1990). Ou *et al.* (1991) did further work characterizing the effects of UV on HIV proviral DNA. From this study, it was clear that the distance of the sample from the UV bulb was very important, with reduced distances resulting in a greater reduction of amplification products. As was noted above, this was attributed to the formation of thymine dimers (Ou *et al.* 1991). Interestingly, the study also concluded that UV irradiation did indeed affect the Taq and primers. The authors further recommended the use of UV to irradiate benchtops, reaction tubes and racks to reduce false positive results.

More recently, Hall and Ballantyne (2004) characterized UVC-induced damage in bloodstains and commented on the forensic implications of this damage. This study investigated the effects of UV on DNA in a number of forms: bloodstains, naked DNA in solution and naked dehydrated DNA. Results of this study showed that naked DNA in solution, when exposed to UV, began to

exhibit migration retardation during electrophoresis when compared to the marker. The authors claimed this to be indicatory of inter- or intra-strand DNA cross-links. Although after one hour of exposure, high molecular weight DNA was still observed during electrophoresis, the authors noted double-strand breakage increased over time, until the sample appeared to be totally damaged after 48 hours. Additionally, single strand breaks increased dramatically after 16 minutes of exposure to UV resulting in a complete lack of retrievable DNA profiles. Bloodstains revealed a different pattern, in that even after 102 hours of exposure to UV; the electrophoresis gel still displayed high molecular weight, non-degraded double-stranded DNA. Although, single-strand breakage did occur in the dried bloodstains after four hours, DNA damage required a much longer exposure time then when compared to the naked DNA in solution. With respect to the dehydrated naked DNA, results indicated that the DNA did not start to degrade until after 1 hour of exposure with complete degradation not occurring until 48 hours of exposure. The naked, dehydrated DNA was capable of producing a full nine loci STR up to 12 hours of exposure, but complete loss of amplification occurred after 24 hours. This study indicated that dehydration or dryness of DNA afforded some protection against the effect of UV irradiation. As a result of the effects of UV light on DNA illustrated by these studies, it was hypothesized that UV light would be an appropriate decontamination method for PCR laboratories and reagents.

Additional studies have examined the use of UV for reagent decontamination and have produced conflicting results (Fox *et al.* 1991, Ou *et al.*

1991). As illustrated above there is conflict revolving around whether UV damages primers or Taq polymerase enzyme. Other conflicts refer to the effect UV has on shorter amplicons: i.e. some studies show amplification is eliminated while others do not (Sakar and Sommer. 1990, Dwyer and Saksena. 1992, Belak and Ballagipordany. 1993, Fox *et al.* 1991).

Note here that all studies that have examined the effects of UV on DNA with respect specifically to decontamination have done so using DNA in solution. As illustrated above dry DNA is protected from UVC rays. This will have implications for the use of UV as a decontamination technique for bone and teeth samples as well as laboratory workspaces, as these surfaces are mainly dry when exposed to UV. This can only lead to one conclusion: DNA within bone will be protected against UV rays. Further, DNA on the surface of dry bone will be less susceptible to the effects of UV, implying that exposure time should be increased to see any viable removal of contaminant DNA. Study to clarify the effects of UV on bone DNA and its surface contaminants is essential.

Although there are a number of ways that bone samples can be decontaminated, there is still much information needed in order to understand the limitations and applicability of each individual method. Physical methods are most common throughout the field, and can be effective in removing contamination on the surface of bone samples but not that which has penetrated the bone tissue. The use of chemical methods could deal with contamination more effectively inside the bone, but these methods are clearly not as prevalent in the literature. This may be due to the concern that the damage that these

chemicals cause may be detrimental to the sought-after authentic DNA. The use of UV irradiation appears to stem from its use in modern laboratories for reagent and laboratory microbial decontamination. Regrettably, this method has not been tested on bone samples and the effects on the DNA housed within the bone are not yet known. As a result, there are no conclusive data for ancient DNA researchers to apply in the decision making process. Consequently, there is no consensus in the field as to which method (or methods) should be used. Systematic study of the various decontamination techniques is necessary to elucidate the effectiveness and destructiveness of each method.

1.3 Objectives

With this being said, five initial objectives were developed for this study, which were designed to systematically evaluate current decontamination methods.

1. **To systematically evaluate the destructiveness of a number of chemicals on DNA.** DNA in solution will be exposed to a number of currently used chemical decontaminators, and the destructiveness of the chemicals will be evaluated based on visualization of presence or absence of DNA samples on electrophoresis gel. Expectations are that some chemicals will be more destructive than others, to the DNA in solution, and that there will be damage to the DNA sequence itself.

Physical removal methods will not be tested in this study for one main reason: these techniques are very difficult to control and test. It is unknown how

much bone should be removed to be sure that the contamination is gone for a particular bone sample. The answer out of necessity is determined case by case; for example, it is easier to remove a large quantity of surface bone when a large sample is used but you cannot follow the same procedure for a very small sample. The use of these contaminant removal techniques follow a common sense approach, as it is logical that the removal of outer surface bone will leave cleaner bone underneath. With that in mind, we suggest that the removal of the surface with a physical method be undertaken prior to the use of other decontamination techniques as an added precaution.

2. **To use an animal model to avoid human contamination**. Human DNA is ubiquitous throughout the environment. To avoid this major source of contamination from the environment as well as from the researcher an animal model will be used. The animal chosen will be of certain antiquity to mimic ancient DNA analysis as well as in a good state of preservation to help ensure that DNA will likely be preserved. It is expected that the use of the animal model will not be problematic but will further aid in the recovery of authentic DNA.

3. **To use an artificially created DNA fragment to quantify the amount of contamination on bone samples.** An artificial DNA fragment will be created that resembles the chosen animal model but differs from anything in nature. This will be done so that the chosen primer set will amplify both fragments and will ensure that the contamination can be tracked and monitored. The use of this artificial DNA is expected to allow for ease of contamination as well as the ability to track and monitor it.

4. **To simulate different contamination levels.** Samples will be subjected to varying amounts of contamination under two different scenarios: soaking and handling. This will be done to determine the limits of the decontamination techniques. These scenarios have been chosen as they represent the spectrum of possible contamination situations with handling being a more realistic, light contaminant case and soaking being the worst possible case. It is expected that if a decontamination technique can remove the contaminant DNA under the worst-case scenario of soaking then under the light case the contaminant will also be removed.

5. **To determine the optimal conditions of decontamination**. Upon determination of the best chemical for decontamination, the optimal conditions for this method will be determined. This will be determined to understand the limits of the method and be sure of the most effective exposure. This will be done by testing the chosen best method under different conditions such as length of exposure and chemical concentration. A difference in the amount of time and concentration necessary to fully decontaminate bone samples is expected.

CHAPTER 2: METHODS AND MATERIALS

2.1 Evaluation of Chemicals for DNA Destruction

As a preliminary evaluation, various chemicals were tested on DNA in solution to investigate their potential for DNA destruction. This step was performed under the reasoning that if these chemicals were unable to destroy the DNA in solution then it would be much more difficult for them to eliminate contaminant DNA in bones. Based on the results of this initial experiment, the chemical that destroyed DNA most readily would then be applied to bone samples in subsequent experiments.

The chemicals tested in these experiments include 1N HCl, 1N NaOH, 10% Clorox™ bleach, 100% Clorox™ bleach, 3% H_2O_2 and DNA Away™. These chemicals were evaluated because they have been used as chemical decontaminants in published research: 1N HCl (Bathurst and Barta. 2004, Yang *et al.* 2004, Wiechmann and Grupe. 2005), 1N NaOH (Bathurst and Barta. 2004, Yang *et al.* 2004) and bleach (Zink *et al.* 2005, Cobb. 2002, Gilbert *et al.* 2005, Lalueza-Fox *et al.* 2003). (See appendix A for a detailed list of references and the decontamination chemicals used). DNA Away™ was included in the experiment because it is commonly used in laboratories to decontaminate surfaces (http://www.mbpinc.com/Store/productpdf/TechReport_205.pdf). Though 3% hydrogen peroxide has not been used as a chemical decontaminator in ancient DNA studies, it has been linked to DNA damage and cell death (Daroui

and Desai *et al.* 2004, Hagensee and Moses. 1986) and was therefore included in the study to test its potential as a new decontamination method.

These experiments were undertaken using three different sources of DNA in solution: 1) PCR products approximately 125 bp in length; 2) 100 BP Ladder™ (Invitrogen) containing 1µg /µl of DNA; and 3) Low Mass Ladder™ (Invitrogen) containing approximately 270ng /µl of DNA. Five µl of PCR product, 2µl of 100bp Ladder (2µg) or 2µl Low Mass Ladder (235ng) were added to six 1.5 ml Eppendorf tubes. Equal amounts of each of the chemicals, 2 or 5µls, were added to each tube respectively and mixed together for between 30 seconds and 1 minute. The solutions were then mixed with pre-stain solution containing Blue Juice (InVitrogen, Carlsbad, CA) and SYBR Green™ (Clare Chemical Research Co.USA), loaded into a 2% agarose gel for visualization and run at 100 volts for 30 minutes.

To ensure that the DNA staining chemical, SYBR Green™ I, accurately reflected the amount and quality of the DNA visualized on the gel, a number of verification procedures were performed. If no DNA was visualized on the electrophoresis gel, the samples were run on a second gel stained with a more sensitive product, SYBR Gold™ that was applied to the entire gel after electrophoresis. If DNA was still unobservable in the lanes, the sample was purified using the Qiaquick™ MinElute Nucleotide Purification kit (Qiagen, Hilden, Germany), as a means of removing the decontaminant chemical prior to staining and electrophoresis.

Tests designed to neutralize the effects of bleach were also performed, for two reasons: first to ensure that the bleach would not unduly affect the gel stain, and second to test whether the effects of the bleach on the DNA could be neutralized. The chemical sodium sulphite (Na_2SO_3) was used for this purpose (Environmental Protection Service. 1985) and created a redox reaction. Sodium sulphite was first applied to the DNA samples to ensure that it had no effect on the DNA itself. When no effect was determined, bleach and sodium sulphite were added to DNA samples separately. After one minute of exposure to the DNA, equimolar concentrations (0.7M) of either sodium sulphite or bleach were added to the samples to nullify the effects of the initial chemical added.

2.2 Detailed DNA Design and Selection

2.2.1 Primer Design

Since the experiment was initially designed using elk as the ancient animal proxy, four sets of species-specific unique primers were designed to amplify elk mitochondrial DNA (mtDNA). It was particularly important that the primers not amplify human DNA to eliminate human DNA as a source of possible contamination. The fours sets of primers were tested for optimal amplification capacity using previously amplified ancient elk DNA, and the most efficient primer set was chosen for use in this study. Whale primers were similarly designed in another separate study. The whale and elk primers used in this study can be found in Table 2.1.

Table 2.1 Primers used for PCR amplifications

Primer	Sequence (5'-3')	bp	Amplicon
F22 (F) - Whale	CCACCATCAGCACCCAAAGC	20	F22 + R258 (237bp D-loop)
R258 (R) - Whale	TGCTCGTGGTGTARATAATTGAATG	25	
F1202 (F) – Elk/Art	ATAGCACTCCAGAGGGAGGTAAG	23	F1202 + R1399 (144bp Elk D-Loop)
R1399 (R) – Elk/Art	CTGGGACCAAACCTATGTGTTT	22	F1202 + R1399 (130bp ArtDNA)

Note: Both sets of primers amplify fragments of the Dloop. F is for the forward and R for the reverse primer. All primers were created by hand and ordered from Invitrogen™. The primers were of desalted purity at a scale of 10 N.

2.2.2 Artificial DNA (ArtDNA) Design

The design of the artificial contaminant DNA required the fulfilment of a number of criteria. The contaminant sequence needed to be similar to the elk reference sequence particularly in the 5′ and 3′ ends so that the set of elk primers would amplify both sequences. The contaminant sequence also needed to be shorter than the elk reference so it would be possible to discern the two amplicons through visualization of an electrophoresis gel. However, the artificial DNA could be no shorter than 100 bps as it would be lost in the extraction/purification process when using Yang *et al.*'s (1998) extraction method. In addition to the size difference between the fragments, restriction enzyme cutting sites that do not exist in the target elk DNA were inserted to facilitate differentiation between the two sequences. Due to technical restrictions, the contaminant DNA needed to be designed as two single-stranded oligonucleotides that could later be joined and extended to form a double-stranded fragment.

Based on the aforementioned criteria, two oligonucleotides (See Figure 2.1), 79 and 80bps in length respectively, were created to resemble the original study subject, elk, and amplify using the elk primer set, producing an amplicon 14 bp shorter than the target elk sequence. The two single-stranded oligonucleotides were combined together to form a 130bp double-stranded fragment of DNA. The two single-stranded oligonucleotides had thirty complimentary bases, to ensure they would bind to each other (but not to the authentic elk). These thirty base pairs were randomly created by use of an online random sequence maker (http://www.cbio.psu.edu/sms/rand_dna.html). The 30 base pairs were created in this way so that they would not prime or bind to the target elk DNA creating hybrid sequences or secondary structures. As a third safe guard against the creation of hybrids, the G/C content of the 30 bps was designed to be approximately 57%, increasing the Tm to 77°C. The elevated Tm would increase the specificity of PCR, annealing the two fragments only to each other (Mullis. 1990).

As an extra precaution, two enzyme restriction sites not included within the authentic elk sequence were created. This allowed for clear visual verification of amplicons without the need for sequencing. The sites Eco RI (G'AATT_C) and HindIII (A'AGCT_T) at bases 52 and 62 respectively were inserted within the 30 complementary base pairs (see Figure 2.2).

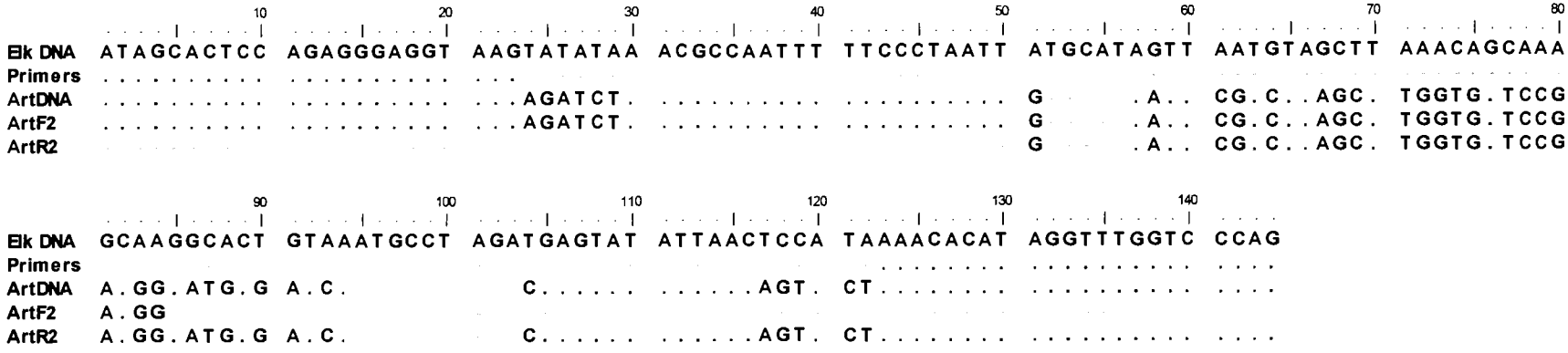

Figure 2.1 Sequence alignment displaying the elk reference sequence (AF016957) primers, complete ArtDNA sequence and Artificial oligonucleotides.

Note: Dots indicate identical base pairs to the elk sequence and dashes represent deletions when compared to the elk sequence. Restriction enzymes sites are located at bases 52 and 62 of the ArtDNA sequence. Note that the ArtF2 and ArtR2 are not shown as complementary.

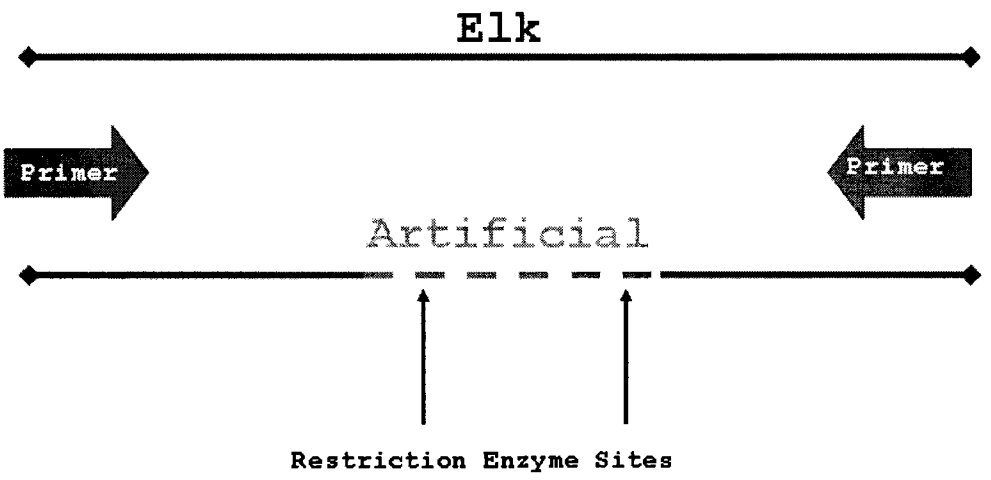

Figure 2.2 Differences between elk DNA and ArtDNA shown in illustration form.

2.2.3 Stovetop PCR

The two single-stranded artificial nucleotides were joined and extended to form a double-stranded fragment using 'stovetop PCR'. To avoid potential PCR contamination caused by using the PCR machine to synthesize double-stranded artificial DNA, a different approach was used in another DNA laboratory. A heat block and ice generated the temperatures needed to simulate a single cycle PCR reaction. A tube containing typical reaction components, including 1X Buffer (Applied Biosystems, Ca., USA), 2mM $MgCl_2$, 0.2mM dNTPs, 1.0 mg/ml BSA, and 1.25 U AmpliTaq Gold™ (Applied Biosystems, Ca., USA) less the primers, plus the addition of 0.3 µM each oligonucleotide (F and R ArtDNA, 15pmol) was used to create double-stranded DNA. The tube was incubated in a heat block at approximately 94°C for 17 minutes and then cooled rapidly in a freezer for 30 seconds. Having anticipated lower efficiency of this stovetop PCR method, the

initial cycle was followed by 10 additional cycles of heating to 94°C followed by rapid cooling.

2.3 Selection of Bone Samples for Testing

Many factors needed to be taken into consideration when choosing bone samples for this study. First, the samples chosen were preferably in a good state of preservation. This allowed for a more even surface for contamination in the hope that the contamination would be absorbed as equally as possible by each sample. Second, cortical bone samples were chosen over very spongy bone, based on the assumption that the contaminant would more deeply penetrate cancellous bone, resulting in skewed results. Third, the bone samples chosen could not be too hard as they may be very difficult to contaminate and process. Fourth, the bone samples chosen needed to be of a certain antiquity to mimic ancient DNA scenarios, but also to have sufficient amounts of preserved DNA available.

Elk (Wapiti – *Cervus elaphus canadensis)* was chosen as the original species of study. The elk specimens used in this study were excavated from Fort d'Epinette (HaRc 27), a 19th century fur trade fort near Fort St. John, British Columbia, in 1975 and 1976 under the supervision of Dr. Knut Fladmark of Simon Fraser University. With the choice made regarding which animal to use, the next decision was which element to use. Although, long bones would have been appropriate for use at the start, the available elements were damaged and fractured, appearing to be in a worse state of morphological preservation than other elements. Ribs would have been an equally good element for use in this

study; however, distinguishing between moose, elk and deer ribs is problematic. Previous species identification of the collection had been undertaken through morphological methods, and several elk vertebrae had been identified. Further comparison was done to confirm the species identification, and as a result, vertebrae were the chosen element.

Elk vertebrae are large elements with a long protruding spinous process. It was with the thought that the spinous process would be an adequate specimen for contamination. Further, they were relatively large bones that allowed for numerous manipulations. These elements, although mainly composed of spongy bone, still contained cortical material. These elements also were not too hard which would allow for ease of contamination and subsequent processing. Lastly, these elements were of certain antiquity, 100 – 150 years old, which would allow for simulation of an ancient DNA situation while allowing for likely amplification of DNA. The spinous process of elk vertebrae were cut, using a handsaw, into pieces approximately 1 cm by 3 cm weighing approximately 1 gram each (See Figure 2.3).

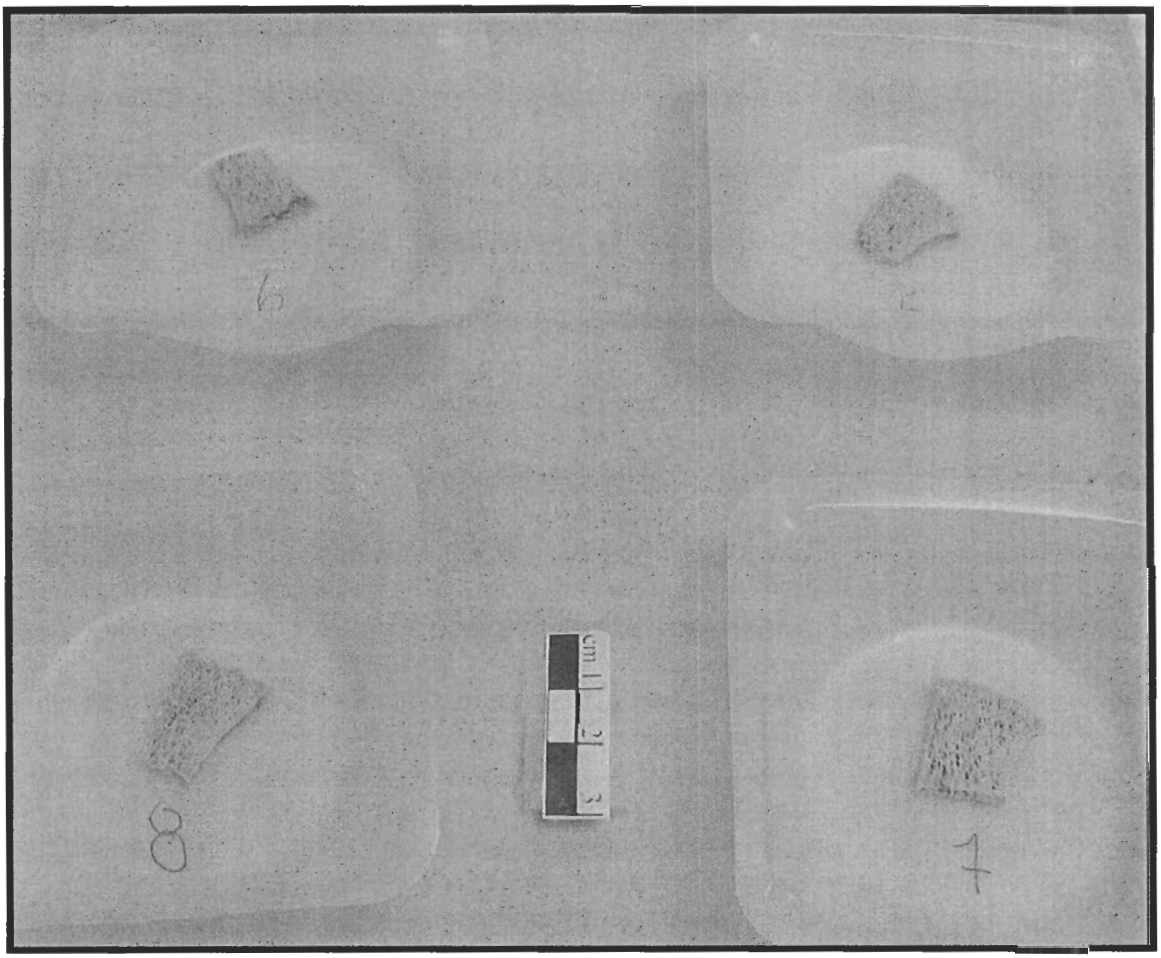

Figure 2.3 Example of elk bone samples used in various decontamination experiments

Unfortunately, initial results indicated that the elk vertebral bone samples could not be evenly contaminated, likely attributable to the porous nature of these elements, their irregular shape and the non-homogeneous composition. Further, upon changing to a long bone element in order to contaminate samples more uniformly, it was determined that it was not possible to grind the bone effectively. Using the protocols and resources of the SFU ancient DNA laboratory (which does not include a commercial grinding mill), the dense bone could not be

efficiently ground into a fine powder, additionally impeding the extraction of the authentic DNA. Due to these limitations with the elk bone, the decision was made to change the species used in the model.

As a substitute it was determined that, a more practical sample would be whale bone. On many whale elements, the cortical portions of the spongy bone are quite thick. In addition, although cortical in nature, whale bone is still quite light and easy to process. Overall, the whale bone surface was more homogeneous and the cortical bone was easier to grind into consistently sized powder. However, one result of changing species was the fact that competitive PCR could not be utilized to quantify the concentration of ArtDNA to use to contaminate the bone.

The whale bone sample used in this study was excavated from the site Ts'ishaa (DfSi 16) on Benson Island, part of the Broken Island Group in Barkley Sound, and dates to 500 +/- 60 BP (Monks and McMillan *et al.* 2001). This sample was in a good state of preservation and has previously shown positive amplification in another project. More importantly, this particular whale bone was DNA identified as Grey Whale (*Eschrichtius robustus)* in a previous study. Mitochondrial DNA sequences from this particular bone represent a unique DNA sequence that was not observed in any other Grey whale bones within that same project. Using a handsaw, the whale bone was cut into 16 equal pieces each weighing 0.35-0.37g (Figure 2.4).

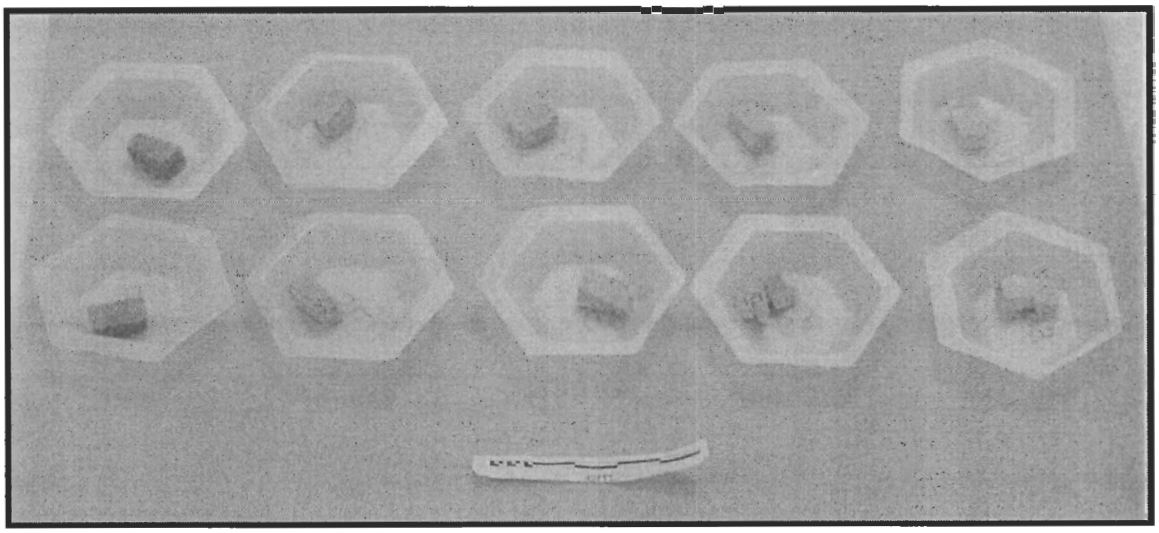

Figure 2.4 Example of whale bone samples used for decontamination experiments.

2.4 Contamination and Decontamination

2.4.1 Elk

Contamination took place based on two different scenarios, the worst case, soaking and a more realistic case, handling. Elk samples were only contaminated by soaking, as it was felt that if the worst case of contamination could be removed via decontamination then contamination via handling would certainly be removed. The aforementioned bone samples were soaked in varying concentrations of ArtDNA and left to dry via air or heat from an oven. The samples were exposed to the solution until the samples were dry. These concentrations were made by dilution of the original stovetop ArtDNA solution 10X, 100X and 10,000X for competitive PCR reaction.

In all, thirteen elk bone samples were processed for DNA extraction and a further twelve were examined via elution, with four samples examined using both techniques. Elution is an easy way to determine whether the decontamination

methods used was successful at removing all the contaminant DNA. Using this technique, bone samples that were contaminated, and then decontaminated were subsequently soaked in water. The water was then included in a PCR reaction and amplified to test for the presence of the artificial DNA. If the artificial DNA was amplified, the decontamination was deemed incomplete. However, if no ArtDNA was amplified, the decontamination might be successful and the bone sample was processed for extraction to confirm the outcome.

In each sample set, two controls were included: one sample was excluded from contamination while another was excluded from decontamination. The former served as a comparative sample control while the latter was used to indicate successful contamination. Elk samples were initially decontaminated via immersion in 50% bleach (approximately 2.65% NaOCl) for 5 minutes. Further tests examined both 50% and 100% bleach for five and ten minutes respectively. These preliminary tests examined DNA eluted from the bone samples with water (Table 2.2). In additional tests, DNA was extracted from samples that were exposed to 100% bleach for 30 seconds, 1.5, 3, 5, 10, 15 and 20 minutes, respectively (Table 2.3).

Table 2.2 Summary of elk bone sample bleach treatments that were examined via elution

		Exposure Time To Bleach - Elutions						
		30 secs.	1.5 mins.	3 mins.	5 mins.	10 mins.	15 mins.	20 mins.
Elk 50%	10^2 X	n/a	n/a	n/a	v	v	n/a	n/a
	10^4 X	n/a	n/a	n/a	v	n/a	n/a	n/a
Elk 100 %	10^2 X	v	v	v	v	v	n/a	n/a
	10^4 X	v	v	v	v	v	v	v

Note: v indicates exposure time tested. % indicates the concentration of the bleach used for decontamination. 10^2X is the concentration of the ArtDNA solution used to contaminate the bone samples.

2.4.2 Whale

Twenty-eight whale bone samples were processed in total. Overall, 21 samples were contaminated with 10^4 X ArtDNA: 14 samples (two sample sets) by handling and seven (one set) by soaking. Two samples from each set were excluded from contamination and decontamination for comparison purposes. Seven samples were contaminated by soaking in the contaminant solution for 5-10 minutes and the other fourteen by handling extensively with contaminated gloved hands. These samples were then left overnight to dry. Four samples from each set were immersed in bleach for 1, 5, 10 and 20 minutes (Table 2.3), and rinsed in ddH_20 for 10 minutes. One sample from each set was rinsed only in ddH_20, one sample from each set was soaked in ddH2O and UV irradiated for 30 minutes on all sides of the bone, and as a positive control for contamination, the remaining samples were left untreated.

Table 2.3 Summary of the bleach treatment for both elk and whale bone samples

		Exposure Time To Bleach				
		1 minute	5 minutes	10 minutes	15 minutes	20 minutes
Whale 100%	Touched	v	v	v	n/a	v
	Soaked	v	v	v	n/a	v
Elk 100%	Touched	n/a	n/a	n/a	n/a	n/a
	Soaked	v	v	v	v	v

Note: v indicates exposure time tested. % indicates the concentration of the bleach used for decontamination. 10^4X is the concentration of the ArtDNA solution used to contaminate the bone samples.

A further test was performed to determine the upper limits of recovery of ancient DNA from 100% bleach treated bones. This was undertaken in much the same manner as previous experiments; however, the bone samples were not contaminated. Four bone samples weighing between 0.35 and 0.37 grams each were soaked in 100% bleach for 1 hour, 3 hours, 6 hours and 24 hours, respectively. The samples were removed from the bleach solution and rinsed in ddH20 for 10 minutes and left to dry. Once dry, the samples were then processed for PCR amplification.

Blank and negative PCR controls serve an important function in an ancient DNA study. A blank extraction control is one in which no bone powder is added to the lysis buffer and this control follows the other samples throughout the process and is indicatory of contamination in the extraction and purification processes. A negative PCR control is a reaction where no DNA extract has been added. This should indicate whether reagents or protocols at the PCR setup stage introduce contaminants. If either sample appears negative after PCR amplification, this indicates that unexpected or systematic contamination is not present in that setup. However if either control shows positive amplification this

indicates that unexpected contamination has taken place. Experiments that result in either control showing positive amplification cannot be trusted and should be eliminated from the study. This is common practice in the field of ancient DNA as each sample in the experiment cannot be trusted because contamination has found its way into the reaction. This protocol was followed in this study.

2.5 DNA Extraction, Amplification and Visualization

2.5.1 DNA Extraction

A modified silica-spin column technique (Yang *et al.* 1998) was used to extract DNA from the bone samples. The bone samples, once dry, were ground into fine powders using both a vice, and a mortar and pestle. The powders were incubated overnight with 3-5ml (dependant upon the amount of bone powder i.e. 1ml of bone powder with 3ml of lysis buffer) of lysis buffer (0.5M EDTA, pH 8.0, 0.5% SDS with 0.5mg/ml proteinase K) in a rotating hybridization oven at 50°C (not 55° because the lid of the plasticware used becomes loose and can cause leakage). After centrifugation (5500 RPM for 20 minutes) to separate the bone powder from the lysis solution, 1.5 – 2.0ml of supernatant was transferred to an Amicon™ Ultra-4 Centrifugal Filter Devices (30KD – 4ml) (Millipore, Billerica, MA., USA) and centrifuged at 5500 RPM for between 40 minutes to an hour for DNA concentration to the 100µl volume or below. Approximately 100µl of the concentrated DNA supernatant from each sample was passed through QIAquick™ (Qiagen) columns for DNA purification (Yang *et al.* 1998). This supernatant was combined with five volumes of PB buffer. The resulting 600µl of

solution was then centrifuged at approximately 10,000 RPM for 60 seconds. After centrifugation, the membrane was washed twice with 400µl of PE buffer (containing ethanol) and centrifuged again (10,000 RPM, 60 seconds). These two steps wash away salts that will interfere with enzymatic reactions. After centrifugation 100µl of EB buffer (10mM Tris-Cl, pH 8.5) was added to each column, incubated at 70°C for 5 minutes and then centrifuged (10,000 RPM, 60 seconds). This step was repeated for a second time resulting in 200µl of DNA being eluted from the column for PCR amplification.

2.5.2 PCR Amplification

PCR amplification was performed using a Mastercycler Personal Thermocycler ™ (Eppendorf, Hamburg, Germany) in a 30µl reaction volume containing 1X Buffer (Applied Biosystems, Ca., USA), 2mM $MgCl_2$, 0.2mM dNTPs, 1.0 mg/ml BSA, 0.3µM each primer, 3µl DNA sample and 1.25 U AmpliTaq Gold™ (Applied Biosystems, Ca., USA). PCR was run for 35-40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 70°C for 30 seconds. Initial denaturation and activation of the enzyme took place at 95°C for 12 minutes and final elongation took place for 7 minutes at 72°.

2.5.3 Electrophoresis and Sequencing

Five µl of PCR product was separated by electrophoresis on a 2% high-resolution agarose gel. Amplified DNA was visualized using SYBR Green I™ (Clare Chemical Research, Co., USA) and samples with visible bands as observed on the gel were purified and sent for direct sequencing to MOBIX

Laboratory at McMaster University in Hamilton, Ontario, Canada. Sequencing took place through use of ABI BigDye terminator chemistry and ABIPRISM® 3100 Genetic Analyzers.

2.5.4 Restriction Enzyme Digestion

Restriction enzyme digestion took place using the HindIII restriction enzyme (InVitrogen) and 2.5µl of PCR product; 0.5U of enzyme was added to 10µl of 1X REact®2 buffer for each sample. Upon addition of PCR product, the tube was incubated at 37°C for one hour, according to the manufacturers' specifications. The digested product was then visualized on a 2% agarose gel.

2.6 Evaluation of Decontamination Techniques Effectiveness

Decontamination techniques were evaluated by four methods. The first method was through visualization of amplicons on agarose gel. This was done by visually assessing presence or absence of the expected amplicons. Secondly, the removal of the contaminant was assessed using restriction enzyme digestion of the ArtDNA. If the amplicon band was digested resulting in two bands on the gel, this represented the additional presence of contaminant DNA and therefore the decontamination method was determined to have failed. Thirdly, to be sure that the contaminant, ArtDNA was indeed not present in extracted DNA, the number of PCR cycles was increased. After this increase, if the ArtDNA amplicon was still not present the decontamination was determined to have been completely and successfully accomplished. As a fourth measure of the decontamination technique's effectiveness, the samples were sent for direct

sequencing. This was intended to determine if there was any damage done to the either of the amplicons because of the decontamination process. As a further verification, all touched whale extractions were repeated once and all amplifications were repeated numerous times.

CHAPTER 3: RESULTS

3.1 Artificial DNA Concentration

The ArtDNA was created according to the criteria set forth in the previous section. The amount of ArtDNA applied to samples was determined by use of a competitive PCR approach. This approach looked at the concentrations at which each fragment, elk and ArtDNA, would begin to compete for reagents. From these experiments it was determined that the original solution of the ArtDNA needed to be diluted approximately 10000X to be preferentially amplified; below this level the elk DNA would overwhelm the ArtDNA, preventing its amplification. As dilution of the ArtDNA 10000X was the lowest concentration to amplify, various concentrations of ArtDNA were applied to bone samples in the first stages of experiments.

3.2 Evaluation of DNA Destruction by Chemicals

Upon exposure to DNA in solution for short periods of time (30 seconds – 1 minute), it became clear that bleach was the best chemical destroyer of DNA. In one minute, 1N HCl and 1N NaOH appeared to damage and denature the DNA, while both 10% and 100% bleach completely eradicated the DNA in solution (Figure 3.1). Further 3% H_2O_2 and DNA Away™ appeared to have little to no effect upon the DNA in solution.

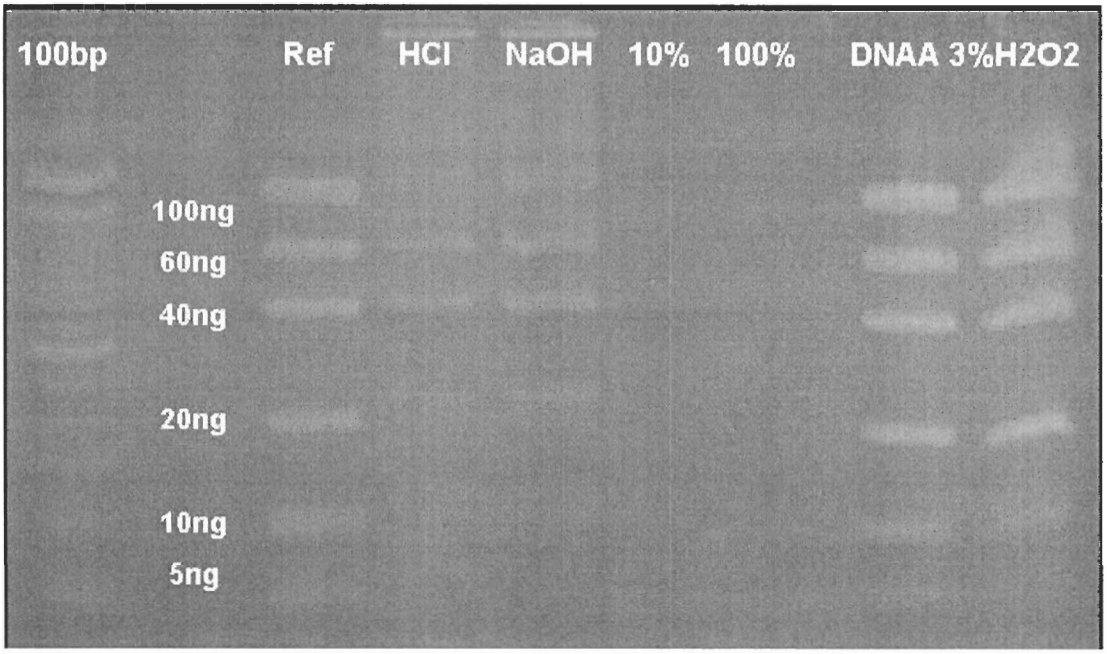

Figure 3.1 Mass ladder exposed for 1 minute to various chemicals.

Note: Ref = 2µl Low Mass Ladder, 10% = 10% Bleach, 100% = 100% Bleach, DNAA = DNA Away (Molecular Bioproducts Inc.)

The concentration of both the bleach and the DNA was a factor in the ability to remove the DNA from solution; 10% bleach was much less efficient when it came to destroying 100bp ladder as it contains much more DNA (2ug) than the Mass Ladder (235ng) shown in Figure 3.3. Even when exposed to 10% bleach for 10 minutes there was still quite a bit of 100bp ladder visible (Figure 3.2.)

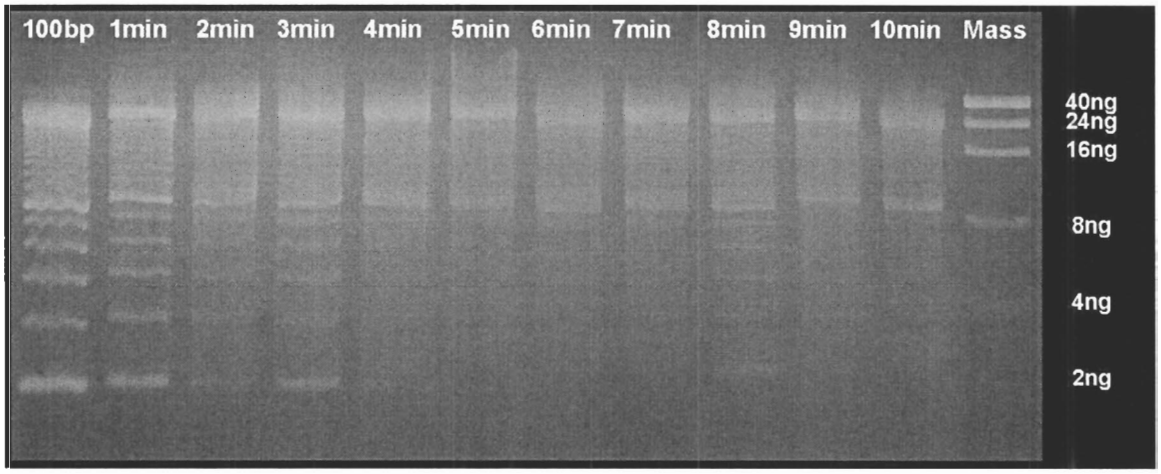

Figure 3.2 Timed series of exposure of 100bp ladder to 10% Bleach.

Note: 2µg of 100bp Ladder (Invitrogen, 1µg/µl). Mass: Untreated mass ladder to show the quantity of DNA in the gel.

As a result of these preliminary experiments, it was decided that the systematic analysis of bone sample decontamination would take place using bleach at concentrations of 50% and 100% for varying time periods.

The effect of bleach on the gel stain (SYBR Green I™) was examined in the three ways described in the previous section, in order to indicate that the chemical was not affecting the efficacy of the gel stain. The first two techniques, sample purification and the substitution of a more sensitive stain, supported the notion that the DNA was indeed absent in the solution visualized by electrophoresis (data not shown). The third technique, the use of sodium sulphite to neutralize the effects of the bleach, was also successful. As displayed in figure 3.3, when bleach was used alone with the mass ladder, no DNA was visible on the gel (lane four). However, figure 3.3 also demonstrates that when bleach and sodium sulphite were applied to the mass ladder in combination, the DNA was visible (lane 10). Further, by comparison of lanes 8 and 10 it is

apparent that the neutralizing affect had taken place because when sodium sulphite was added to the solution after the mass ladder was treated by bleach there was little/no DNA visible. However when the bleach was neutralized and the DNA was then added to the tube the mass ladder was present. Clearly, sodium sulphite was able to neutralize the effects of the bleach on DNA in solution,

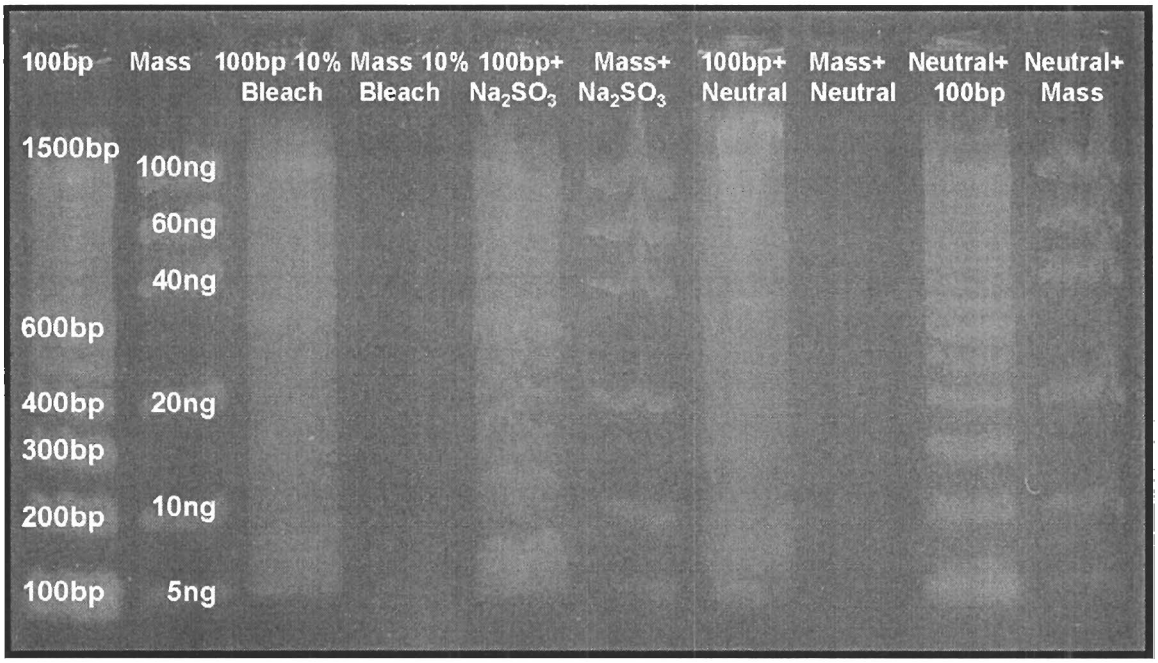

Figure 3.3 Electrophoresis gel showing the effects of 10% bleach after 30 seconds of treatment and Na_2SO_3 on both 100bp ladder and low mass ladder.

3.3 Contamination

The joining of the two oligonucleotides to create one double-stranded 130bp fragment was successful. Very strong amplification was shown on electrophoresis (Figure 3.4.)

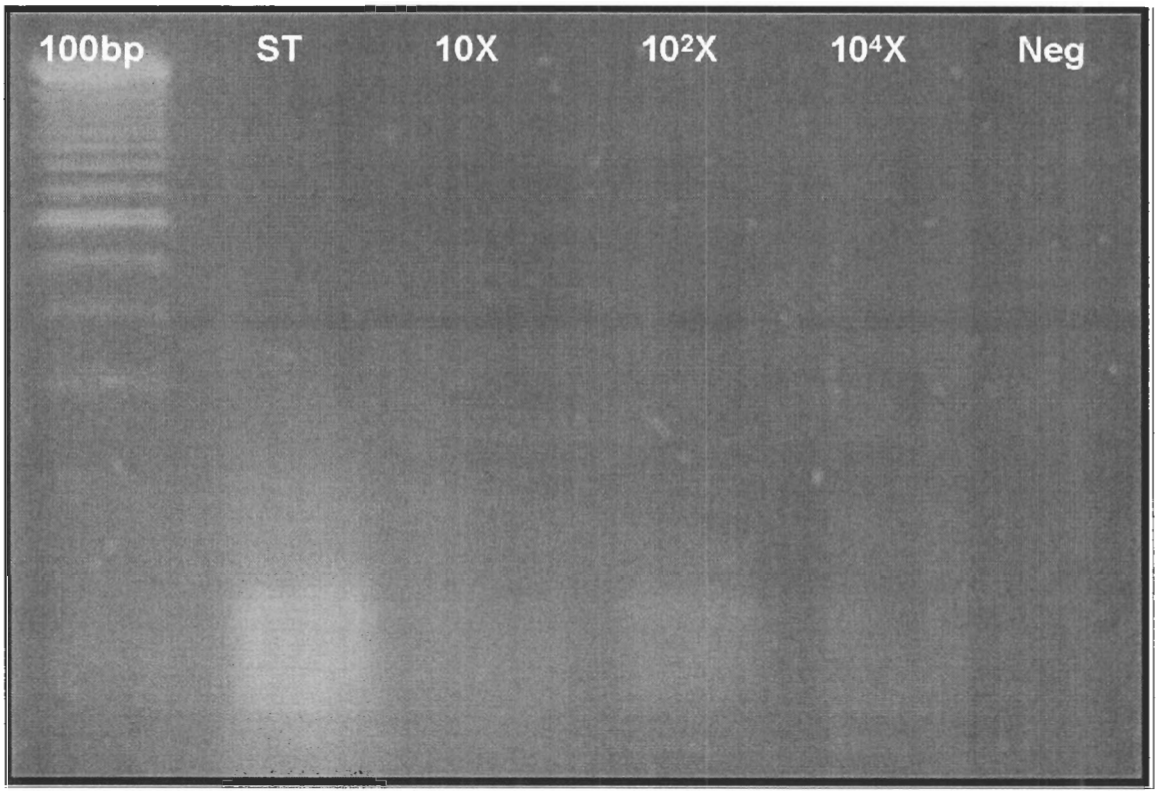

Figure 3.4 Stovetop PCR dilution electrophoresis gel

Note: The first lane after the ladder indicates positive synthesis of double-stranded DNA after Stovetop PCR. ST= Original synthesized ArtDNA, 10X = ST diluted 10X, 10^2X= ST diluted 10^2X, 10^4X= ST diluted 10^4X

The method used to contaminate bone samples under either scenario, handled or soaked, was successful. All bone samples that were soaked resulted in positive amplification of ArtDNA. It is assumed that the bone samples that were handled were also contaminated sufficiently because the positive controls (sample not decontaminated), as well as the samples treated with water and UV only (Figure 3.5) showed amplification of the artificial DNA. Systematic contamination from the environment or human error is assumed to have not occurred, as both the negative PCR setup and blank extraction are clear of any amplification (Figure 3.5). The gel also demonstrated that soaking the bone sample in artificial DNA contaminated the bone tissue much more thoroughly

than merely touching the bone surface with contaminant DNA, as the relative amount of amplified ArtDNA is much higher in the soaked samples.

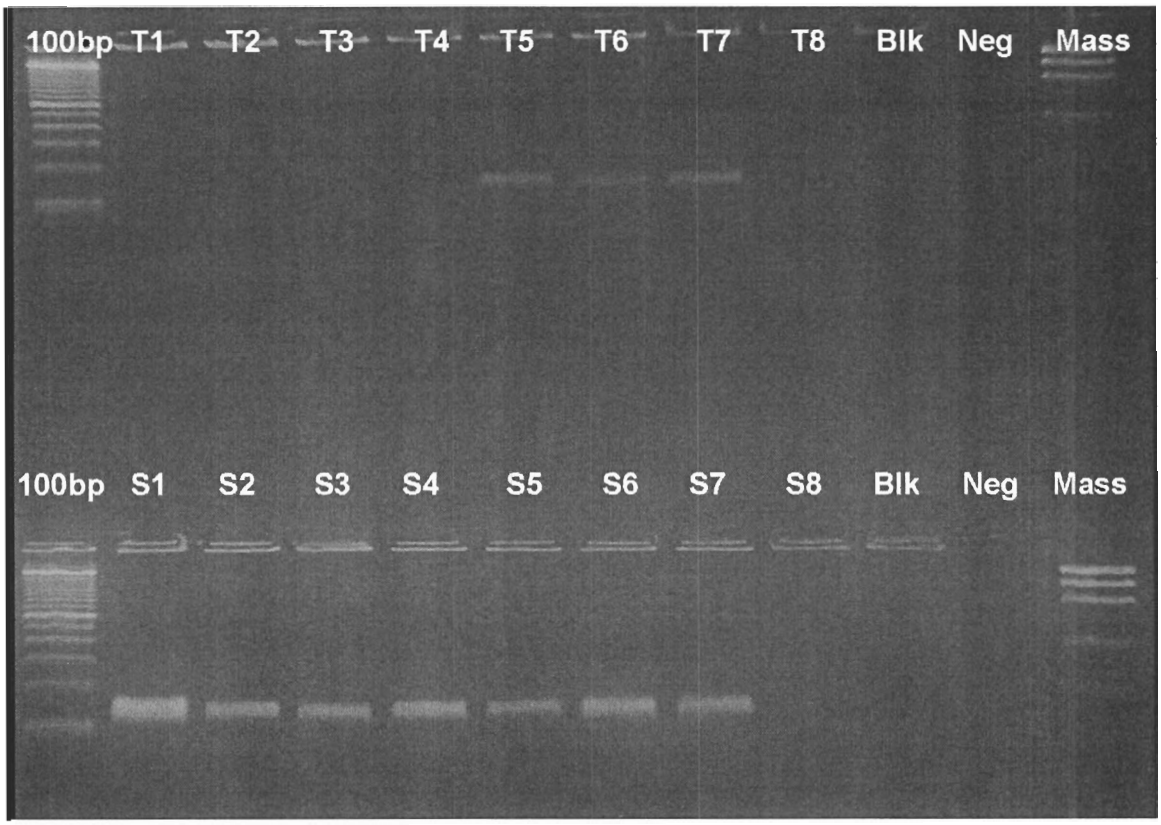

Figure 3.5 Amplified ArtDNA from whale bone after contamination and decontamination

Note: T represents touched and S represents soaked. B = blank extraction and N = negative PCR Setup. These times all represent the length of bleach exposure for decontamination: T1/S1=5mins, T2/S2=10mins, T3/S3=15mins, T4/S4=20mins, T5/S5=10minsH20, T6/S6=UV, T7/S7=Contaminated, T8/S8=Not Contaminated

3.4 Decontamination

All 25 elk samples were contaminated by soaking. Seven of the twelve elk samples amplified after elution of DNA from the surface displayed positive amplifications of ArtDNA. Thirteen elk samples were extracted and amplified in thirty reactions, twenty-four of those reactions displayed positive amplification of the contaminant DNA (Table 3.1).

Table 3.1 Results of the elk experiments excluding those showing visible signs of unintentional contamination

Elution (EL) / Extraction (EX)	# Amps.	ArtDNA Concentration	Bleach Concentration	Time in Bleach	ArtDNA Amp.
EL	1	10^2X ST	50%	5 minutes	1
EL	1	10^2X ST	50%	10 minutes	0
EL	1	10^2X ST	100%	5 minutes	0
EL	1	10^2X ST	100%	10 minutes	0
EL	1	10^4X ST	50%	5 minutes	1
EL	1	10^4X ST	100%	30 seconds	1
EL	1	10^4X ST	100%	1.5 minutes	1
EL	1	10^4X ST	100%	3 minutes	1
EL	1	10^4X ST	100%	5 minutes	1
EL	1	10^4X ST	100%	10 minutes	1
EL	1	10^4X ST	100%	15 minutes	0
EL	1	10^4X ST	100%	20 minutes	0
EX (2)	5	10^4X ST	100%	5 minutes	3
EX (2)	5	10^4X ST	100%	10 minutes	3
EX (2)	5	10^4X ST	100%	15 minutes	4
EX (2)	5	10^4X ST	100%	20 minutes	4
EX (1)	2	10^3X ST	100%	1 minute	2
EX (1)	2	10^3X ST	100%	5 minutes	2
EX (1)	2	10^3X ST	100%	10 minutes	2
EX (1)	2	10^3X ST	100%	15 minutes	2
EX (1)	2	10^3X ST	100%	20 minutes	2
Total	42				31

Note: The number in brackets in the first column indicates the number of extractions performed. ST = Original synthesized ArtDNA solution. Elution is the bone sample soaked in water with the water PCR amplified. Extracted is DNA extracted from the pulverized bone samples. The column titled ArtDNA Amp. indicates the number of times that the contaminant ArtDNA was amplified.

It is clear that some elk samples indicated removal of the contaminant while others, exposed to the same concentration of bleach, for the same time period, still amplified the contaminant. This unexpected inconsistency may be due to uneven contamination or uneven decontamination which was assumed to be caused by the non-homogeneous elk bone texture. However, overall an inability to remove the soaked in contamination was revealed.

Positive amplification of ArtDNA in blank and PCR negative controls also appeared on the electrophoresis gels produced from some of the initial PCR setups in elk experiments. As the decontamination techniques used in this study deal with contamination that is already present on bone samples, the method did not prevent this unexpected contamination from taking place. Although the results of the experiments which included contaminated blanks and negative were not incorporated in the study, they demonstrated how easily contamination could take place even with artificially designed and controlled contamination in a dedicated lab facility. Extra precautions were taken in subsequent elk and whale experiments, resulting in the absence of such unexpected contamination.

Whale bone samples soaked in ArtDNA solution and decontaminated with 100% bleach for up to 20 minutes still showed strong amplification of the contaminant DNA (see figure 3.5). Samples soaked in ArtDNA and exposed only to UV demonstrated a reduction in the amount of amplified contaminant when compared to the positive control, but not its eradication. A different pattern was seen in the samples that were handled with, rather than soaked in, the contaminant DNA. Unlike the soaked samples, 100% bleach removed all

amplifiable contaminant DNA in all four timed experiments of surface contaminated samples. In the initial experiment, the ArtDNA was not amplifiable after as little as one-minute exposure to 100% bleach. However, in a repeated extraction, minimal contamination was amplified after one minute of bleach exposure (data not shown), though amplification was not evident after exposure for 5, 10 or 20 minutes. As in the soaking experiments, UV irradiation did not eliminate the amplification of contaminant DNA from handled samples, although amplification strength was reduced. Water was incapable of contaminant removal in both the soaking and handling experiments. Those samples not treated with bleach (i.e., samples treated with UV and water only) resulted in weaker amplification of whale and a stronger presence of ArtDNA when compared to bleach treated samples. The positive whale control sample, left completely untreated by any contamination or decontamination method never resulted in amplification of either whale or ArtDNA (Figure 3.5). In experiments using whale bone, 100% bleach removed contamination from 92% touched samples, while in 0% of the soaked samples could contamination be removed from the bone (Table 3.2).

Results also indicated that the authentic whale DNA was still amplifiable after 20 minutes of exposure to 100% bleach. Surprisingly, whale DNA in samples treated with 100% bleach for any length of time was also observed to be more readily amplified than those not treated with bleach (Figure 3.6). Further, the negative and blanks appeared clear of contamination occurring from laboratory reagents or human error (Figure 3.6).

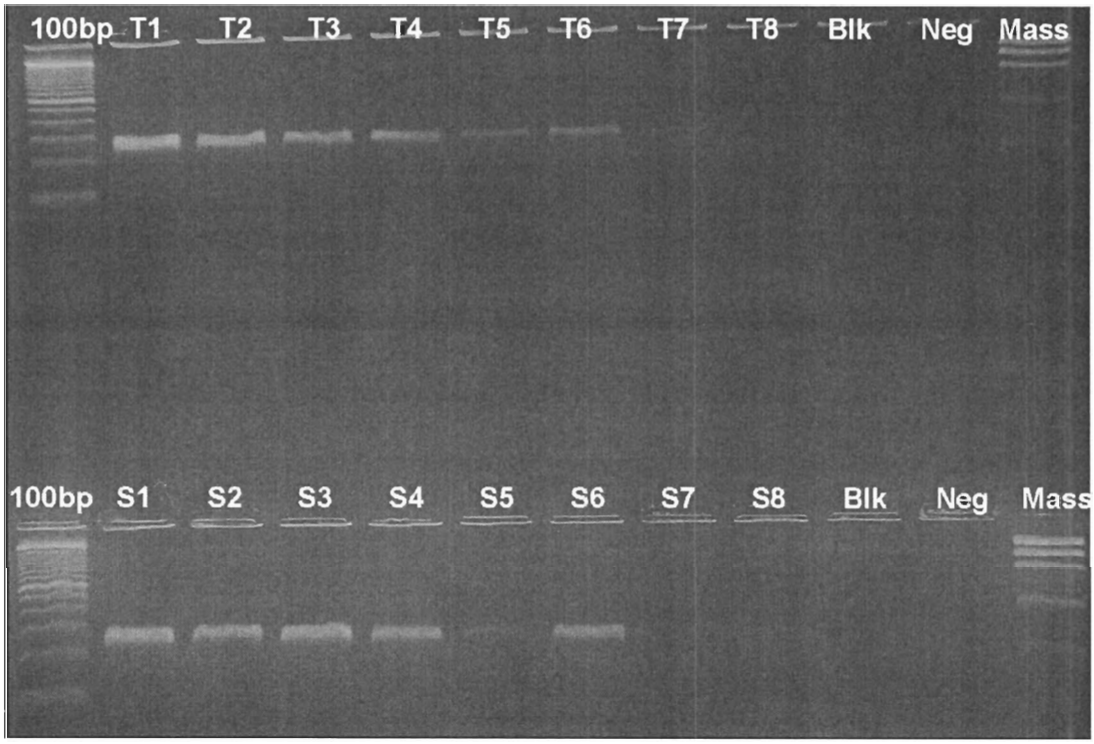

Figure 3.6 Amplified whale DNA after contamination and decontamination with 100% bleach for varying time periods

Note: T represents touched and S represents soaked. B = blank extraction and N = negative PCR Setup. These times all represent the length of 100% bleach exposure for decontamination: T1/S1=5mins, T2/S2=10mins, T3/S3=15mins, T4/S4=20mins, T5/S5=10minsH20, T6/S6=UV, T7/S7=Contaminated, T8/S8=Not Contaminated

Table 3.2 Summary of the results of whale experiments

Extraction	# Amps.	Time in Bleach	ArtDNA Amplified
Soaked 1	2	1 minute	2
1	2	5 minutes	2
1	2	10 minutes	2
1	2	20 minutes	2
Total	8		8
Touched 2	3	1 minute	1
2	3	5 minutes	0
2	3	10 minutes	0
2	3	20 minutes	0
Total	12		1

Note: The number in the first column indicates the number of extractions performed. ArtDNA concentration of 10^3X and 100% bleach were used in all tests.

Further experiments were performed in order to determine the limits on recovery of amplifiable ancient DNA after treatment with 100% bleach. The results of this experiment clearly show that ancient DNA is amplifiable after being submerged in 100% bleach for periods of 1 hour, 3 hours, 6 hours, and even 24 hours (Figure 3.7). Additionally both the blank extraction and negative PCR setup were clear of visible contamination.

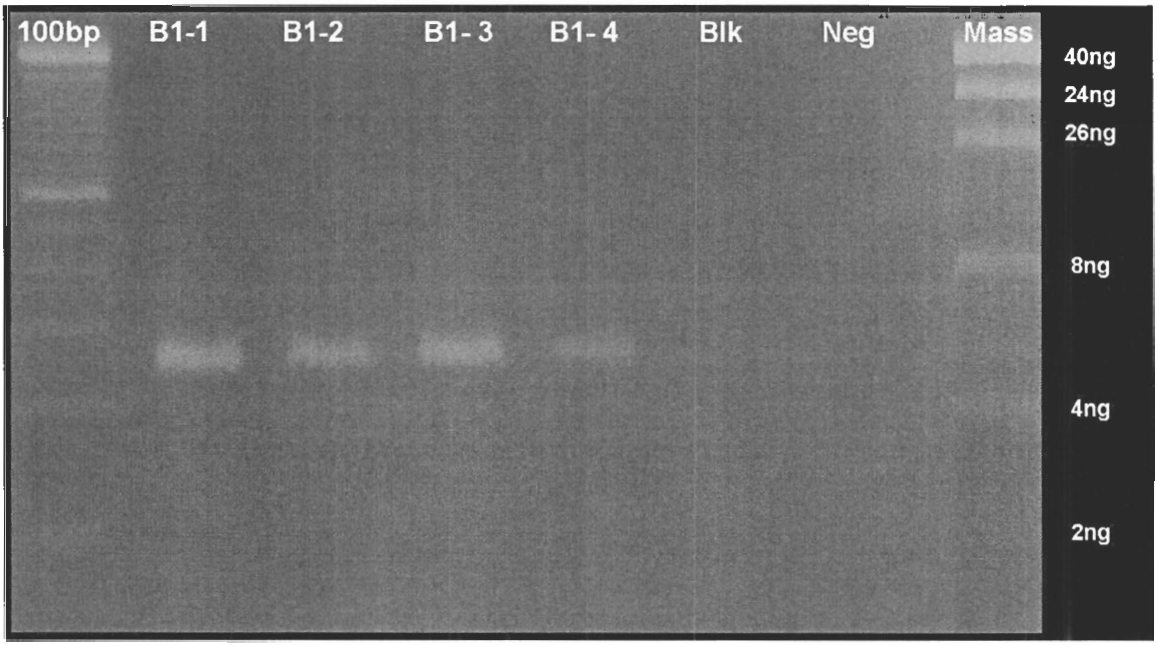

Figure 3.7 Electrophoresis gel showing results of bone exposed to 100% bleach for long periods of time.

Note: B1-1 = 1 hour, B2-1= 3 hours, B3-1=6 hours, B4-1= 24 hours, BB-1=blank extraction, N=negative setup. PCR Conditions: 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 70°C for 30 seconds.

Of the 40 uncontaminated amplifications of elk (n=16) and whale (n=24) samples included in the study that were treated with bleach, 100% resulted in the successful recovery and amplification of the authentic target DNA. This was assessed by the presence of a band on electrophoresis gel. The negative and blank extraction controls from these experiments did not result in the

amplification of any product. This pattern as shown (Table 3.3) was also repeated from different extractions of whale bone.

Table 3.3 Summary of recovery and amplification of target ancient DNA after 100% bleach treatment of various lengths of time.

Species	Extraction	# Amps.	Time in Bleach	Ancient DNA Amplified
Elk	1	4	5 minutes	4
Elk	1	4	10 minutes	4
Elk	1	4	15 minutes	4
Elk	1	4	20 minutes	4
Whale	3	5	1 minute	5
Whale	3	5	5 minutes	5
Whale	3	5	10 minutes	5
Whale	3	5	20 minutes	5
Whale	1	1	1 hour	1
Whale	1	1	3 hours	1
Whale	1	1	6 hours	1
Whale	1	1	24 hours	1
Total		40		40

Note: The number in the second column indicates the number of extractions performed.

The amplified PCR products from all handled samples were purified and sent for direct sequencing using the whale primers, while the PCR products of the soaked samples were purified and sent for sequencing with the ArtDNA primer. The soaked whale samples were not sequenced using the whale primer because it was felt that it was unlikely that they would offer any further information regarding destruction to DNA. Upon receipt, sequence analysis was carried out through visual editing, and multiple alignments were created using ClustalW (Thompson, *et al.* 1994), through BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html). As expected, for samples prepared

from the same whale individual, all whale amplicon sequences matched each other exactly and were correctly identified as Grey whale (*Eschrichtius robustus)*. All ArtDNA amplicon sequences matched each other as well as the original created sequence. All sequences were of similar quality and were clear of background noise.

CHAPTER 4: DISCUSSION

4.1 DNA Selection

There were a number of criteria that were involved in the design and selection of the DNA marker used to contaminate bone samples in this study. The use of an animal model as opposed to a human model was important in order to rule out contamination introduced at unknown times by unknown individuals. This also lessened the concern about researchers contributing to the contamination problem. Further, as human remains of any antiquity are valuable materials, the use of them for such a project raised ethical concerns.

Controlling and monitoring the contamination throughout this project was also of utmost interest. As a result, the creation of a fragment of DNA that resembled a natural entity, yet not found in the environment was necessary. The use of restriction enzyme sites as well as randomly synthesized fragments not only fulfilled this need but also allowed for ease of ArtDNA identification after amplification.

Competitive PCR was used to determine the amount of contaminant DNA that was to be applied to bone samples in order to simulate contamination in an ancient DNA study. This was done by assessing the amplification strength of the two fragments when they were added to the PCR reaction in different concentrations. This allowed for visualization of the point at which one fragment out competed the other during amplification. This method was appropriate when

the subjects of the study were elk, as the two DNA fragments allowed for co-amplification, and competed against each other.

Unfortunately, it was necessary to change the species of the animal model due to technical problems. Obviously, one drawback of changing the study animal from elk to whale was that competitive PCR could no longer be used as a technique to determine how much contamination to apply to bone samples. Consequently, it was decided that no matter how much DNA was preserved in the whale bone samples, the amount of contaminant determined from the competitive PCR experiments would likely be sufficient. This is due to the fact that ancient DNA would be out-competed by modern undamaged DNA no matter how much of either was present in the samples.

Along with the lack of competitive PCR, came an inability to use one primer set for amplification of both ArtDNA and the animal DNA. The latter problem was overcome by the use of two primer sets: a whale primer set that did not amplify the artificial DNA and the elk/Art set that did not amplify the whale DNA. Although the ArtDNA and authentic target did not have to compete for amplification within a single PCR reaction, this new technique did allow for clear verification of both contaminant and authentic DNA amplification.

4.2 Bone Selection

Bone sample selection appears to have been one of the most important steps in the design of a project of this nature. The fulfilment of the criteria set out in a previous section regarding bone preservation, homogeneity, and antiquity

were necessary for success of the design and completion of this project. At the beginning of this project, both elk and whale were suggested as possible subjects for the animal proxy as both were readily available. However, because of concerns regarding the use of a marine mammal as opposed to a terrestrial mammal Elk was the original chosen animal proxy. Unfortunately, because of the limitations involved with the elk bone, whale was substituted as the animal proxy.

There has been little study regarding how contamination affects different bone samples and the correlation, if any, between different types of bone and the amount of contamination present (Gilbert *et al.* 2005). The elk experiments in this study demonstrated that not all bones may absorb contamination evenly, and that contamination may vary significantly between samples.

The particular specimen of whale that was incorporated into this project had been successfully analyzed previously in the ancient DNA laboratory for another project. As whales are extremely large marine mammals, single elements are often quite large, with substantial portions of cortical bone, resulting in sizeable homogeneous bone samples for contamination experimentation. As was noted before, concerns were raised regarding the use of a marine mammal for this type of study. As an adaptation to life in water, whale bone is typically more porous, (less dense) than terrestrial mammal bone (Pabst and Rommel *et al.* 1999), a characteristic which could unduly affect the amount of contamination the bone may take up when handled, and particularly when soaked. The results of this study seem to indicate that fortunately, this does not seem to be the case

with the handled samples, since surface contamination was easily removed with bleach. However, some of the soaked samples still appeared to contain a significant amount of ArtDNA even after longer exposure to bleach (See Figure 3.5, Sample S4). Further study needs to be undertaken to determine the relationship between contamination and bone porosity.

The issues revolving around contamination and bone sample selection also raises concerns regarding project design. This study demonstrated that elk bones were not able to reveal any repeatable pattern in terms of contamination and decontamination while whale bones clearly demonstrate a recognizable and repeatable pattern. The results of this study, though preliminary, seem to indicate that the effects of contamination are variable between bone type, element and species; ancient DNA researchers should acknowledge this variability and design their project accordingly, in order to monitor and counteract potential inconsistencies of contamination.

4.3 Evaluation of Chemicals for DNA Destruction

The results of the first set of experiments in this study indicated that bleach was the most effective chemical in destroying DNA in solution. However, the effectiveness of bleach was affected by the concentration of DNA contained in the solution. When higher concentrations of DNA were used, a higher concentration of bleach, or a longer exposure time was also necessary to destroy the DNA. Although other chemical methods appeared to be ineffective or inefficient at destroying the DNA in solution, this does not mean that they are not capable of doing so. 1N HCl is a very strong acid, which is known to hydrolyse

DNA (Savage and Plaut. 1958). The results of this experiment are likely due to the length of time with which the DNA was exposed to this chemical; thirty seconds or 1 minute may not be a sufficient time period to effectively depurinate and denature the DNA. The same is likely true for the other chemicals examined, particularly NaOH and 3% hydrogen peroxide. The commercial product examined in these tests, DNA Away™ (Molecular Bioproducts Inc.) appeared to have no effect upon the DNA in solution. However, this does not indicate that the product is ineffective on surfaces or dry DNA, which according to the manufacturer's specifications, is the intended use of the product (http://www.mbpinc.com/Store/productpdf/TechReport_205.pdf).

4.4 Staining Chemicals for Evaluating DNA Destruction

Bleach was determined to be the best chemical for use in the destruction of DNA. However, three concerns resulted from this series of experiments when SYBR Green was used to pre-stain the bleach-treated DNA: 1) was SYBR Green/Gold affected by bleach in concentrations greater that 10%; 2) can we neutralize the effects of bleach in the samples to be sure that its effects were completely stopped prior to staining; and 3) is SYBR Green sensitive enough to reveal remnant DNA? The first concern was examined by purifying a DNA sample that was bleached, and visualizing the sample on a gel, as well as by after-staining (rather than pre-staining) an electrophoresis gel loaded with bleached DNA samples. No DNA was visualized on the gel in either experiment, suggesting that bleach was indeed destroying the DNA and not merely counter-acting the effects of the SYBR green. The second concern, whether the effects of

bleach could be neutralized, was tested using sodium sulphite (Na2S03) a chemical commonly used in water treatment (Environmental Protection Service. 1985). Experiments were first run to see whether this chemical would affect the DNA greatly and if it would indeed neutralize the bleach. It was shown clearly on the gel that 10% bleach could eradicate the mass ladder within 1 minute (Figure 3.3.) however, when Na_2SO_3 was added to bleach prior to being added to the DNA, the mass ladder was still present. Clearly, the bleach could be neutralized by sodium sulphite. The third concern, the sensitivity of the stain, was addressed by substituting with SYBR Gold™, a staining chemical that is sensitive to less than 100pg of double-stranded DNA (dsDNA). When this stain was used, it was assumed that a lack of DNA visualized on the gel meant that no DNA was present in the sample. In sum, the study indicated that SYBR Green I™ staining was both effective and sensitive enough to visualize bleach-treated DNA on the agarose gel.

4.5 Contamination

The method used here for creating an artificial source of DNA that could be monitored throughout the laboratory was very successful. The use of an artificially designed fragment of DNA has previously been used to quantify the number of templates in ancient DNA studies through use of competitive PCR (Marota, *et al.* 2002, Jehaes, *et al.* 2001) and it can also be used to design contaminant DNA. Future studies in this lab could attempt to use this fragment as a marker for systematic laboratory contamination by checking reagents, amplifications and surfaces for its presence. The technique used for the creation

of a fragment that mimics contamination of a specific entity could also be used in other studies because the number of safeguards incorporated within the fragment allows for ease of identification.

One potential problem however could arise from the synthesis of the double-stranded DNA: carry-over contamination. This was counteracted by strict control over the travel of PCR products. As stated earlier, movement of personnel or PCR products from the post PCR laboratory to the extraction lab is under no circumstances allowed. The reason for this is that as has been noted PCR products can cause detrimental contamination to a study. This is further exemplified by the fact that laboratory areas must be geographically separated to prevent this. In this experiment, the creation of the double-stranded fragment of ArtDNA was performed in a neutral area and primers were excluded from the reaction; this allowed for only the joining and extension of the two strands and not the exponential amplification of them.

The techniques used for contamination of bone samples appeared to have been successful based on the appearance of ArtDNA in amplifications where both negative and blank extractions were clear. Although, the possibility always exists that some samples were not contaminated as much as others, because all of the whale samples were soaked in the same solution they should contain similar amounts of contaminant DNA. Accordingly, all of the whale samples that were soaked revealed contaminant DNA upon amplification (Figure 3.5). For the samples that were handled, there is a real possibility that some samples could have been contaminated more than others. However, it seems unlikely that only

the samples untreated with bleach were successfully contaminated with ArtDNA through handling, particularly because these bone samples were picked randomly after contamination for each process. Further, it seems highly improbable that only the samples that were treated with bleach would not contain any amplifiable ArtDNA in repeated extractions and amplifications. Nevertheless, this study has illustrated that the use of these two techniques, i.e., blank extractions, and negative controls, are suitable and effective for tracking systematic contamination.

Another important question to consider is the amount of contamination used. Unfortunately, there is currently no knowledge regarding the amount of contamination that is actually deposited on bone via touching even with a known quantity of DNA applied to the bone. The quantity used here may in fact be an excessive amount of contamination; however, if this is the case, the outlook for decontamination may be quite good, as even this unreasonably high amount of contamination was eradicated from bone surfaces. However, as we can see from the results of the soaking study, the problem of deeply imbedded contaminant DNA still exists. It is believed that the technique used for contaminating the bone samples was effective (both in handling and soaking) and could be used again for other studies.

4.6 Decontamination

This study has demonstrated that the use of 100% household bleach is the most effective decontamination chemical available for destroying surface contaminant DNA. However, it is also clear from this study that when a sample

has been soaked in a contaminant DNA, even this concentration of bleach for upwards of 20 minutes will not remove the contaminant. Further, the presence of only ArtDNA in some of the elk extractions illustrates that exogenous modern contaminant DNA can clearly out-compete the authentic ancient DNA within bone. This observation indicates that when attempting to examine ancient DNA, we must take all possible precautions to remove contamination prior to extraction. We must treat all samples as if they are contaminated because you can never be sure that the sample is contaminant free.

The bone samples exposed to UV rays were not clean of ArtDNA. As had been stated UV cross-links DNA when in solution, however apparently this takes a much longer time when dry (Hall and Ballantyne. 2004). Although this study is not the first to use UV as a technique for decontamination of bone samples, it clearly demonstrates that UV is ineffective at removing/inactivating contaminant DNA after 30 minutes of exposure on all sides of the bone. The bone samples were wet when exposed to UV to help create a suitable environment for the reaction to occur. However, irradiation was not completely effective as the ArtDNA was clearly amplifiable. Studies examining bone DNA and the effects of UV are clearly lacking in the literature, however many laboratories use this technique, whether alone or in combination, for decontamination. Further examination of how bone DNA and UV react, as well as the conditions under which UV irradiation is successful and the appropriate time needed for effective decontamination are required.

The soaking experiments illustrated a situation where contaminant DNA can penetrate deeply into bone. Unfortunately, this type of contamination could not be removed by exposure to 100% bleach for up to 20 minutes. This raises some serious issues for studies that use remains that have been collected prior to the advent of ancient DNA studies. This is because many excavations take place under less than clean conditions. Many archaeologists wash bone fragments in dirty water, which for human remains, could mean that they are deeply contaminated by a variety of DNA sources. Bleach, apparently even after 20 minutes of exposure is not able to remove this type of contamination. Many factors may be involved in the process of soaked in contamination, including the porosity of bone samples and the nature of the relationship between DNA and bone mineral; these variables must be further explored. If the results from this study are any indication of the likelihood of removing this type of contamination, then clearly there needs to be further exploration into removing deeply invading contaminant DNA.

Due to the duration of exposure of bone to a high concentration of bleach, questions of safety to the DNA housed within skeletal remains were raised. To address this issue, experiments were performed that exposed non-contaminated samples to 100% bleach for varying periods of time. The results of these tests clearly indicate that although the template number may be reduced, positive amplification of ancient samples still occurs not only after 20 minutes of treatment with 100% bleach but up to 24 hours after. This indicates that the bone mineral must serve a protective function for the DNA housed inside it. There is little

known about this mechanism, but it has been hypothesized that DNA binds to the mineral hydroxyapatite (Okazaki *et al.* 2001). The idea of bone mineral as a protective barrier is also supported by the data that the samples soaked in the contaminant solution positively amplified contaminant DNA even after 20 minutes in 100% bleach, while the DNA in solution, without the protection of bone mineral was eradicated by exposure to similar durations and concentrations of bleach. The results suggest that even the ArtDNA may be protected in a similar manner by the bone mineral or that uneven decontamination has taken place due to bone porosity though further study of this phenomenon is needed.

Contaminant DNA on the surface of bones however is clearly not protected in the same manner as either the soaked ArtDNA or the authentic DNA within the bone. Contaminant DNA that lay naked upon the surface of bone samples appears to be readily eliminated after approximately one minute of exposure to 100% bleach. Repeated extraction however, displayed that a weak band of ArtDNA amplified after one-minute exposure to bleach (data not shown). This discrepancy is likely due to two possibilities: the randomness of the contamination application and/or the stochastic nature of PCR amplification of low copy samples. These results indicate the necessity of repeated extractions and the use of at least 5 minutes of bleach for decontamination.

Sequencing results were expected to demonstrate that high concentrations of bleach for varying lengths of exposure would indeed cause damage to the authentic DNA housed inside the bone, as indicated by the varying levels of PCR amplification. However this was not the case, all

sequences were identical, indicating that little damage was being caused through base pair modification. It is now believed that it is not the final sequence that shows the damage but the net effective template that is available for PCR amplification. For example, let us say that a piece of bone begins with 20 complete template strands. After decontamination with 100% bleach for 1 minute three strands are nicked and broken leaving 17 net effective templates for PCR amplification. It would be logical to assume that this would get worse as exposure time increases causing only five net effective template strands left for PCR amplification. This would in turn not result in modified sequences at all because those five net effective template strands are still ample models for PCR amplification. The only time this effect would be visible is when amplifying at a high cycle number with extremely low original template number or in clones of PCR products.

Recently, Kemp and Smith (2005) published online the first systematic study of bleach as a surface contaminant remover using human bone. The study contaminated Native American bone samples via handling by the author for approximately 30 minutes. The results of the current study are consistent with those in Kemp and Smiths study. Their data show that the high concentration of bleach (50% - 100%) is adequate to remove surface contamination in all tested samples. This result is also observed here. It is clear that in this study the use of an animal model, controlled amounts of contamination and more sensitive staining chemicals have helped to address issues more effectively. For example to ensure that low amounts of amplifiable contaminant DNA left after treatment

could still be visualized, this study used SYBR Green I™ and SYBR Gold™ which are much more sensitive than ethidium bromide(Jin *et al.* 1994, Schneeberger *et al.* 1995). Although our soaked tests may "exaggerate" the real amount of contamination, it clearly points to the possibility that the heavily and deeply contaminated bone samples might not be easily decontaminated by bleach for 10 minutes. More research is needed to determine if a longer time in, or stronger concentration of sodium hypochlorite should be used to remove the contamination.

From this study, it is recommended that bone samples first have the surface removed through abrasion then each sample should be soaked in 100% household bleach for 5 – 10 minutes, with the exposure time determined by the state of bone preservation, and the probable amount of contamination. This is recommended for several reasons. Although quite clearly most of the contaminant DNA has been eliminated after one minute of exposure to 100% bleach we feel that 5 minutes is a more realistic and manageable time frame to work within, particularly when processing many samples. Secondly, although 10 minutes could be considered quite a lengthy exposure, it still falls well within the time limits for recovery of authentic DNA and if the samples are heavily contaminated a long time of exposure may be necessary to remove all the contaminant DNA. Unfortunately, there is no clear-cut answer regarding decontamination. Nevertheless, it is important to be sure that the method used is effective enough to retrieve authentic results. The use of a rigorous decontamination technique does not preclude researchers who are studying

ancient DNA from following standards of authentication (see Cooper and Poinar. 2000, Pääbo *et al.* 2004). The knowledge gained through testing decontamination techniques can allow for more confidence that ancient DNA results are authentic, but is not cause to abandon previous criteria for authentication. Ancient DNA researchers still need to hold results to the standards that have been previously put forth.

4.7 Bleach and Removal of PCR Inhibitors

Additionally, it was observed that when 100% bleach was used for any amount of time stronger amplification of authentic DNA was seen (Figure 3.5). Based on band strength comparison to those samples that were not treated at all or those not treated with bleach it is suggested that bleach performs a second function, as an inhibition remover. This was tested by a few means; first, the extracts were spiked with known amounts of human DNA and second extra Taq enzyme was added to increase PCR amplification efficiency. The first experiment revealed that those samples untreated by bleach were indeed suffering from some inhibition (some samples more than others). The second experiment confirmed this result as all samples amplified more strongly and even a sample that had never been previously amplified showed a strong band.

Inhibition is a part of every ancient DNA extract, and may originate from a variety of sources including Maillard reaction products of altered carbohydrates (Brown and Brown. 1992) products found in soil: fulvic and humic acids, tannins and metal ions (Tuross. 1994, Hanni *et al.* 1995), DNA itself due to damage (Pääbo *et al.* 2004, Pusch *et al.* 1998), bacterial DNA (Pääbo. 1989), and

collagen I, (Scholz *et al.* 1998), may also cause PCR inhibition. Inhibitors affect the efficiency of the Taq polymerase enzyme, producing only faint bands or no bands during electrophoresis (Hummel. 2003). Ancient DNA extracts are plagued by the fact that if we do not purify the DNA, and sometimes even if we do, the resulting extract will be a brownish colour (Pääbo. 1990, Yang *et al.* 1997). If this colour is not removed via purification and extraction procedures, the likelihood is that amplification will not occur. During our extraction process, it was noted that those samples that were bleached all resulted in a much lighter colour following hybridization in lysis buffer. This is possibly evidence of inhibitor removal. Other techniques have been used to combat inhibition including the use of Bovine Serum Albumin (BSA) in the PCR reaction (Cooper. 1994), addition of more enzyme or dilution of extract (Brown and Brown. 1992), Chelex purification (DelRio *et al.* 1996) and silca-spin column purification (Yang *et al.* 1997). It is hypothesized here, that the stronger amplification observed is the result of a variety of possibilities. These include the removal or elimination of bacterial and fungal DNA, the elimination of chemicals that are leached into the bone via burial allowing for a clear view of the ancient DNA, the breakdown of the bone mineral including collagen, or a combination of these, allowing more DNA to be released. Further study of this phenomenon needs to be undertaken to determine what exactly is taking place.

CHAPTER 5: CONCLUSIONS

This study has illustrated an effective way to evaluate decontamination techniques. It has demonstrated that using an animal bone model and ArtDNA we can thoroughly appraise decontamination methods. An animal model eliminates the omnipresent human contamination in the environment and allows for clearer evaluation of the technique's capabilities. The use of an artificially created DNA fragment that closely resembles an existing species but does not exist in nature allows for clear control and manipulation of that fragment. Stemming from this are clearer observations regarding the effectiveness of decontamination techniques.

This study has determined that the use of 100% bleach for duration of between 5 and 10 minutes should be the most effective decontamination technique available as it is a cost-effective and relatively easy procedure to undertake. The length of time however is up to the researchers' discretion and should be determined based on the amount of probable contamination and the morphological preservation of the sample. This rigorous protocol was not determined to be of great detriment to the authentic DNA housed within the bone even after 24 hours of exposure.

In this study, the use of 100% bleach was actually observed to have increased the DNA yield after PCR amplification, probably due to the removal of

inhibitors. Further studies need to be undertaken to examine the interaction between bleach and bone to determine what is actually occurring.

There is much to be gained by the understanding and optimization of decontamination procedures. We are more able to be confident in the authenticity of ancient DNA results because we can be surer that we have eliminated surface contamination. Additionally we can eliminate extra steps that may allow for the introduction of other contamination. Further, we may gain a better understanding of the interaction between bone mineral and the DNA within it.

REFERENCE LIST

Adachi, N., K. Umetsu, W. Takigawa, and K. Sakaue
2004 Phylogenetic analysis of the human ancient mitochondrial DNA. *Journal of Archaeological Science* 31(10):1339-1348.

Adcock, G.J., E.S. Dennis, S. Easteal, G.A. Huttley, L.S. Jermiin, W.J. Peacock, and A. Thorne
2001 Mitochondrial DNA sequences in ancient Australians: Implications for modern human origins. *Proceedings of the National Academy of Sciences of the United States of America* 98(2):537-542.

Albrich, J.M., C.A. Mccarthy, and J.K. Hurst
1981 Biological Reactivity of Hypochlorous Acid - Implications for Microbicidal Mechanisms of Leukocyte Myeloperoxidase. *Proceedings of the National Academy of Sciences of the United States of America-Biological Sciences* 78(1):210-214.

Alonso, A., P. Martin, C. Albarran, P. Garcia, O. Garcia, L.F. de Simon, J. Garcia-Hirschfeld, M. Sancho, C. de la Rua, and J. Fernandez-Piqueras
2004 Real-time PCR designs to estimate nuclear and mitochondrial DNA copy number in forensic and ancient DNA studies. *Forensic Science International* 139(2-3):141-149.

Alonso, A., S. Andelinovic, P. Martin, D. Sutlovic, I. Erceg, E. Huffine, L.F. de Simon, C. Albarran, M. Definis-Gojanovic, A. Fernandez-Rodriguez, P. Garcia, I. Drmic, B. Rezic, S. Kuret, M. Sancho, and D. Primorac
2001 DNA typing from skeletal remains: evaluation of multiplex and megaplex STR systems on DNA isolated from bone and teeth samples. *Croatian Medical Journal* 42(3):260-266.

Arismendi, J.L., L.E. Baker, and K.J. Matteson
2004 Effects of processing techniques on the forensic DNA analysis of human skeletal remains. *Journal of Forensic Sciences* 49(5):930-934.

Arndt, A., W. Van Neer, B. Hellemans, J. Robben, F. Volckaert, and M. Waelkens
2003 Roman trade relationships at Sagalassos (Turkey) elucidated by ancient DNA of fish remains. *Journal of Archaeological Science* 30(9):1095-1105.

Austin, J.J., E.N. Arnold, and R. Bour
2002 The Provenance of Type Specimens of Extinct Mascarene Island Giant Tortoises (*Cylindraspis*) Revealed by Ancient Mitochondrial DNA Sequences. *Journal of Herpetology* 36(2):280-285.

Austin, J.J., E.N. Arnold, and C.G. Jones
2004 Reconstructing an island radiation using ancient and recent DNA: the extinct and living day geckos (Phelsuma) of the Mascarene islands. *Molecular Phylogenetics and Evolution* 31(1):109-122.

Austin, J.J., and E.N. Arnold
2001 Ancient mitochondrial DNA and morphology elucidate an extinct island radiation of Indian Ocean giant tortoises (Cylindraspis). *Proceedings of the Royal Society of London Series B-Biological Sciences* 268(1485):2515-2523.

Austin, J.J., A.J. Ross, A.B. Smith, R.A. Fortey, and R.H. Thomas
1997 Problems of reproducibility - Does geologically ancient DNA survive in amber-preserved insects? *Proceedings of the Royal Society of London Series B-Biological Sciences* 264(1381):467-474.

Bar-Gal, G.K., P. Ducos, and L.K. Horwitz
2003 The application of ancient DNA analysis to identify Neolithic caprinae: A case study from the site of Hatoula, Israel. *International Journal of Osteoarchaeology* 13(3):120-131.

Barnes, I., J.P.W. Young, and K.M. Dobney
2000 DNA-based identification of goose species from two archaeological sites in Lincolnshire. *Journal of Archaeological Science* 27(2):91-100.

Barnes, I., K.M. Dobney, and J.P.W. Young
1998 The molecular palaeoecology of geese: Identification of archaeological goose remains using ancient DNA analysis. *International Journal of Osteoarchaeology* 8(4):280-287.

Baron, H., S. Hummel, and B. Herrmann
1996 *Mycobacterium tuberculosis* Complex DNA in Ancient Human Bones. *Journal of Archaeological Science* 23(5):667-671.

Bathurst, R.R., and J.L. Barta
2004 Molecular evidence of tuberculosis induced hypertrophic osteopathy in a 16th-century Iroquoian dog. *Journal of Archaeological Science* 31(7):917-925.

Belak, S., and A. Ballagipordany
1993 Experiences on the Application of the Polymerase Chain-Reaction in a Diagnostic Laboratory. *Molecular and Cellular Probes* 7(3):241-248.

Bender, K., P.M. Schneider, and C. Rittner
2000 Application of mtDNA sequence analysis in forensic casework for the identification of human remains. *Forensic Science International* 113(1-3):103-107.

Beraud-Colomb, E., R. Roubin, J. Martin, N. Maroc, A. Gardeisen, G. Trabuchet, and M. Goossens
1995 Human beta-globin gene polymorphisms characterized in DNA extracted from ancient bones 12,000 years old. *American Journal of Human Genetics* 57(6):1267-1274.

Bouwman, A.S., and T.A. Brown
2005 The limits of biomolecular palaeopathology: ancient DNA cannot be used to study venereal syphilis. *Journal of Archaeological Science* 32(5):703-713.

Bouwman, A.S., and T.A. Brown
2002 Comparison Between Silica-based Methods for the Extraction of DNA from Human Bones from 18th to Mid-19th Century London. *Ancient Biomolecules* 4(4):173-178.

Braun, M., D.C. Cook, and S. Pfeiffer
1998 DNA from Mycobacterium-tuberculosis complex identified in North American, pre-Columbian human skeletal remains (Archaeology and human biology). *Journal of Archaeological Science* 25(3):271-277.

Brown, T.A., and K.A. Brown
1992 Ancient DNA and the Archaeologist. *Antiquity* 66(250):10-23.

Burger, J., S. Hummel, B. Herrmann, and W. Henke
1999 DNA preservation: A microsatellite-DNA study on ancient skeletal remains. *Electrophoresis* 20(8):1722-1728.

Butler, V., and N.J. Bowers
1998 Ancient DNA from Salmon Bone: a Preliminary Study. *Ancient Biomolecules* 2(1):17-26.

Cadet, J., E. Sage, and T. Douki
2005 Ultraviolet radiation-mediated damage to cellular DNA. *Mutation Research-Fundamental and Molecular Mechanisms of Mutagenesis* 571(1-2):3-17.

Cano, R.J., H.N. Poinar, and G.O. Poinar Jr.
1992a Isolation and partial characterisation of DNA from the bee *Proplebeia dominicana* (Apidae: Hymenoptera) in 25-40 million year old amber. *Medical Science Research* 20:249-251.

Cano, R.J., H.N. Poinar, D.W. Roublik, and G.O. Poinar Jr.
1992b Enzymatic amplification and nucleotide sequencing of portions of the 18S rRNA gene of the bee Proplebeia dominicana (Apidae: Hymenoptera). *Medical Science Research* 20619-622.

Cano, R.J.
1996 Analysing ancient DNA. *Endeavour* 20(4):162-167.

Cappellini, E., B. Chiarelli, L. Sineo, A. Casoli, A. Di Gioia, C. Vernesi, M.C. Biella, and D. Caramelli
2004 Biomolecular study of the human remains from tomb 5859 in the Etruscan necropolis of Monterozzi, Tarquinia (Viterbo, Italy). *Journal of Archaeological Science* 31(5):603-612.

Choi, J.H., and G.P. Pfeifer
2005 The role of DNA polymerase in UV mutational spectra. *DNA Repair* 4(2):211-220.

Cipollaro, M., G. Di Bernardo, A. Forte, G. Galano, L. De Masi, U. Galderisi, F.M. Guarino, F. Angelini, and A. Cascino
1999 Histological analysis and ancient DNA amplification of human bone remains found in Caius Iulius Polybius house in Pompeii. *Croatian Medical Journal* 40(3):392-397.

Cipollaro, M., G. Di Bernardo, G. Galano, U. Galderisi, F. Guarino, F. Angelini, and A. Cascino
1998 Ancient DNA in human bone remains from Pompeii archaeological site. *Biochemical and Biophysical Research Communications* 247(3):901-904.

Clisson, I., C. Keyser, H.P. Francfort, E. Crubezy, Z. Samashev, and B. Ludes
2002 Genetic analysis of human remains from a double inhumation in a frozen kurgan in Kazakhstan (Berel site, Early 3rd Century BC). *International Journal of Legal Medicine* 116(5):304-308.

Cobb, J.C.
2002 Ancient DNA Recovered by a Non-destructive Method. *Ancient Biomolecules* 4(4):169-172.

Colson, I.B., M.B. Richards, J.F. Bailey, B.C. Sykes, and R.E.M. Hedges
1997 DNA analysis of seven human skeletons excavated from the tern of Wijnaldum. *Journal of Archaeological Science* 24(10):911-917.

Cooper, A.
1994 DNA from Museum Specimens. In *Ancient DNA,* edited by B. Herrmann and S. Hummel, pp. 149-165. Springer-Verlag, New York.

Cooper, A., C. Lalueza-Fox, S. Anderson, A. Rambaut, J. Austin, and R. Ward
2001 Complete mitochondrial genome sequences of two extinct moas clarify ratite evolution. *Nature* 409(6821):704-707.

Cooper, A., A. Rambaut, V. Macaulay, E. Willerslev, A.J. Hansen, and C. Stringer
2001 Human origins and ancient human DNA. *Science* 292(5522):1655-1656.

Cooper, A., and H.N. Poinar
2000 Ancient DNA: Do it right or not at All. *Science* 289(5482):1139-1139.

Cooper, A.
1997 Ancient DNA - How do you really know when you have it? Reply. *American Journal of Human Genetics* 60(4):1001-1002.

Daroui, P., S.D. Desai, T.K. Li, A.A. Liu, and L.F. Liu
2004 Hydrogen peroxide induces topoisomerase I-mediated DNA damage and cell death. *Journal of Biological Chemistry* 279(15):14587-14594.

Debruyne, R., V. Barriel, and P. Tassy
2003 Mitochondrial cytochrome b of the Lyakhov mammoth (Proboscidea, Mammalia): new data and phylogenetic analyses of Elephantidae. *Molecular Phylogenetics and Evolution* 26(3):421-434.

DelRio, S.A., M.A. Marino, and P. Belgrader
1996 PCR-based human leukocyte antigen (HLA) DQ alpha typing of blood stained light and dark blue denim fabric. *Journal of Forensic Sciences* 41(3):490-492.

DeSalle, R.
1994 Implications of Ancient DNA for Phylogenetic Studies. *Experientia* 50(6):543-550.

Di Bernardo, G., U. Galderisi, S. Del Gaudio, A. D'Aniello, C. Lanave, M.T. De Robertis, A. Cascino, and M. Cipollaro
2004 Genetic characterization of Pompeii and Herculaneum Equidae buried by Vesuvius in 79 AD. *Journal of Cellular Physiology* 199(2):200-205.

Di Bernardo, G., S. Del Gaudio, M. Cammarota, U. Gaiderisi, A. Cascino, and M. Cipollaro
2002 Enzymatic repair of selected cross-linked homoduplex molecules enhances nuclear gene rescue from Pompeii and Herculaneum remains. *Nucleic Acids Research* 30(4):e16.

Douki, T., G. Laporte, and J. Cadet
2003 Inter-strand photoproducts are produced in high yield within A-DNA exposed to UVC radiation. *Nucleic Acids Research* 31(12):3134-3142.

Drancourt, M., G. Aboudharam, M. Signoli, O. Dutour, and D. Raoult
1998 Detection of 400-year-old Yersinia pestis DNA in human dental pulp: An approach to the diagnosis of ancient septicemia. *Proceedings of the National Academy of Sciences of the United States of America* 95(21):12637-12640.

Dudar, J.C., J.S. Waye, and S.R. Saunders
2003 Determination of a kinship system using ancient DNA, mortuary practice, and historic records in an Upper Canadian pioneer cemetery. *International Journal of Osteoarchaeology* 13(4):232-246.

Dwyer, D.E., and N. Saksena
1992 Failure of ultra-violet irradiation and autoclaving to eliminate PCR contamination. *Molecular and Cellular Probes* 6(1):87-88.

Edwards, C.J., D.E. MacHugh, K.M. Dobney, L. Martin, N. Russell, L.K. Horwitz, S.K. McIntosh, K.C. MacDonald, D. Helmer, A. Tresset, J.D. Vigne, and D.G. Bradley
2004 Ancient DNA analysis of 101 cattle remains: limits and prospects. *Journal of Archaeological Science* 31(6):695-710.

Environmental Protection Service
1985 *Sodium Hypochlorite: Environmental and Technical Tips for Problem Spills.* Environment Canada, Ottawa, Canada.

Eshleman, J., and D.G. Smith
2001 Use of DNase to eliminate contamination in ancient DNA analysis. *Electrophoresis* 22(20):4316-4319.

Faerman, M., R. Jankauskas, A. Gorski, H. Bercovier, and C.L. Greenblatt
1997 Prevalence of human tuberculosis in a medieval population of Lithuania studied by ancient DNA. *Ancient Biomolecules* 1(3):205-214.

Faerman, M., A. Nebel, D. Filon, M.G. Thomas, N. Bradman, B.D. Ragsdale, M. Schultz, and A. Oppenheim
2000 From a dry bone to a genetic portrait: A case study of sickle cell anemia. *American Journal of Physical Anthropology* 111(2):153-163.

Faerman, M., G.K. Bar-Gal, D. Filon, C.L. Greenblatt, L. Stager, A. Oppenheim, and P. Smith
1998 Determining the sex of infanticide victims from the late Roman era through ancient DNA analysis. *Journal of Archaeological Science* 25(9):861-865.

Fleischer, R.C., S.L. Olson, H.F. James, and A.C. Cooper
2000 Identification of the extinct Hawaiian eagle (Haliaeetus) by mtDNA sequence analysis. *Auk* 117(4):1051-1056.

Fox, J.C., M. Aitkhaled, A. Webster, and V.C. Emery
1991 Eliminating PCR Contamination - is UV Irradiation the Answer. *Journal of Virological Methods* 33(3):375-382.

Freitas, F.O., G. Bendel, R.G. Allaby, and T.A. Brown
2003 DNA from primitive maize landraces and archaeological remains: implications for the domestication of maize and its expansion into South America. *Journal of Archaeological Science* 30(7):901-908.

Gabriel, M.N., E.F. Huffine, J.H. Ryan, M.M. Holland, and T.J. Parsons
2001 Improved MtDNA sequence analysis of forensic remains using a "mini-primer set" amplification strategy. *Journal of forensic sciences* 46(2):247-253.

Garcia-Bour, J., A. Perez-Perez, S. Alvarez, E. Fernandez, A.M. Lopez-Parra, E. Arroyo-Pardo, and D. Turbon
2004 Early population differentiation in extinct aborigines from Tierra del Fuego-Patagonia: Ancient mtDNA sequences and Y-chromosome *STR* characterization. *American Journal of Physical Anthropology* 123(4):361-370.

Gerstenberger, J., S. Hummel, and B. Herrmann
1998 Assignment of an Isolated Skeletal Element to the Skeleton of Duke Christian II through STR Typing. *Ancient Biomolecules* 2(1):63-68.

Gilbert, M.T.P., L. Rudbeck, E. Willerslev, A.J. Hansen, C. Smith, K.E.H. Penkman, K. Prangenberg, C.M. Nielsen-Marsh, M.E. Jans, P. Arthur, N. Lynnerup, G. Turner-Walker, M. Biddle, B. Kjolbye-Biddle, and M.J. Collins
2005 Biochemical and physical correlates of DNA contamination in archaeological human bones and teeth excavated at Matera, Italy. *Journal of Archaeological Science* 32(5):785-793.

Gilbert, M.T.P., J. Cuccui, W. White, N. Lynnerup, R.W. Titball, A. Cooper, and M.B. Prentice
2004 Absence of Yersinia pestis-specific DNA in human teeth from five European excavations of putative plague victims. *Microbiology-Sgm* 150:341-354.

Gill, P., P.L. Ivanov, C. Kimpton, R. Piercy, N. Benson, G. Tully, I. Evett, E. Hagelberg, and K. Sullivan
1994 Identification of the remains of the Romanov family by DNA analysis. *Nature Genetics* 6(2):130-135.

Ginther, C., L. Issel-Tarver, and M.C. King
1992 Identifying individuals by sequencing mitochondrial DNA from teeth. *Nature Genetics* 2(2):135-138.

Golenberg, E.M.
1991 Amplification and Analysis of Miocene Plant Fossil *DNA. Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 333(1268):419-427.

Gotherstrom, A., M.J. Collins, A. Angerbjorn, and K. Liden
2002 Bone preservation and DNA amplification. *Archaeometry* 44:395-404.

Gutierrez, G., and A. Marin
1998 The most ancient DNA recovered from an amber-preserved specimen may not be as ancient as it seems. *Molecular Biology and Evolution* 15(7):926-929.

Haas, C.J., A. Zink, G. Palfi, U. Szeimies, and A.G. Nerlich
2000 Detection of leprosy in ancient human skeletal remains by molecular identification of Mycobacterium leprae. *American Journal of Clinical Pathology* 114(3):428-436.

Hagelberg, E.
1993 Ancient DNA Studies. *Evolutionary Anthropology* 2(6):199-207.

Hagelberg, E., L.S. Bell, T. Allen, A. Boyde, S.J. Jones, and J.B. Clegg
1991 Analysis of ancient bone DNA: techniques and applications. *Philosophical transactions of the Royal Society of London. Series B: Biological sciences* 333(1268):399-407.

Hagelberg, E., and J.B. Clegg
1991 Isolation and characterization of DNA from archaeological bone. *Proc.Biol.Sci.* 244(1309):45-50.

Hagensee, M.E., and R.E. Moses
1986 Repair response of Escherichia coli to hydrogen peroxide DNA damage. *Journal of Bacteriology* 168(3):1059-1065.

Hall, A., and J. Ballantyne
2004 Characterization of UVC-induced DNA damage in bloodstains: forensic implications. *Analytical and Bioanalytical Chemistry* 380(1):72-83.

Handt, O., M. Krings, R.H. Ward, and S. Pääbo
1996 The retrieval of ancient human DNA sequences. *American Journal of Human Genetics* 59(2):368-376.

Handt, O., M. Hoss, M. Krings, and S. Pääbo
1994 Ancient DNA - Methodological Challenges. *Experientia* 50(6):524-529.

Handt, O., M. Richards, M. Trommsdorff, C. Kilger, J. Simanainen, O. Georgiev, K. Bauer, A. Stone, R. Hedges, W. Schaffner, G. Utermann, B. Sykes, and S. Pääbo
1994 Molecular-Genetic Analyses of the Tyrolean Ice Man. *Science* 264(5166):1775-1778.

Hanni, C., A. Begue, V. Laudet, D. Stehelin, T. Brousseau, P. Amouyel, and H. Duday
1995 Molecular Typing of Neolithic Human Bones. *Journal of Archaeological Science* 22(5):649-658.

Hawkins, C.L., D.I. Pattison, and M.J. Davies
2003 Hypochlorite-induced oxidation of amino acids, peptides and proteins. *Amino Acids* 25(3-4):259-274.

Hawkins, C.L., and M.J. Davies
2002 Hypochlorite-induced damage to DNA, RNA, and polynucleotides: Formation of chloramines and nitrogen-centered radicals. *Chemical Research in Toxicology* 15(1):83-92.

Hayatsu, H., S. Pan, and T. Ukita
1971 Reaction of sodium hypochlorite with nucleic acids and their constituents. *Chemical & Pharmaceutical Bulletin* 19(10):2189-2192.

Haynes, S., J.B. Searle, A. Bertman, and K.M. Dobney
2002 Bone preservation and ancient DNA: The application of screening methods for predicting DNA survival. *Journal of Archaeological Science* 29(6):585-592.

Hedges, S.B., and M.H. Schweitzer
1995 Detecting Dinosaur DNA. *Science* 268(5214):1191-1192.

Hidalgo, E., R. Bartolome, and C. Dominguez
2002 Cytotoxicity mechanisms of sodium hypochlorite in cultured human dermal fibroblasts and its bactericidal effectiveness. *Chemico-biological Interactions* 139(3):265-282.

Higuchi, R., B. Bowman, M. Freiberger, O.A. Ryder, and A.C. Wilson
1984 DNA sequences from the quagga, an extinct member of the horse family. *Nature* 312(15):282-284.

Hochmeister, M.N., B. Budowle, U.V. Borer, U. Eggmann, C.T. Comey, and R. Dirnhofer
1991 Typing of deoxyribonucleic acid (DNA) extracted from compact bone from human remains. *Journal of Forensic Sciences* 36(6):1649-1661.

Hofreiter, M., D. Serre, H.N. Poinar, M. Kuch, and S. Pääbo
2001 Ancient DNA. *Nature Reviews Genetics* 2(5):353-359.

Hongo, H., N. Ishiguro, T. Watanobe, N. Shigehara, T. Anezaki, V.T. Long, D.V. Binh, N.T. Tien, and N.H. Nam
2002 Variation in mitochondrial DNA of Vietnamese pigs: relationships with Asian domestic pigs and Ryukyu wild boars. *Zoological Science* 19(11):1329-1335.

Hoss, M., A. Dilling, A. Currant, and S. Pääbo
1996 Molecular phylogeny of the extinct ground sloth Mylodon darwinii. *Proceedings of the National Academy of Sciences of the United States of America* 93(1):181-185.

Hoss, M., P. Jaruga, T.H. Zastawny, M. Dizdaroglu, and S. Pääbo
1996 DNA damage and DNA sequence retrieval from ancient tissues. *Nucleic Acids Research* 24(7):1304-1307.

Hoss, M., and S. Pääbo
1993 DNA Extraction from Pleistocene Bones by a Silica-Based Purification Method. *Nucleic Acids Research* 21(16):3913-3914.

Hummel, S.
2003 *Ancient DNA Typing: Methods, Strategies and Applications.* Springer-Verlag, Berlin Heidelberg.

Hummel, S., B. Herrmann, J. Rameckers, D. Muller, K. Sperling, H. Neitzel, and H. Tonnies
1999 Proving the authenticity of ancient DNA by comparative genomic hybridization. *Naturwissenschaften* 86(10):500-503.

Huynen, L., C.D. Millar, R.P. Scofield, and D.M. Lambert
2003 Nuclear DNA sequences detect species limits in ancient moa. *Nature* 425(6954):175-178.

Innis, M.A.
1990 *PCR protocols : a guide to methods and applications.* Academic Press, San Diego.

Jehaes, E., R. Decorte, A. Peneau, J.H. Petrie, P.A. Boiry, A. Gilissen, J.P. Moisan, H. Van den Berghe, O. Pascal, and J.J. Cassiman
1998 Mitochondrial DNA analysis on remains of a putative son of Louis XVI, King of France and Marie-Antoinette. *European Journal of Human Genetics* 6(4):383-395.

Jin, X., S. Yue, K.S. Wells, and V.L. Singer
1994 Sybr Green-i and Sybr Green-Ii - New Ultrasensitive Fluorescent Stains for Detecting Picogram Levels of Nucleic-Acids in Polyacrylamide *or* Agarose Gels. *Faseb Journal* 8(7):A1266-A1266.

Kalmar, T., C.Z. Bachrati, A. Marcsik, and I. Rasko
2000 A simple and efficient method for PCR amplifiable DNA extraction from ancient bones. *Nucleic Acids Research* 28(12):E67.

Ke, W.Z., D.F. Zhou, and J.Z. Wu
2005 Effects of UV irradiation on calf thymus DNA in aqueous solution. A Raman spectroscopic study. *Journal of Raman Spectroscopy* 36(1):39-44.

Kemp, B.M., and D.G. Smith
2005 Use of bleach to eliminate contaminating DNA from the surface of bones and teeth. *Forensic Science International* in press

Knight, A., L.A. Zhivotovsky, D.H. Kass, D.E. Litwin, L.D. Green, P.S. White, and J.L. Mountain
2004 Molecular, forensic and haplotypic inconsistencies regarding the identity of the Ekaterinburg remains. *Annals of Human Biology* 31(2):129-138.

Kolman, C.J., and N. Tuross
2000 Ancient DNA analysis of human populations. *American Journal of Physical Anthropology* 111(1):5-23.

Kovatsi, L., S. Triantafyllou, J. Eliopoulos, M. Besios, S. Andreou, A.S. Tsaftaris, A. Dimitriadou, and S. Kouidou
2003 In search of mothers and daughters in 3000 year old burials in Northern Greece. *Forensic Science International* 13:642-43.

Krings, M., H. Geisert, R.W. Schmitz, H. Krainitzki, and S. Pääbo
1999 DNA sequence of the mitochondrial hypervariable region II from the neandertal type specimen. *Proceedings of the National Academy of Sciences of the United States of America* 96(10):5581-5585.

Krings, M., A. Stone, R.W. Schmitz, H. Krainitzki, M. Stoneking, and S. Pääbo
1997 Neandertal DNA sequences and the origin of modern humans. *Cell* 90(1):19-30.

Kumar, S.S., I. Nasidze, S.R. Walimbe, and M. Stoneking
2000 Brief communication: Discouraging prospects for ancient DNA from India. *American Journal of Physical Anthropology* 113(1):129-133.

Kwok, S., and R. Higuchi
1989 Avoiding false positives with PCR. *Nature* 339(6221):237-238.

Lalueza-Fox, C., M.L. Sampietro, M.T. Gilbert, L. Castri, F. Facchini, D. Pettener, and J. Bertranpetit
2004 Unravelling migrations in the steppe: mitochondrial DNA sequences from ancient central Asians. *Proceedings of the Royal Society of London. Series B. Biological sciences* 271(1542):941-947.

Lalueza-Fox, C., M.T.P. Gilbert, A.J. Martinez-Fuentes, F. Calafell, and J. Bertranpetit
2003 Mitochondrial DNA from pre-Columbian Ciboneys from Cuba and the prehistoric colonization of the Caribbean. *American Journal of Physical Anthropology* 121(2):97-108.

Lalueza-Fox, C., F.L. Calderon, F. Calafell, B. Morera, and J. Bertranpetit
2001 MtDNA from extinct Tainos and the peopling of the Caribbean. *Annals of Human Genetics* 65:137-151.

Lassen, C., and S. Hummel
1996 PCR based sex identification of ancient human bones by amplification of X-and Y-chromosomal sequences: A comparison. *Ancient Biomolecules* 1(1):25-33.

Lassen, C., S. Hummel, and B. Herrmann
1994 Comparison of DNA Extraction and Amplification from Ancient Human Bone and Mummified Soft-Tissue. *International Journal of Legal Medicine* 107(3):152-155.

Leonard, J.A., C. Vila, and R.K. Wayne
2005 Legacy lost: genetic variability and population size of extirpated US grey wolves (Canis lupus). *Molecular Ecology* 14(1):9-17.

Lindahl, T.
1993 Instability and decay of the primary structure of DNA. *Nature* 362(6422):709-715.

Linville, J.G., and J.D. Wells
2002 Surface sterilization of a maggot using bleach does not interfere with mitochondrial DNA analysis of crop contents. *Journal of Forensic Sciences* 47(5):1055-1059.

Lister, A.M., M. Kadwell, L.M. Kaagan, W.C. Jordan, M.B. Richards, and H.E. Stanley
1998 Ancient and modern DNA in a study of horse domestication. *Ancient Biomolecules* 2(2/3):267-280.

MacHugh, D.E., C.J. Edwards, J.F. Bailey, D.R. Bancroft, and D.G. Bradley
2000 The Extraction and Analysis of Ancient DNA From Bone and Teeth: a Survey of Current Methodologies. *Ancient Biomolecules* 3:81-102.

MacHugh, D.E., C.S. Troy, F. McCormick, I. Olsaker, E. Eythorsdottir, and D.G. Bradley
1999 Early medieval cattle remains from a Scandinavian settlement in Dublin: Genetic analysis and comparison with extant breeds. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 354(1379):99-108.

Malhi, R.S., K.E. Breece, B.A. Shook, F.A. Kaestle, J.C. Chatters, S. Hackenberger, and D.G. Smith
2004 Patterns of mtDNA diversity in northwestern North America. *Human Biology; an International Record of Research* 76(1):33-54.

Marota, I., C. Basile, M. Ubaldi, and F. Rollo
2002 DNA decay rate in papyri and human remains from Egyptian archaeological sites. *American Journal of Physical Anthropology* 121(2):109-11.

Matheson, C.D., and T.H. Loy
2001 Genetic sex identification of 9400-year-old human skull samples from Cayonu Tepesi, Turkey. *Journal of Archaeological Science* 28(6):569-575.

MatisooSmith, E., J.S. Allen, T.N. Ladefoged, R.M. Roberts, and D.M. Lambert
1997 Ancient DNA from Polynesian rats: Extraction, amplification and sequence from single small bones. *Electrophoresis* 18(9):1534-1537.

Mays, S., and M. Faerman
2001 Sex identification in some putative infanticide victims from Roman Britain using ancient DNA. *Journal of Archaeological Science* 28(5):555-559.

Mays, S., G.M. Taylor, A.J. Legge, D.B. Young, and G. Turner-Walker
2001 Paleopathological and biomolecular study of tuberculosis in a medieval skeletal collection from England. *American Journal of Physical Anthropology* 114(4):298-311.

Melton, T.L., and K. Nelson
2001 Forensic mitochondrial DNA analysis: Two years of commercial casework experience in the United States. *Croatian Medical Journal* 42(3):298-303.

Meyer, E., M. Wiese, H. Bruchhaus, M. Claussen, and A. Klein
2000 Extraction and amplification of authentic DNA from ancient human remains. *Forensic Science International* 113(1-3):87-90.

Monks, G.G., A.D. McMillan, and D.E.S. Claire
2001 Nuu-chah-nulth whaling: Archaeological insights into antiquity, species preferences, and cultural importance. *Arctic Anthropology* 38(1):60-81.

Montiel, R., A. Malgosa, and P. Francalacci
2001 Authenticating ancient human mitochondrial DNA. *Human Biology* 73(5):689-713.

Mullis, K.B.
1990 The unusual origin of the polymerase chain reaction. *Scientific American* 262(4):56-61, 64-5.

Newman, M.E., J.S. Parboosingh, P.J. Bridge, and H. Ceri
2002 Identification of archaeological animal bone by PCR/DNA analysis. *Journal of Archaeological Science* 29(1):77-84.

Nias, A.H.W.
1998 *An introduction to Radiobiology.* 2nd ed. John Wiley & Sons, Baffin Lane, Chichester, West Sussex, England.

Nicholls, A., E. Matisoo-Smith, and M.S. Allen
2003 A novel application of molecular techniques to Pacific archaeofish remains. *Archaeometry* 45:133-147.

Ohnishi, S., M. Murata, and S. Kawanishi
2002 DNA damage induced by hypochlorite and hypobromite with reference to inflammation-associated carcinogenesis. *Cancer letters* 178(1):37-42.

Okazaki, M., Y. Yoshida, S. Yamaguchi, M. Kaneno, and J.C. Elliott
2001 Affinity binding phenomena of DNA onto apatite crystals. *Biomaterials* 22(18):2459-2464.

Olsen, L.E., and A. Hassanin
2003 Contamination and chimerism are perpetuating the legend of the snake-eating cow with twisted horns (*Psedonovibos spiralis*). A case study of the pitfalls of ancient DNA. *Molecular Phylogenetics and Evolution* 27:545-548.

Orlando, L., J.A. Leonard, A. Thenot, V. Laudet, C. Guerin, and C. Hanni
2003 Ancient DNA analysis reveals woolly rhino evolutionary relationships. *Molecular Phylogenetics and Evolution* 28(3):485-499.

Ou, C.Y., J.L. Moore, and G. Schochetman
1991 Use of UV Irradiation to Reduce False Positivity in Polymerase Chain-Reaction. *BioTechniques* 10(4):442-&.

Ovchinnikov, I.V., A. Gotherstrom, G.P. Romanova, V.M. Kharitonov, K. Liden, and W. Goodwin
2000 Molecular analysis of Neanderthal DNA from the northern Caucasus. *Nature* 404(6777):490-493.

Ozawa, T., S. Hayashi, and V.M. Mikhelson
1997 Phylogenetic position of Mammoth and Steller's sea cow within Tethytheria demonstrated by mitochondrial DNA sequences. *Journal of Molecular Evolution* 44(4):406-413.

Pääbo, S.
1990 Amplifying Ancient DNA. In *PCR Protocols,* edited by M.A. Innis, D.H. Gelfand, J.J. Sninsky and T.J. White, pp. 159-166. Academic Press, San Diego.

Pääbo, S.
1985 Molecular cloning of ancient Egyptian mummy DNA. *Nature* 31:4644-645.

Pääbo, S., H. Poinar, D. Serre, V. Jaenicke-Despres, J. Hebler, N. Rohland, M. Kuch, J. Krause, L. Vigilant, and M. Hofreiter
2004 Genetic analyses from ancient DNA. *Annual Review of Genetics* 38:645-679.

Pääbo, S.
1989 Ancient DNA - Extraction, Characterization, Molecular-Cloning, and Enzymatic Amplification. *Proceedings of the National Academy of Sciences of the United States of America* 86(6):1939-1943.

Pääbo, S., R.G. Higuchi, and A.C. Wilson
1989 Ancient DNA and the Polymerase Chain-Reaction - the Emerging Field of Molecular Archaeology. *Journal of Biological Chemistry* 264(17):9709-9712.

Pabst, D.A., S.A. Rommel, and W.A. McLellan
1999 The functional morphology of marine mammals. In *Biology of Marine Mammals,* edited by J.E. Reynolds and S.A. Rommel, pp. 15-72. Smithsonian Institution Press, Washington and London.

Parson, W., K. Pegoraro, H. Niederstatter, M. Foger, and M. Steinlechner
2000 Species identification by means of the cytochrome b gene. *International Journal of Legal Medicine* 114(1-2):23-28.

Pfeiffer, H., J. Huhne, B. Seitz, and B. Brinkmann
1999 Influence of soil storage and exposure period on DNA recovery from teeth. *International Journal of Legal Medicine* 112(2):142-144.

Poinar, H.N.
2003 The top 10 list: criteria of authenticity for DNA from ancient and forensic samples. *International Congress Series* 1239:575-579.

Poinar, H.N., R.J. Cano, and G.O. Poinar
1993 DNA from an extinct plant. *Nature* 36:3677.

Poinar, H.N., and B.A. Stankiewicz
1999 Protein preservation and DNA retrieval from ancient tissues. *Proceedings of the National Academy of Sciences of the United States of America* 96(15):8426-8431.

Poinar, H.N., M. Hoss, J.L. Bada, and S. Pääbo
1996 Amino acid racemization and the preservation of ancient DNA. *Science* 272(5263):864-866.

Poulakakis, N., G.E. Theodorou, E. Zouros, and M. Mylonas
2002 Molecular phylogeny of the extinct pleistocene dwarf elephant Palaeoloxodon antiquus falconeri from Tilos Island, Dodekanisa, Greece. *Journal of Molecular Evolution* 55(3):364-374.

Prado, M., C.M. Franco, C.A. Fente, A. Cepeda, B.I. Vazquez, and J. Barros-Velazquez
2002 Comparison of extraction methods for the recovery, amplification and species-specific analysis of DNA from bone and bone meals. *Electrophoresis* 23(7-8):1005-1012.

Prince, A.M., and L. Andrus
1992 PCR: how to kill unwanted DNA. *BioTechniques* 12(3):358-360.

Prutz, W.A.
1998 Interactions of hypochlorous acid with pyrimidine nucleotides, and secondary reactions of chlorinated pyrimidines with GSH, NADH, and other substrates. *Archives of Biochemistry and Biophysics* 349(1):183-191.

Prutz, W.A.
1996 Hypochlorous acid interactions with thiols, nucleotides, DNA, and other biological substrates. *Archives of Biochemistry and Biophysics* 332(1):110-120.

Pruvost, M., and E.M. Geigl
2004 Real-time quantitative PCR to assess the authenticity of ancient DNA amplification. *Journal of Archaeological Science* 31(9):1191-1197.

Pusch, C., and M. Scholz
1998 Detecting Prehistoric Human DNA: Construction and Screening of a Small-Scale Plasmid Library Reveals a New Repetitive and Tandemly Organized 25 bp Motif. *Ancient Biomolecules* 2(1):43-62.

Pusch, C.M., I. Giddings, and M. Scholz
1998 Repair of degraded duplex DNA from prehistoric samples using Escherichia coli DNA polymerase I and T4 DNA ligase. *Nucleic Acids Research* 26(3):857-859.

Raoult, D., G. Aboudharam, E. Crubezy, G. Larrouy, B. Ludes, and M. Drancourt
2000 Molecular identification by "suicide PCR" of Yersinia pestis as the agent of Medieval Black Death. *Proceedings of the National Academy of Sciences of the United States of America* 97(23):12800-12803.

Ravanat, J.L., T. Douki, and J. Cadet
2001 Direct and indirect effects of UV radiation on DNA and its components. *Journal of Photochemistry and Photobiology B-Biology* 63(1-3):88-102.

Ricaut, F.X., C. Keyser-Tracqui, J. Bourgeois, E. Crubezy, and B. Ludes
2004 Genetic analysis of a Scytho-siberian skeleton and its implications for ancient Central Asian migrations. *Human Biology* 76(1):109-125.

Ricaut, F.X., S. Kolodesnikov, C. Keyser-Tracqui, A.N. Alekseev, E. Crubezy, and B. Ludes
2004 Genetic analysis of human remains found in two eighteenth century Yakut graves at At-Dabaan. *International Journal of Legal Medicine* 118(1):24-31.

Richards, M.B., B.C. Sykes, and R.E.M. Hedges
1995 Authenticating DNA Extracted from Ancient Skeletal Remains. *Journal of Archaeological Science* 22(2):291-299.

Rosenbaum, H.C., M.G. Egan, P.J. Clapham, R.L. Brownell, and R. Desalle
1997 An effective method for isolating DNA from historical specimens of baleen. *Molecular Ecology* 6(7):677-681.

Sakar, G., and S.S. Sommer
1990 Shedding light on PCR Contamination. *Nature* 34:327.

Savage, R.E., and W. Plaut
1958 The effect of HCl hydrolysis on the retention of thymidine in DNA. *J.Biophys.Biochem.Cytol.* 4(6):701-706.

Schmerer, W.M., S. Hummel, and B. Herrmann
1999 Optimized DNA extraction to improve reproducibility of short tandem repeat genotyping with highly degraded DNA as target. *Electrophoresis* 20(8):1712-1716.

Schmitz, R.W., D. Serre, G. Bonani, S. Feine, F. Hillgruber, H. Krainitzki, S. Pääbo, and F.H. Smith
2002 The Neandertal type site revisited: Interdisciplinary investigations of skeletal remains from the Neander Valley, Germany. *Proceedings of the National Academy of Sciences of the United States of America* 99(20):13342-13347.

Schneeberger, C., P. Speiser, F. Kury, and R. Zeillinger
1995 Quantitative Detection of Reverse Transcriptase-PCR Products by Means of a Novel and Sensitive DNA Stain. *PCR-Methods and Applications* 4(4):234-238.

Scholz, M., I. Giddings, and C.M. Pusch
1998 A Polymerase Chain Reaction Inhibitor of Ancient Hard and Soft Tissue DNA Extracts is Determined as Human Collagen Type I. *Analytical Biochemistry* 259:283-286.

Scholz, M., L. Bachmann, G.J. Nicholson, J. Bachmann, I. Giddings, B. Ruschoff-Thale, A. Czarnetzki, and C.M. Pusch
2000 Genomic differentiation of neanderthals and anatomically modern man allows a fossil-DNA-based classification of morphologically indistinguishable hominid bones. *American Journal of Human Genetics* 66(6):1927-1932.

Serre, D., A. Langaney, M. Chech, M. Teschler-Nicola, M. Paunovic, P. Mennecier, M. Hofreiter, G. Possnert, and S. Pääbo
2004 No evidence of neandertal mtDNA contribution to early modern humans. *Plos Biology* 2(3):313-317.

Sica, M., S. Aceto, A. Genovese, and L. Gaudio
2002 Analysis of Five Ancient Equine Skeletons by Mitochondrial DNA Sequencing. *Ancient Biomolecules* 4(4):179-185.

Sidow, A., A.C. Wilson, and S. Pääbo
1991 Bacterial DNA in Clarkia fossils. *Philosophical transactions of the Royal Society of London. Series B: Biological sciences* 333(1268):429-32; discussion 432-3.

Smith, K.C.
1964 The Photochemical Interaction of Deoxyribonucleic Acid and Protein *In Vivo* and its Biological Importance. *Photochemistry and Photobiology* 3:415-427.

Smith, C.I., A.T. Chamberlain, M.S. Riley, C. Stringer, and M.J. Collins
2003 The thermal history of human fossils and the likelihood of successful DNA amplification. *Journal of Human Evolution* 45(3):203-217.

Soltis, P.S., D.E. Soltis, and C.J. Smiley
1992 An rbcL sequence from a Miocene Taxodium (bald cypress). *Proceedings of the National Academy of Sciences of the United States of America* 89(1):449-451.

Spencer, M., and C.J. Howe
2004 Authenticity of ancient-DNA results: A statistical approach. *American Journal of Human Genetics* 75(2):240-250.

Stone, A.C., and M. Stoneking
1996 Genetic analyses of an 8000 year-old native American skeleton. *Ancient Biomolecules* 1(1):83-87.

Stone, A.C., and M. Stoneking
1999 Analysis of ancient DNA from a prehistoric Amerindian cemetery. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* 354(1379):153-159.

Stoneking, M.
1995 Ancient DNA - how do You Know when You have it and what can You do with it. *American Journal of Human Genetics* 57(6):1259-1262.

Taylor, G.M., M. Crossey, J. Saldanha, and T. Waldron
1996 DNA from Mycobacterium tuberculosis identified in Mediaeval human skeletal remains using polymerase chain reaction. *Journal of Archaeological Science* 23(5):789-798.

Thompson, J. D., D. G. Higgins and T. J. Gibson
1994 CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22:4673-4680.

Tuross, N.
1994 The Biochemistry of Ancient DNA in Bone. *Experientia* 50(6):530-535.

Vernesi, C., D. Caramelli, I. Dupanloup, G. Bertorelle, M. Lari, E. Cappellini, J. Moggi-Cecchi, B. Chiarelli, L. Castri, A. Casoli, F. Mallegni, C. Lalueza-Fox, and G. Barbujani
2004 The Etruscans: A population-genetic study. *American Journal of Human Genetics* 74(4):694-704.

von Wurmb-Schwark, N., M. Harbeck, U. Wiesbrock, I. Schroeder, S. Ritz-Timme, and M. Oehmichen
2003 Extraction and amplification of nuclear and mitochondrial DNA from ancient and artificially aged bones. *Leg.Med.(Tokyo)* 5 Suppl 1S169-72.

Wandeler, P., S. Smith, P.A. Morin, R.A. Pettifor, and S.M. Funk
2003 Patterns of nuclear DNA degeneration over time - a case study in historic teeth samples. *Molecular Ecology* 12(4):1087-1093.

Watanabe, T., N. Ishiguro, N. Okumura, M. Nakano, A. Matsui, H. Hongo, and H. Ushiro
2001 Ancient mitochondrial DNA reveals the origin of Sus scrofa from Rebun Island, Japan. *Journal of Molecular Evolution* 52(3):281-289.

Watanobe, T., N. Ishiguro, M. Nakano, H. Takamiya, A. Matsui, and H. Hongo
2002 Prehistoric introduction of domestic pigs onto the Okinawa islands: Ancient mitochondrial DNA evidence. *Journal of Molecular Evolution* 55(2):222-231.

Whiteman, M., H.S. Hong, A. Jenner, and B. Halliwell
2002 Loss of oxidized and chlorinated bases in DNA treated with reactive oxygen species: implications for assessment of oxidative damage in vivo. *Biochemical and Biophysical Research Communications* 296(4):883-889.

Whiteman, M., J.P.E. Spencer, A. Jenner, and B. Halliwell
1999 Hypochlorous acid induced DNA base modification: Potentiation by nitrite: Biomarkers of DNA damage by reactive oxygen species. *Biochemical and Biophysical Research Communications* 257(2):572-576.

Whiteman, M., A. Jenner, and B. Halliwell
1997 Hypochlorous acid-induced base modifications in isolated calf thymus DNA. *Chemical Research in Toxicology* 10(11):1240-1246.

Wiechmann, I., and G. Grupe
2005 Detection of Yersinia pestis DNA in two early medieval skeletal finds from Aschheim (Upper Bavaria, 6th century A.D.). *American Journal of Physical Anthropology* 126(1):48-55.

Willerslev, E., and A. Cooper
2005 Ancient DNA. *Proceedings of the Royal Society of London B-Biology* 2723-16.

Willerslev, E., A.J. Hansen, and H.N. Poinar
2004 Isolation of nucleic acids and cultures from fossil ice and permafrost. *Trends in Ecology & Evolution* 19(3):141-147.

Willerslev, E., A.J. Hansen, R. Ronn, T.B. Brand, I. Barnes, C. Wiuf, D. Gilichinsky, D. Mitchell, and A. Cooper
2004 Long-term persistence of bacterial DNA. *Current Biology* 14(1):R9-R10.

Woodward, S.R., N.J. Weyland, and M. Bunnell
1994 DNA Sequence from Cretaceous Period Bone Fragments. *Science* 266(5188):1229-1232.

Yang, D.Y., and K. Watt
2005 Contamination controls when preparing archaeological remains for ancient DNA analysis. *Journal of Archaeological Science* 32:331-336.

Yang, D.Y., J.R. Woiderski, and J.C. Driver
2005 DNA analysis of archaeological rabbit remains from the American Southwest. *Journal of Archaeological Science* 32(4):567-578.

Yang, D.Y., B. Eng, J.C. Dudar, S.R. Saunders, and J.S. Waye
1997 Removal of PCR Inhibitors Using Silica-Based Spin Columns: Application to Ancient Bones. *Canadian Society of Forensic Science* 30(1):1-6.

Yang, D.Y., B. Eng, and S.R. Saunders
2003 Hypersensitive PCR, ancient human mtDNA, and contamination. *Human Biology* 75(3):355-364.

Yang, D.Y., B. Eng, J.S. Waye, J.C. Dudar, and S.R. Saunders
1998 Technical note: improved DNA extraction from ancient bones using silica-based spin columns. *American Journal of Physical Anthropology* 105(4):539-543.

Yang, D.Y., A. Cannon, and S.R. Saunders
2004 DNA species identification of archaeological salmon bone from the Pacific Northwest Coast of North America. *Journal of Archaeological Science* 31(5):619-631.

Yang, H., E.M. Golenberg, and J. Shoshani
1997a Proboscidean DNA from museum and fossil specimens: An assessment of ancient DNA extraction and amplification techniques. *Biochemical Genetics* 35(5-6):165-179.

Yang, H., E.M. Golenberg, and J. Shoshani
1997b A blind testing design for authenticating ancient DNA sequences. *Molecular Phylogenetics and Evolution* 7(2):261-265.

Zink, A., C.J. Haas, U. Reischl, U. Szeimies, and A.G. Nerlich
2001 Molecular analysis of skeletal tuberculosis in an ancient Egyptian population. *Journal of Medical Microbiology* 50(4):355-366.

Zink, A.R., W. Grabner, and A.G. Nerlich
2005 Molecular identification of human tuberculosis in recent and historic bone tissue samples: The role of molecular techniques for the study of historic tuberculosis. *American Journal of Physical Anthropology* 126(1):32-47.

Zink, A.R., C. Sola, U. Reischl, W. Grabner, N. Rastogi, H. Wolf, and A.G. Nerlich
2004 Molecular identification and characterization of Mycobacterium tuberculosis complex in ancient Egyptian mummies. *International Journal of Osteoarchaeology* 14(5):404-413.

Zink, A.R., W. Grabner, U. Reischl, H. Wolf, and A.G. Nerlich
2003 Molecular study on human tuberculosis in three geographically distinct and time delineated populations from ancient Egypt. *Epidemiology and Infection* 130(2):239-249.

Zischler, H., M. Hoss, O. Handt, A. von Haeseler, A.C. van der Kuyl, and J. Goudsmit
1995 Detecting dinosaur DNA. *Science* 268(5214):1192-3; author reply 1194.

APPENDICES

Appendix 1

Surveyed ancient DNA and forensic DNA articles noting the decontamination techniques used.

	Decontamination Technique													
	Physical Methods					Chemical						Other		
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Leonard *et al.* 2005)												*		
(Zink *et al.* 2005)					*	*								
(Wiechmann and Grupe. 2005)							*		*	*	*			*
(Yang *et al.* 2005)						*	*	*			*		*	
(Gilbert *et al.* 2005)						*								
(Bouwman and Brown. 2005)			*								*			
(Cappellini *et al.* 2004)				*							*			
(Yang *et al.* 2004).	*					*	*	*			*			
(Garcia-Bour *et al.* 2004)		*												
(Knight *et al.* 2004)												*		
(Gilbert *et al.* 2004)						*					*			
(Pruvost and Geigl. 2004)			*								*			
(Malhi *et al.* 2004)						*								

	Decontamination Technique														
	Physical Methods					Chemical						Other			
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Serre *et al.* 2004)												*		
(Ricaut *et al.* 2004)		*												
(Lalueza-Fox *et al.* 2004)			*											
(Edwards *et al.* 2004)		*												
(Austin *et al.* 2004)		*												
(Di Bernardo *et al.* 2004)				*		*					*			
(Vernesi *et al.* 2004)				*							*			
(Ricaut *et al.* 2004)		*												
(Alonso *et al.* 2004)				*		*			*	*			*	
(Zink *et al.* 2004)					*	*								
(Arismendi *et al.* 2004)	*					*								
(Adachi *et al.* 2004)		*				*					*			
(Bathurst and Barta. 2004)						*	*	*			*			
(Arndt *et al.* 2003)		*												
(Bar-Gal *et al.* 2003)		*												
(Dudar *et al.* 2003)	*													
(Kovatsi *et al.* 2003)					*						*			

	Decontamination Technique														
	Physical Methods					Chemical					Other				
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Nicholls *et al.* 2003)	*								*					
(Lalueza-Fox *et al.* 2003)						*			*					
(Orlando *et al.* 2003)												*		
(Debruyne *et al.* 2003)					*									
(Huynen *et al.* 2003)		*												
(Yang *et al.* 2003)											*			
(von Wurmb-Schwark *et al.* 2003)									*					
(Haynes *et al.* 2002)	*													
(Newman *et al.* 2002)	*													
(Cobb. 2002)				*		*				*				
(Gotherstrom *et al.* 2002).												*		
(Bouwman and Brown. 2002)			*								*			
(Austin *et al.* 2002)		*												
(Di Bernardo *et al.* 2002)				*		*					*			
(Sica *et al.* 2002)			*			*							*	
(Clisson *et al.* 2002)			*											

	Decontamination Technique														
	Physical Methods					Chemical						Other			
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Poulakakis *et al.* 2002)										*				
(Watanobe *et al.* 2002)			*											
(Hongo *et al.* 2002)			*											
(Prado *et al.* 2002)			*											
(Matheson and Loy. 2001)											*			
(Mays and Faerman. 2001)			*											
(Lalueza-Fox *et al.* 2001)			*											
(Alonso *et al.* 2001)	*			*	*	*			*	*			*	
(Gabriel *et al.* 2001)	*													
(Austin and Arnold. 2001)		*												
(Watanabe *et al.* 2001)			*											
(Barnes *et al.* 2000)		*												
(Kolman and Tuross. 2000)						*				*				
(Kalmar *et al.* 2000)		*				*					*			
(Kumar *et al.* 2000)			*											
(Bender *et al.* 2000)		*												

	Decontamination Technique														
	Physical Methods					Chemical						Other			
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Parson *et al.* 2000)	*								*					
(Meyer *et al.* 2000)		*												
(Burger *et al.* 1999)					*						*			
(Cipollaro *et al.* 1999)				*		*					*			
(Schmerer *et al.* 1999)											*			
(MacHugh *et al.* 1999)		*				*					*			
(Pfeiffer *et al.* 1999)														*
(Krings *et al.* 1999)		*				*								
(Yang *et al.* 1998)	*													
(Faerman *et al.* 1998)		*		*										
(Braun *et al.* 1998)			*											
(Cipollaro *et al.* 1998)				*		*					*			
(Barnes *et al.* 1998)			*											
(Butler and Bowers. 1998)		*				*								
(Pusch and Scholz. 1998)		*												
(Gerstenberger *et al.* 1998)					*						*			

	Decontamination Technique														
	Physical Methods					Chemical						Other			
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Lister *et al.* 1998)	*								*				*	
(Jehaes *et al.* 1998)					*									
(Colson *et al.* 1997)						*								
(Yang *et al.* 1997)	*													
(Krings *et al.* 1997)		*				*								
(MatisooSmith *et al.* 1997).	*													
(Ozawa *et al.* 1997)					*									
(Rosenbaum *et al.* 1997)						*			*		*			
(Yang *et al.* 1997a)						*								
(Faerman *et al.* 1997)		*		*										
(Austin *et al.* 1997)						*			*				*	*
(Baron *et al.* 1996)											*			
(Taylor *et al.* 1996)			*										*	
(Hoss *et al.* 1996)		*												
(Lassen and Hummel. 1996)											*			
(Hoss *et al.* 1996)		*												

	Decontamination Technique														
	Physical Methods					Chemical						Other			
Ref.	Sandpaper	Dremel/Drill/ Shotblasting	Scalpel	Brushed	O	Bleach	HCl	NaOH	Ethanol	O	UV	None	H20	O
(Stone and Stoneking. 1996)		*												
(Richards *et al.* 1995)		*				*								
(Hanni *et al.* 1995)			*											
(Lassen *et al.* 1994)											*			
(Gill *et al.* 1994)		*												
(Handt *et al.* 1994)					*									
(Woodward *et al.* 1994)			*											
(Hoss and Pääbo. 1993)		*												
(Ginther *et al.* 1992)						*			*	*			*	
(Hochmeister *et al.* 1991)	*		*											
(Hagelberg and Clegg. 1991)				*	*									
(Hagelberg *et al.* 1991)				*	*									
Totals	14	28	19	13	12	31	4	3	11	7	27	5	8	3