Improving Genetic Profiling Techniques For Low Copy Number DNA

By

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Abbreviations

A	Adenine	**ABI**	Applied Biosystems
aDNA	ancient DNA	**Amel**	Amelogenin
APS	Ammonium Persulfate	**ARY**	Alphoid Repeat for Y Chromosome
bp	base pair		
BP	Before Present	**C**	Cytosine
CE	Capillary Electrophoresis	**Ct**	Cycle Threshold
CTAB	Cetyl-trimethylammonium Bromide	**dATP**	deoxyadenine triphosphate
		dCTP	deoxycytosine triphosphate
ddNTP	dideoxynucleotide	**dGTP**	deoxyguanine triphosphate
dNTP	deoxynucleotide triphosphate triphosphate	**DTT**	Dithiothreitol
		dsDNA	double stranded DNA
dTTP	deoxythymine triphosphate	***E. coli***	*Escherichia coli*
EDTA	Ethylene-Diamine-Tetra Acetic acid	**EtBr**	Ethidium Bromide
		EtOH	Ethanol
FRET	Fluorescent Resonance Energy Transfer	**G**	Guanine
		GuSCN	Guanidinium Thiocyanate
HCl	Hydrochloric Acid	**HV1**	Hypervariable Region 1
HV2	Hypervariable Region 2	**KCl**	Potassium Chloride
Mg	Magnesium	**$MgCl_2$**	Magnesium Chloride
mtDNA	mitochondrial DNA	**NaCl**	Sodium Chloride
nDNA	nuclear DNA	**PAGE**	Polyacrylamide Gel Electrophoresis
PCR	Polymerase Chain Reaction		
PK	Proteinase K	**PCT**	Pressure Cycling Technology
RNA	Ribonucleic acid	**SDS**	Sodium Dodecyl Sulfate
SEM	Scanning Electron Microscopy	**STR**	Short Tandem Repeat
		SEM-EDS	Scanning Electron Microscopy- Energy Dispersive Spectrometer
T	Thymine		
Taq	*Thermus acquaticus*		
TBE	Tris-Boric acid-EDTA	**UV**	Ultraviolet

Abstract

The extraction of DNA from ancient samples presents many obstacles to the analyst. These samples are often subjected to many years of harsh environmental conditions and many forms of damage resulting in low copy number and highly fragmented DNA. Furthermore, due to their degraded nature, these samples may only be present in small quantities and thus limit the analysis to only one or two extractions.

Therefore strategic methodological approaches must be designed to accommodate these limiting factors, while maximizing the results that may be achieved. The initial stages of analysis are the most crucial, since they are responsible for isolating the DNA. Therefore, the assessment of different decontamination, sample preparation and extraction techniques to determine their ability to yield high quality DNA was conducted. Three types of tissue, 50 bone extractions, 49 teeth extractions and 10 soft tissue extractions were evaluated using two sample preparation methods and four extraction methods. Homogenization or pulverization of the sample increased the overall surface area of the sample, and resulted in a higher success of retrieving DNA for all three tissue types. Both bone and teeth were found to be reliable sources for DNA, however the success of the extraction method was dependent upon the preservation of the sample. Proteinase K and Guanidinium Thiocyanate were determined to be the most reliable methods for ancient samples from all three types of tissue with 45% and 36% success respectively. A novel technique, Pressure Cycling Technology was found to have promising implications for modern samples, with 100% detection. mtDNA sequence analysis concluded these aDNA samples were of low copy number and highly fragmented.

I. Introduction

The macromolecule, deoxyribonucleic acid (DNA), has propelled our understanding of the function of an organism at the cellular level and how organisms replicate and pass on information from generation to generation. In fact, DNA has often been referred to as the genetic "blueprint" of an organism, meaning that all the information required for the organism to survive is found within that blueprint, and with time can be identified. Within the past thirty years, the focus of research has been on developing techniques for the detection of DNA in order to determine the presence of unique traits between different organisms or individuals. Examples of these fundamental techniques include the polymerase chain reaction (PCR), short tandem repeat (STR) profiling and mitochondrial DNA (mtDNA) sequencing. These techniques are extremely sensitive allowing for the detection of minute concentrations of degraded DNA.

Techniques such as Short Tandem Repeat (STR) and mtDNA profiling have been applied to criminal investigations as well as, human population biology, anthropological and evolutionary studies. Even more so, they have provided valuable information in solving investigations of the ethnic origins of populations of the past.

i. Structure of DNA

It was in 1953, that James Watson and Francis Crick first discovered the structure of DNA (Watson and Crick 1953b; Watson and Crick 1953a). Using experimental X-ray diffraction data, they determined DNA is comprised of two helical chains that spiral in a right handed conformation around a central axis (Watson and Crick 1953b). These two chains are built from three biochemical components; deoxyribose sugars, phosphates and

nitrogenous bases. When combined, these components make up what is referred to as a nucleotide (Watson and Crick 1953b; Watson and Crick 1953a). The sugar and the phosphate alternate along the chain and build the "backbone" of the DNA molecule (Watson and Crick 1953a). The phosphate binds at the 5' and 3' carbons of the sugars through the formation of an ester bond. The deoxyribose sugar is also attached to one of four possible nitrogenous bases at the 1' carbon through a N-glycosyl bond (Watson and Crick 1953b; Alberts 2002). The four possible bases are; adenine (A), cytosine (C), guanine (G) or thymine (T) (Watson and Crick 1953a; Watson and Crick 1953b; Alberts 2002). It is these four nitrogenous bases that are responsible for linking the two helical chains. In order to create the double helical structure, a purine being either adenine or guanine must pair with a pyrimidine, either thymine or cytosine through hydrogen bonding (Watson and Crick 1953a). However, these purines and pyrimidines will not bind in any combination. Adenine may only bind with thymine and cytosine only with guanine due to their tautomeric structure (Watson and Crick 1953a). Two hydrogen bonds bind adenine with thymine, while cytosine binds with guanine through three hydrogen bonds (Watson and Crick 1953b; Hummel 2003) (see Figure 1). The complementary base pairing results in the two helical chains running antiparallel to each other in the 5' to 3' direction, allowing the DNA molecule to be in its most stable and energetically favorable conformation (Watson and Crick 1953a; Alberts 2002). This directionality is observed in Figure 1. The order in which the bases exist on the DNA is not a regular sequence, allowing a large number of possible sequence combinations to exist. It is these sequences that generate what is known as the genetic code, and are responsible for all genetically determined traits within a living organism (Hummel 2003).

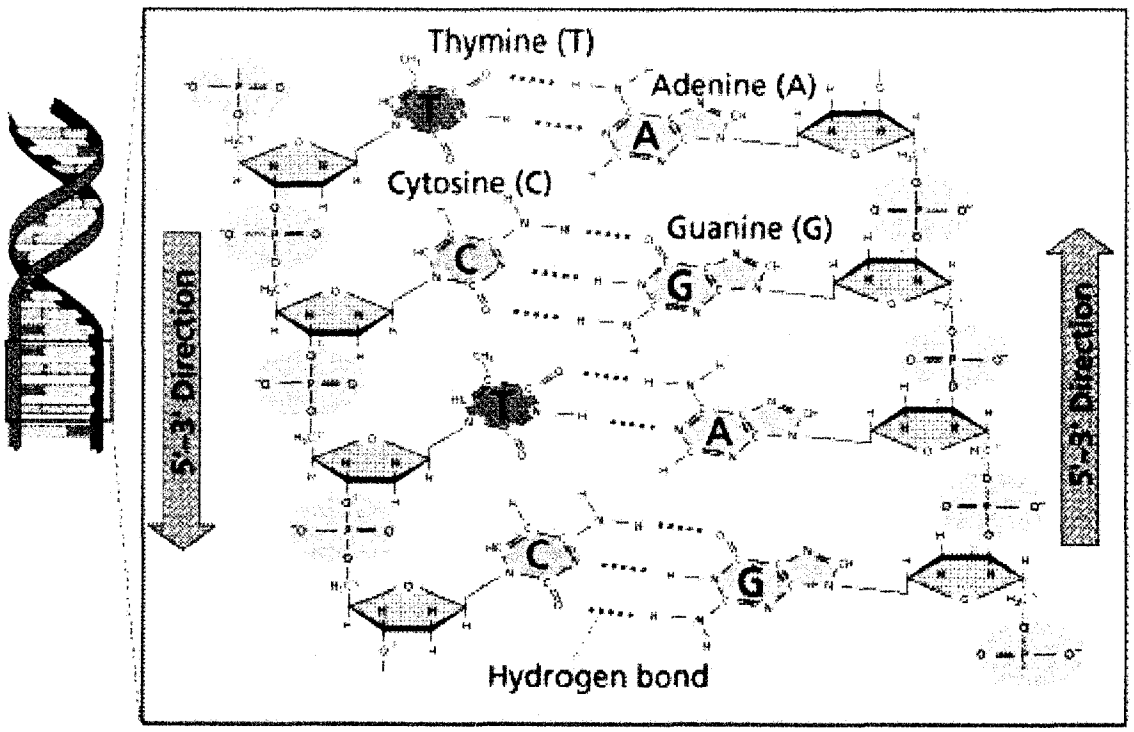

Figure 1: The basic structure of the DNA double helix
(Image taken from: alfin2100.blogspot.com/2005_12_01_archive.html)

ii. Types of DNA

The most common two types of DNA that are used for analytical purposes are nuclear DNA (nDNA) and mitochondrial DNA (mtDNA). As the names imply, nuclear DNA is found within the nucleus of a cell and mitochondrial DNA is found in the mitochondria. Although both of these types of DNA are responsible for encoding proteins necessary for the cell's proper function, they differ in many ways. These differences include the number of copies per cell, the size and structure of the DNA and their mode of inheritance. Each type of DNA has diagnostic advantages for the researcher, depending on the characteristics or polymorphisms that are of interest for study.

a. Nuclear DNA

Nuclear DNA exists as a single copy within the nucleus of the cell. This type of DNA can be found highly compacted for many reasons, including protection and size. It is essential that nDNA have some form of protection, as the survival of the cell is dependent upon the information contained within its sequence. Also, if the DNA were not compacted, the strands would be too long to fit within the nucleus of the cell. DNA wraps around a histone complex comprised of eight histones (Alberts 2002). This forms the most basic form of compaction known as a nucleosome. Approximately 146 base pairs are wrapped around each histone complex and each nucleosome is joined through linker regions that are approximately 80 base pairs in length (Alberts 2002). Nucleosomes are stacked to allow further compaction and form chromatin which then supercoils, resulting in the formation of a chromosome (Alberts 2002). Figure 2 shows the nature in which nDNA is wrapped around the histones and further compacted. Within the nuclear genome there are a total of 46 chromosomes forming 22 pairs of autosomal chromosomes (autosomes) and one pair of sex chromosomes. When all the chromosomes are combined, the nuclear genome is 3 billion bases long (Butler 2001).

The nuclear genome is highly conserved between individuals, with approximately 99.9% of nDNA being shared from one person to the next. It is the remaining 0.1%, approximately three million bases that differ (with the exception of identical twins which share 100% of their DNA) between individuals (Butler 2001). This difference can be used for analysis in determining the presence of unique polymorphisms. It is a result of the mode of inheritance in which nDNA is passed on from generation to generation. As mentioned, nDNA is comprised of 24 pairs of chromosomes (Butler 2001; Hummel

2003). Each individual inherits one chromosome from each pair from their mother and the other from their father. The only exception is the sex chromosomes. The mother only passes on an X chromosome, whereas the father has the ability to pass on either the X or Y chromosome (Hummel 2003). The X chromosome is therefore inherited much like the autosomal chromosomes. However, the Y chromosome is inherited only from the father and when it is passed to the son it does not recombine (Herrmann and Hummel 1994). Therefore, the Y chromosome provides a paternal mode of inheritance.

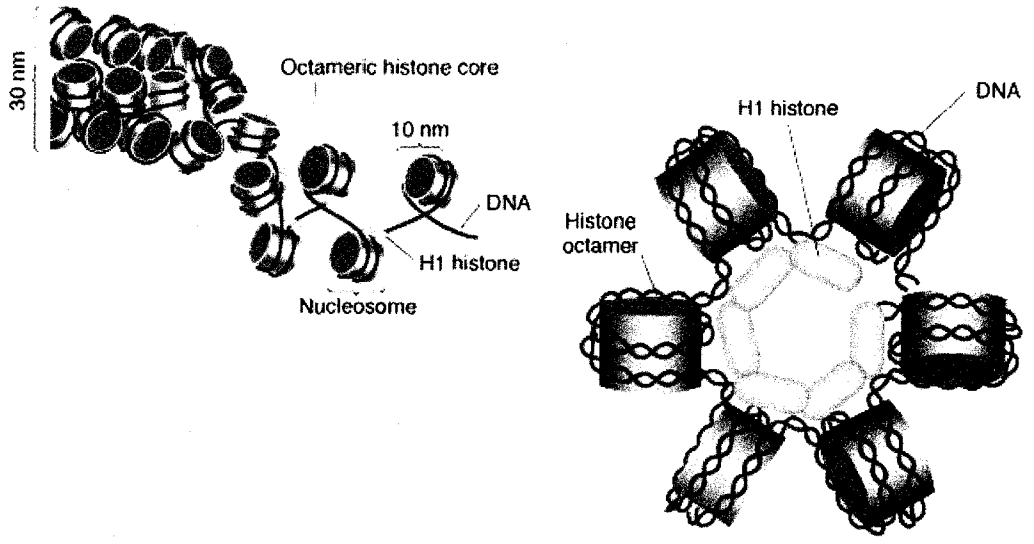

Figure 2: The compaction of nuclear DNA forming a nucleosome (a histone octamer) which further compacts to form chromatin.
(Image taken from: http://www.researchintelligentdesign.org/wiki/Image:Histone-nucleosome.jpg)

b. Mitochondrial DNA

Within each cell, there may exist up to 1 000 mitochondria (Wallace 1994; Budowle et al. 2003). The mitochondrion is a subcellular organelle responsible for the production of energy to maintain the cell's function through the use of the oxidative phosphorylation pathway (Kang 2002).

In the mitochondrion an extrachromosomal genome exists that is entirely distinct from the nuclear genome, known as mitochondrial DNA (mtDNA). Mitochondrial DNA exists in high copy number and may vary anywhere from two to ten copies per mitochondrion (Budowle et al. 2003). Human mtDNA is highly compact, histone free, double stranded in a circular conformation and is 16 569 base pairs in length (Anderson et al. 1981; Budowle et al. 2003; Hummel 2003). Of the two strands of mtDNA, one is purine rich and is referred to as the heavy strand, while the other is pyrimidine rich and called the light strand (Wallace 1994). This genome is responsible for encoding 37 genes, 24 necessary for intramitochondrial protein synthesis and the remaining 13 are for essential subunits for enzymes involved in the oxidative phosphorylation pathway (Wallace 1994; Budowle et al. 2003; Hummel 2003). The circular structure of mtDNA and the distribution of the encoding genes are observed in Figure 2.

Unlike nDNA, the mode of inheritance for mtDNA is only maternal (Budowle et al. 2003; Hummel 2003). Therefore, all maternally related individuals, in theory, possess the same mtDNA genome. This indicates that the discriminatory power of mtDNA for the identification of individuals is not as great as nDNA, however, mtDNA is extremely useful for population and migratory studies. Mitochondrial DNA can also be used in assisting missing persons cases, since maternal relatives may be used to create a mtDNA profile for the missing individual or for a comparison to unknown individuals.

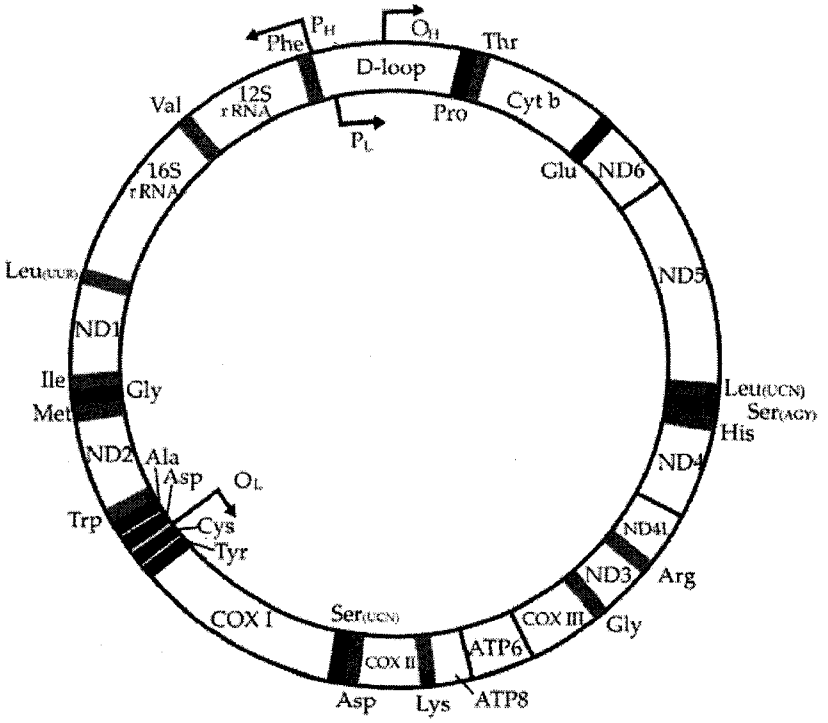

Figure 2: The human mitochondrial DNA genome.
(Image taken from: http://herkules.oulu.fi/isbn9514268490/html/c347.html)

mtDNA is more susceptible to mutation and damage than nDNA, since it is not wrapped around histones for protection. Also, mtDNA has a polymerase with low fidelity and lacks repair mechanisms to correct any damage or mutation (Budowle et al. 2003). It is for these reasons that there is a higher mutation rate within the mtDNA genome (Budowle et al. 2003). Furthermore, the mitochondrial genome contains several non-coding regions, called control regions (Hummel 2003). Within these regions a higher rate of sequence variation between individuals exists. Some regions have been observed to evolve five to ten times faster than a single copy of a nuclear gene (Budowle

et al. 2003). The polymorphic variability that exists in these regions is not distributed uniformly, but rather appears to cluster in regions referred to as "hot-spots" (Hummel 2003). One region in particular, located near the nomenclatorial origin of the mitochondrial genome, known as the D-Loop or control region, has been found to account for approximately 3% of the variability observed between individuals (Stoneking 2000; Budowle et al. 2003; Hummel 2003) (see Figure 2). This non-coding region spans approximately 1 100 base pairs and is divided into two segments, hypervariable region 1 (HV1) and hypervariable region 2 (HV2) (Budowle et al. 2003). HV1 spans nucleotide positions 16 024 to 16 365 and HV2 covers nucleotide positions 73 to 340 (Hummel, 2003; Budowle et al 2003). Due to the maternal mode of inheritance, sequence polymorphisms within these two regions enable identification of individuals only at the family level. Other regions such as the cytochrome c oxidase subunit II, although less variable than HV1 and HV2, are capable of identifying individuals at the population level (Hummel 2003).

In 1981, Anderson et al. sequenced the entire mitochondrial genome, known as the Cambridge Reference Sequence (Anderson et al. 1981). Since then, some modifications have been made to the sequence, however it is still used as the reference in which other mtDNA sequences may be compared. The sequence is displayed as the light strand and only the differences between the sequence being compared to the reference are noted (Budowle et al. 2003).

iii. Sources of DNA

Any biological source can be used to extract DNA, however, some sources are more commonly encountered in criminal or anthropological investigations. Also, some sources are better preserved or contain a higher copy number of DNA based on their structural integrity. A few biological sources that have been known to be successful in retrieving DNA include bone, teeth and soft tissue.

a. Bone

The use of skeletal remains is often used to aid in the determination of characteristics such as stature, sex and age for identification purposes. These attributes would be determined using metric and morphological traits at certain points on the bone; however, osseous tissue also provides an excellent source of DNA. It is considered to be one of the strongest types of tissue found within the human body and therefore can withstand a variety of environmental conditions.

In every adult human body there are 206 major bones. These bones vary in both their structure and size. Each bone contains two types of osseous tissue; compact bone and cancellous bone (see Figure 3). The compact bone is the dense outer surface of the bone that forms the protective layer. The cancellous bone is an open network of struts and plates and is often referred to as spongy bone (Martini 2001). The proportion of these two types of bone is dependent on the bone itself. In Figure 3, both types of bone are observed with the compact bone in a greater proportion than the cancellous. It is also important to note, that the transition from compact to cancellous bone is continuous with no barrier formed between. To better understand, it is easiest to consider two groups of bones; long bones (for example the femur or humerus) and flat bone (such as those found

in the cranium) (Martini 2001). In the instance of long bones, the shaft of the bone is referred to as the diaphysis and the expanded areas at each end are the epiphyses. The diaphysis has a layer of compact bone surrounding a marrow cavity within the central space of the shaft. The epiphyses at each end consist mostly of cancellous bone with a thin layer of compact bone (Martini 2001). Dispersed throughout the marrow cavity and the cancellous bone is a loose connective tissue called bone marrow. There are two types of marrow, yellow, which is made up of fat cells and red, which has a combination of mature and immature red and white blood cells, and stem cells (Martini 2001). For flat bones, there is a thin layer of compact bone that covers a layer of spongy bone, while the marrow cavity is absent (Martini 2001).

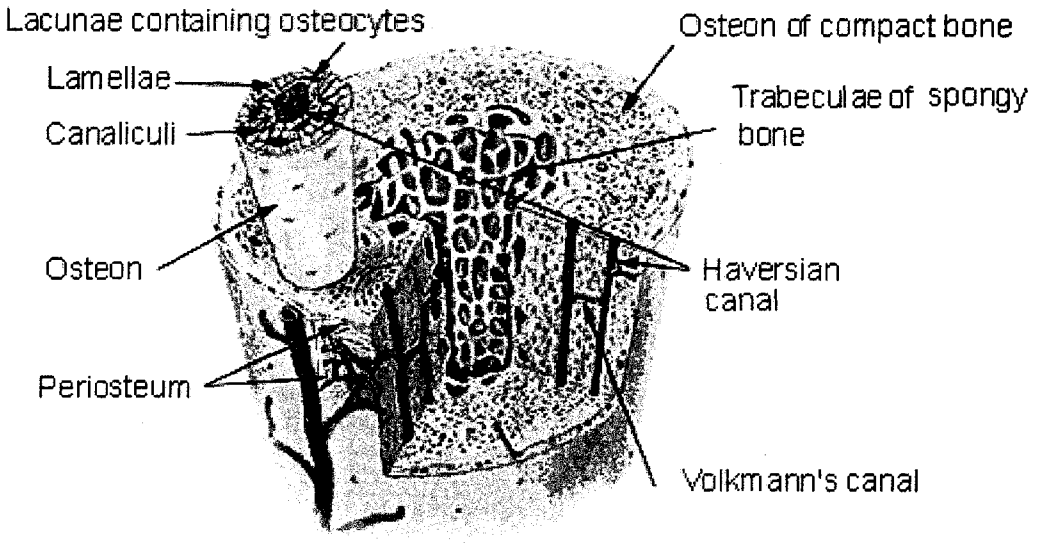

Figure 3: The structure of compact bone and cancellous (spongy) bone.
(Image taken from: http://en.wikipedia.org/wiki/Osteon)

Both types of osseous tissue are comprised of the same type of matrix, with calcium phosphate and calcium hydroxide as the two primary components. When these

two compounds interact, they form a crystalline structure known as hydroxyapatite. This compound is hard and able to withstand compression, however is easily susceptible to breakage when exposed to bending or sudden impact. Therefore, there is a third component of the matrix, collagen. These protein fibres are flexible, however, not resistant to compression forces. This combination allows bone to be strong while still somewhat flexible (Martini 2001).

There are four types of cells that are responsible for the formation and remodeling of bone. The first type, osteocytes, is the most abundant type of cell found in bone. These cells are mature bone cells that maintain and monitor the protein and mineral content and can participate in the repair of damaged bone (Martini 2001). The second cell type is the osteoprogenitor cell. These are stem cells that differentiate into the third cell type, osteoblasts. The osteoprogenitor cells are responsible for maintaining the population of osteoblasts, especially when repair to damaged bone is required. Osteoblasts produce new bone matrix (Martini 2001). They make and release proteins and organic materials to form the matrix. The fourth type of bone cell is the osteoclast. These are large cells that can contain up to fifty nuclei within one cell. They secrete acidic and proteolytic enzymes that dissolve the bone matrix. This is necessary to regulate the concentrations of calcium and phosphates within bodily fluids (Martini 2001).

Bone is an advantageous type of tissue for the analysis of DNA, especially for ancient or degraded DNA, primarily because it is commonly found at archeological sites. The outer layer of bones, especially long bones, is made up of compact bone. This layer is dense and strong allowing the inner trabeculae and marrow to be protected and preserved from the surrounding environment. The majority of the DNA in compact bone

is in the osteocytes. As mentioned, these cells are remnants of the osteoblasts that have become isolated from the extracellular matrix. In fact, it has been suggested that there are 20 000 to 26 000 osteoblasts per cubic millimeter of calcified bone matrix (Frost, 1960; Frost, 1961; Martin and Burr, 1989).

Even though osteocytes embedded within the calcified matrix are advantageous for the protection of DNA from the environment, it may actually restrict access to the DNA during extraction. Depending on the extraction method used, certain treatments can be added to the procedure in attempt to avoid this (Hochmeister et al. 1991). Also, bone may be highly resistant to physical and chemical erosion, but DNA degradation will still occur within the sample since it is no longer alive and functioning. Furthermore, if the bone is fractured the inner cavity can be exposed. This would create an opening in which contaminants such as bacteria, chemicals, water and severe weather conditions can enter and break down the integrity of the inner bone. Since the inner portion of the bone is more porous due to the trabeculae of the spongy bone, the compact portion of the bone may also absorb these contaminants. These factors may accelerate the rate of degradation of the DNA or introduce substances that may inhibit the extraction or amplification of DNA. Furthermore, factors such as humidity and temperature can affect the rate of auto-degradation (Perry et al, 1988;Ye, 2004).

b. Teeth

The use of teeth in identification of remains is well known (Schwartz et al. 1991; Ginther et al. 1992; Woodward et al. 1994; Pfeiffer et al. 1999; Pfeiffer et al. 2003; Cerri 2004). In the past, they have been used for comparison purposes with antemortem dental records, where teeth are compared for similar dental restorations. This approach is not

always successful. In order for a comparison to occur, the investigation needs to have a lead as to possible individuals. Also, many individuals do not regularly visit the dentist or are unable to receive dental care and may not have current dental records or radiographies.

Alternatively, the anatomy and composition of teeth makes them an ideal source for the recovery of DNA. In fact the successful recovery of high molecular weight DNA from both the nucleus and the mitochondria, has been reported throughout the past two decades (Schwartz et al. 1991; Ginther et al. 1992; Woodward et al. 1994; Mornstad et al. 1999). Unlike soft tissue, teeth have an outer enamel matrix making themresistant to the degradation effects caused by decomposition and putrefaction post mortem, leaving the structure of teeth well intact for long periods of time.

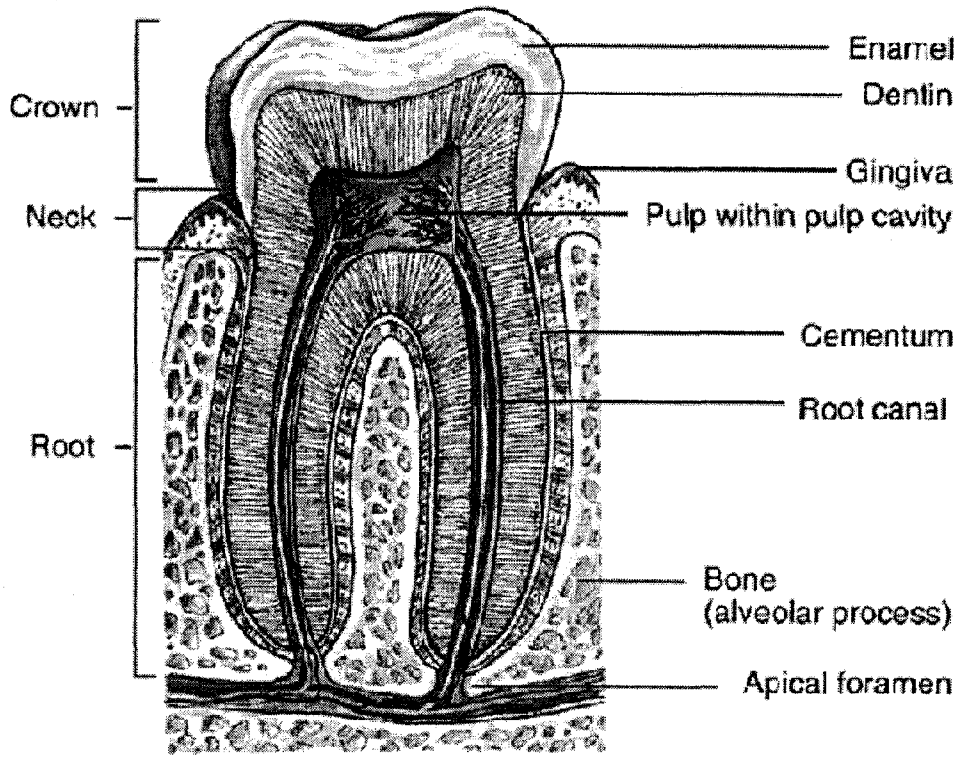

Figure 4: The structure of a tooth in a longitudinal section view.
(Image taken from: http://www.mse.cornell.edu/.../structure_of_teeth.html)

Teeth are comprised of three mineralized tissues; enamel, dentine and cementum which all surround and protect the inner connective tissue making up the dental pulp (Berkovitz 2002) (seeFigure 4). The dentine makes up the bulk of the tooth, where the enamel forms the outer layer of the crown and the cementum is the outer layer of the root. Enamel is the hardest biological tissue; it is highly resistant to abrasion and shearing (Berkovitz 2002). Conversely, it has no repair or regeneration properties and therefore wears down slowly over time. The primary mineral component of enamel, about 90%, is hydroxyapatite, while the remaining components are water and an organic matrix comprised of peptides and amino acids (Berkovitz 2002). Although the enamel is dense, there is some porosity within this layer. These pores are filled with water molecules and create a pathway for ions like fluoride, to help maintain the health of the tooth (Berkovitz 2002). The cementum is a thin layer of calcified tissue that covers the root. It is made up of both inorganic and organic materials and water. The primary inorganic material again is hydroxyapatite, however calcium exists in higher levels in this layer than in the enamel or dentine (Berkovitz 2002). The third calcified tissue, dentine, is harder than bone and cementum, but softer than enamel. The dentine is a permeable matrix made of inorganic compounds such as calcium and hydroxyapatite, and organic compounds including collagen. The formation of dentine is created by odontoblasts, which extend their processes into the edge of the already formed dentine (Berkovitz 2002). The fourth component of a tooth is the specialized connective tissue known as the dental pulp. It is made up of a combination of cells, which embed in an extracellular matrix. These types of cells include odontoblasts, defence cells and possibly fibroblasts. These cells however, are unable to differentiate (Berkovitz 2002).

As mentioned, the enamel coating surrounds the inner pulp cavity where the DNA rich cells reside (Ginther et al. 1992). The unique environment within the pulp cavity allows the DNA to remain relatively stable in comparison to the DNA found in other cellular components (Ginther et al. 1992). These DNA rich cells, odontoblasts, contain a high copy number of mitochondria. In fact, it has been suggested by Mornstad *et al.* (1999) that as the tooth ages, these processes disintegrate leaving mitochondria containing mtDNA, even though fragmented, within the calcium phosphate crystals. Therefore acting as a preserving agent, protecting the mtDNA from degradation.

In particular with ancient specimens, the purity of the DNA is just as important as the quantity of DNA. Not only does the hard outer surface protect the endogenous DNA within the pulp chamber, but it also acts as a natural barrier preventing exogenous DNA and other organic materials from entering and introducing contaminants or inhibition during amplification (Ginther et al. 1992; Woodward et al. 1994). Environmental factors such as temperature, humidity, pH and the exposure to seawater have failed to have any significant influence on the recovery of DNA from teeth (Schwartz et al. 1991). As reported by Woodward *et al.* (Woodward et al. 1994), the DNA recovered from teeth of Egyptian Mummies resulted in successful amplification without any further modification, unlike that of the soft tissue from the same sources.

Although the use of teeth can be effective in obtaining high molecular weight DNA with a high degree of purity, the condition and preservation of the tooth is very influential on the success. The presence of dental restorations or lack there of, may affect the purity and presence of inhibitors. If the tooth has the presence of dental caries or has been subjected to some sort of infection or damage by breakage, an opening may be

created in which environmental insult and exogenous DNA can enter. The use of dental restorations such as amalgams, fill or cover these openings to once again provide a barrier for the pulp cavity from the surrounding environment. However, the composition of these amalgams can have inhibitory effects during the extraction and amplification of the DNA. Over the past couple of centuries, the components and quality of dental amalgams have changed. Prior to and in the first quarter of the 20th century the primary components of an amalgam were tin and silver. Other elements would be added in small quantities such as mercury to provide the proper consistency (Soler et al. 2002). However, mercury is also known to inhibit the activity of proteinase K (PK), one commonly used DNA extraction method (Muller et al. 1999).

Not only do the condition and preservation of the tooth affect the yield of DNA recovered, but so can the type of tooth. Since the size of the pulp cavity is relative to the size of the tooth, an incisor would have a much smaller cavity than a molar.

c. Soft Tissue

Intact and preserved DNA can not only be found in hard tissues like bone and teeth, but also in the soft tissue comprising the muscular system, internal organs and epithelial layers. These types of tissues will vary in the amount of DNA and the type of DNA present depending on which type of soft tissue is available and the state of preservation. The preservation of these samples is further dependent upon the depositional environment. Soft tissue samples excavated from dry environments are typically the most well preserved as the tissue undergoes natural desiccation, thus protecting it from certain kinds of damage such as hydrolytic deamination (O'Rourke 2000). However, it does not protect it from all forms of damage such as oxidative damage. Two geographic

regions in which this type of preservation is common, due to the large number of preserved individuals found and the extended period of time these individuals have been exposed, are representative of are Egypt and the central Andean region (Paabo 1986). When soft tissue is excavated from moist or humid environments, the yield may not be as great since it is more likely to contain impurities from other components within the human body such as acids or proteins, or from the surrounding environment (O'Rourke 2000). For example, brain tissue from Windover peat bogs yielded only 1% of the expected yield from fresh tissue. Furthermore, this yield is likely even less as the extraction co-extracted peat (O'Rourke 2000).

As mentioned, the yield of DNA, more specifically mtDNA, may differ depending on the type of soft tissue being analyzed. Different tissues have different metabolic and morphologic rates, and thus may have a greater amount of mitochondria within each cell or the tissue may contain more cellular layers resulting in a greater number of nuclei in some tissues. The muscular system is one example of a soft tissue that requires a high quantity of mitochondria due to their high-energy expenditure. Muscles require energy to contract, extend and grow (Martini 2001). In contrast, internal organs such as the stomach or intestines may not yield as high quantity of DNA since the presence of bacteria and acids found within these organs may cause cells to rupture and damage DNA with the onset of decomposition. Epithelial tissues more commonly referred to as skin may not yield as high quality DNA as other soft tissues as it is exposed to various environmental insults throughout an individual's lifetime, which may cause damage to the DNA.

Soft tissue is more permeable or absorbent then other harder biological tissues used for analysis, certain decontamination techniques such as solution washes with sodium hypochlorite and ethanol may actually cause more damage or inhibition to the sample. Therefore an alternative form of decontamination would be to sample subsurface tissue, removing the surface layer which is more susceptible to contamination from improper handling or environmental insult (O'Rourke 2000). Since the techniques for decontamination are limited, it is often advantageous to incorporate a sample preparation method that will increase the probability of extracting high quality DNA. Examples of such types of methods could include tearing the tissue into smaller fragments or using a mortar and pestle to grind the sample into smaller pieces or a paste.

iv. Preservation and Age of DNA

So far, DNA has been classified based on the source it is collected from and the location in which it is found in the cell. However, another form of classifying DNA samples is often used, and in terms of the analysis approach, may possibly be the most significant; the age of DNA. A DNA sample typically falls into one of two categories in regards to age, ancient or modern. Ancient DNA (aDNA) refers to DNA fragments that are found in preserved or fossilized biological material (Brown 1994). Whereas modern DNA is considered to be DNA extracted from "fresh" material, or in a forensic context, samples that are less than 50 years old.

At the onset of death, DNA begins to be degraded by endogenous nucleases (Hofreiter et al. 2001b). These nucleases can become inactivated or destroyed in certain environmental conditions, such as low temperatures, rapid desiccation or high salt

concentrations (O'Rourke 2000; Hofreiter et al. 2001b). Simultaneously, other cellular processes such as nucleases, although degrade at a much slower rate, occur and begin to accumulate. Therefore, although modern DNA samples become susceptible to these damaging processes, the damage is still typically minimal in comparison to the number of copies of DNA available. This is not the case with aDNA. Since ancient samples have been exposed to variable environmental conditions over an extended period of time (100s to 1000s of years), these damaging processes have had more time to accumulate and ultimately result in DNA fragments less than 300 base pairs in length (O'Rourke 2000).

DNA degradation is caused by a gradual breakdown of the nucleic acids over an extended period of time and can be caused by different degradative processes. Such examples of degradation processes include both hydrolysis and oxidation (Lindahl 1993; O'Rourke 2000). Hydrolytic damage can cause degradation in two ways, the cleavage of the phosphodiester bonds formed in the phosphate-sugar backbone, resulting in single stranded nicks, and the cleavage of glycosidic bonds between the sugar backbone and the nitrous base, creating abasic sites (Lindahl 1993; Paabo et al. 2004). Oxidation occurs when water-derived hydroxyl or superoxide radicals distort the helix and modify bases (Lindahl 1993; O'Rourke 2000). These degrading processes occur in living cells but are repaired and controlled by repair mechanisms found within the nucleus, however after death, the repair ceases while the degrading processes continue.

The analysis of aDNA was first described by Higuchi*et. al* in 1984 (Saiki et al. 1988), in which mitochondrial DNA fragments extracted from 140 year old tissue of a quagga were cloned. Shortly after, the first human analysis was from a 2400 year old mummy of an Egyptian boy, in which a sequence of an *Alu* element was retrieved (Paabo

1985). Since then, the field of aDNA analysis has been growing (Paabo 1986; Paabo 1989; Hoss and Paabo 1993; Handt et al. 1996; Hofreiter et al. 2001a; Pfeiffer et al. 2003). However, this area of research has also undergone much scrutiny in regards to the authenticity of the data within these various studies. As previously mentioned, aDNA is subjected to various damaging and thus degrading processes resulting in very short fragments of DNA. Also, these processes cause a decrease in the copy number of intact DNA available for analysis. Furthermore, the sample collected is often only available in limited amount, preventing extensive analysis from being performed. Thus it is often not possible to replicate the results to determine if the profile achieved is the endogenous DNA. A second factor that affects the successful detection of DNA is inhibition. Inhibition can influence the DNA analysis at two stages, extraction and amplification. During extraction, inhibitors may interfere with the chemistry of the extraction technique, preventing the active reagents from solubilizing proteins and releasing DNA. Such an example would be mercury inhibiting PK, as previously mentioned (Muller et al. 1999). Certain compounds co-purified with the DNA may inhibit the amplification of DNA; these will typically affect the chemistry of the activity of the polymerase within the PCR. PCR inhibitors mostly encountered in aDNA analysis are tannins, humic acids, and fulvic acids, all of which are soil-derived degradation products (O'Rourke 2000).

Since aDNA is typically only present in low copy numbers and highly fragmented, specific criteria have been developed to ensure all possible precautions are taken to prevent any further contamination and to ensure authentic results are achieved. Typically, to ensure reliable results, the analysis on any sample would be performed in duplicate or reproduced, but with aDNA analysis this is often not possible because there

is only limited sample available. Therefore, decontamination procedures of laboratory equipment must be enforced, as well as utilizing extraction and PCR controls to ensure no modern contaminants from the laboratory are introduced (Handt et al. 1996; Hofreiter et al. 2001b). Furthermore, the pre- and post-amplification analyses should be physically separated (O'Rourke 2000). Finally, the results should be phylogenetically consistent and an inverse relationship between fragment size and amplification success should be observed (Carlyle et al. 2000; O'Rourke 2000; Hofreiter et al. 2001b).

II.Methodological Background

i.Sample Pretreatment

When samples are received for DNA analysis; the previous collection, handling and storage procedures may not be known. Furthermore, the conditions of the depositional environment may contain compounds that can be co-purified with the endogenous DNA and cause inhibition. Therefore, in order to produce high quality results the sample must undergo some form of pretreatment prior to DNA extraction. This pretreatment can be divided into two categories, decontamination and sample preparation. Decontamination techniques are employed to remove surface contaminants, while sample preparation techniques are used to increase the surface area of the sample to facilitate the extraction process. The application of these steps is significant especially if any surfaces of the sample have potentially been cross-contaminated or have been in contact in any way with cellular material from another individual or any other type of biological specimen (including animals, plants, or bacteria) (Hummel 2003). These procedures are particularly important with aDNA studies since the endogenous DNA exists only in low copy numbers, and thus cannot compete with any modern exogenous DNA present through handling or even bacterial DNA present from the depositional environment.

a. Decontamination

The removal of surface contaminants is important in order to prevent exogenous DNA from contaminating the endogenous DNA sample and to avoid the introduction of inhibitors that may prevent the extraction or the amplification of DNA. There are various decontamination techniques that are successful, however, which one to apply is

dependent on the type and size of the sample. The most obvious technique to remove surface contaminants is a wash or rinse step. This can be performed using a variety of solutions including detergents, sodium hypochlorite, ethanol or distilled water. In fact, these solutions are often used in combination with each other. The samples are then allowed to air dry and are either ready for extraction or undergo an additional decontamination method. The use of a solution will physically wash contaminants off the surface of the sample. When washes of sodium hypochlorite or ethanol are used, they can actually kill organisms on the surface and even degrade exogenous DNA. Sodium hypochlorite destroys DNA by oxidative damage through base modifications and the formation of chlorinated base derivatives (Kemp and Smith 2005). At high concentrations of sodium hypochlorite, the DNA strands will cleave into smaller fragments, eventually resulting in single bases (Kemp and Smith 2005). In fact a study conducted by Prince and Andrus suggests that only a five minute treatment of sodium hypochlorite would cleave fragments into such a small size that a 76 base pair amplicon could not be amplified (Prince and Andrus 1992). It is important to note that the use of the aforementioned solutions for decontamination is only advisable for hard-calcified tissues such as bone or teeth. The porosity of these tissues is very small, therefore limiting the possibility of the wash solution being absorbed. If the tissue is soft tissue such as muscle, blood or other bodily fluids the wash solution would be mixed or absorbed and thus may interfere with the analysis.

An alternative approach to solution washes and one of the most common decontamination techniques is the use of shortwave ultraviolet (UV) light that induces covalent binding of opposite thymine bases causing strand breaks and preventing double

stranded DNA from denaturing during PCR (Niederhauser 1994; Hummel 2003). Therefore these double stranded DNA fragments are inaccessible for amplification during analysis. The length of exposure, the distance between the sample and the wavelength of the UV source are all factors that have been considered in previous studies (Sarkar and Sommer 1991; Prince and Andrus 1992; Niederhauser 1994). Unfortunately, many studies focus UV irradiation decontamination for PCR reagents, however, a recent study by Bouwman et al. in 2006 assessed the efficacy of UV irradiation in removing surface contaminants from ancient bone in obtaining authentic DNA sequences (Sarkar and Sommer 1991; Prince and Andrus 1992; Niederhauser 1994; Bouwman et al. 2006).

The use of sonication is another technique for decontaminating the surface of the sample. Sonication utilizes high frequency sound waves to disrupt the cell membrane. The sound waves travel through a solution, typically distilled water, however any of the solutions mentioned could be used as well. By having the sample in the solution, it will rinse off any surface contaminants, furthermore, the sound waves disrupt cell membranes from other contaminating sources so that the DNA will be released into solution which will then themselves be sheared by the high frequency sound waves.

b.Sample Preparation

Prior to DNA extraction the sample must be prepared in order to increase the probability of yielding a high quantity of DNA. There are a variety of sample preparation techniques that can be used and are dependent on the type of sample being analyzed. However, each technique serves one purpose, to increase the surface area of the sample so that the extraction buffer will have a greater chance in retrieving DNA during lysis. One common technique is the homogenization of the sample (Hummel 2003). When

dealing with mummified or soft and brittle tissues this can be achieved using a mortar and a pestle. For bone and teeth samples, a grinding mill is necessary, where the samples are broken into small fragments and then ground into a fine powder. An alternative to completely pulverizing and destroying the entire bone or tooth sample is drilling. This technique still allows the homogenization of the sample however, without complete destruction of the sample. It also prevents the inclusion of the outer surface that may contain remnants of contaminants. Other sample preparation methods involve fragmentation of the sample rather than complete homogenization. For soft tissue, the sample can be torn or cut into smaller pieces and with bone or teeth samples they can be broken into smaller fragments. This technique still increases the surface area, but requires less time.

ii. DNA Extraction

The extraction of DNA is the most critical step within the entire analytical process; if the wrong method is performed it can reduce and even destroy the potential genetic information that may have been retained within the sample (Herrmann and Hummel 1994). There are many extraction methods that are available to use and each has been designed to perform the following: the lysis or fractionation of the cell membrane of the tissue being analyzed; the use of a detergent or salt to dissociate the DNA from proteins within the cell; the use of an extraction buffer to separate the bulk of protein from DNA and finally, to employ the use of enzymes to remove any RNA and polysaccharides (Travaglini 1973). The determination of which extraction method to use is influenced primarily by the type of sample to be analyzed, however the depositional

environment from which the sample was recovered may also affect the success of the extraction. Although careful selection of an extraction method is significant, previous research has been able to draw conclusions as to which are the most reliable and robust (Herrmann and Hummel 1994). For example, in forensic investigations, the proteinase K (PK) extraction method (Hansen 1974) is usually the standard method used, whereas in aDNA research facilities a common technique employed is the guanidinium thiocyanate (GuSCN) chemical extraction method (Boom et al. 1990). It is important to note that these are not the only two extraction methods that can be used. Since the primary purpose of this step is to liberate nucleic acids from the cells or cellular remains into solution, it is necessary to understand the biochemical premise of each extraction method in order to know when its use is appropriate (Hummel 2003).

No matter which extraction method is used, each will contain some common reagents that comprise the extraction buffer. The presence of the buffer, typically Tris-HCl maintains the optimal pH to allow the extraction to progress at its highest efficiency. Also, Tris-HCl in combination with ethylene-diaminetetra-acetic acid (EDTA) allows the lysis of cell walls by chelating with calcium ions, while stabilizing the DNA at the same time (Herrmann and Hummel 1994). A third common component is a sodium or potassium salt which creates an isotonic environment to stabilize the released DNA (Herrmann and Hummel 1994). An optional reagent is a nonionic detergent to further stabilize the extraction buffer and disrupt the phospholipid bilayer of the cell wall to gain access to internal cellular components (Herrmann and Hummel 1994).

a. Proteinase K

The PK method is an enzymatic extraction method first described by Hansen *et al.* in 1974 (Hansen 1974). Proteinase K can be added to the extraction buffer to digest the protein fraction of the cell, as well as release any nucleic acids adhering to proteins (Herrmann and Hummel 1994). In addition to digesting proteins, the proteinase also prevents DNA degradation presumably because it inactivates nucleases released during cell lysis (Hansen 1974). Aside from the aforementioned components of the extraction buffer, additional detergents including sodium dodecyl sulfate (SDS) are also added. Another reagent, dithiothreitol (DTT), can also be added to disrupt protein disulfide bonds within the cell membrane (Butler 2001). These reagents help to disrupt the cell wall in order to release the DNA into solution.

As mentioned, this type of extraction technique is considered the standard method in forensic investigations. The successful extraction of DNA from a variety of different samples using PK has been well documented (Hochmeister et al. 1991; Pfeiffer et al. 1999) and therefore, is considered to be both a reliable and robust method. However, because PK is an enzyme, the presence of certain metal ions or other environmental compounds may have inhibitory effects on the activity of the enzyme and thus affect the success of the extraction.

b. Guanidinium Thiocyanate

As previously mentioned, GuSCN is one of the most common extraction methods used in aDNA research facilities (in particular aDNA facilities). There are many variations of this extraction method, but the most common or the founding technique for nucleic acids is the method first described by Boom *et al.* in 1990 (Boom et al. 1990).

The presence of GuSCN, a chaotropic agent results in the denaturation of cellular proteins releasing DNA. This result is achieved because the chaotropic environment interferes with the hydrogen bonding and polar interactions of the protein and thus reduces the energy required for unfolding of the protein.

Although this extraction method has been available for quite some time, it has not been standardized and thus, is not used in forensic investigations. However, it has been used in many studies and has been found to be a reliable and versatile technique (O'Rourke 2000).

c. Cetyl-trimethylammonium Bromide

Although the use of Cetyl-trimethylammonium Bromide (CTAB) is typically used for the extraction of DNA from plant material (Murray and Thompson 1980), the application to human samples has also been reported (Yang et al. 1998; Ye et al. 2004). Jones first described the initial application of CTAB in 1953 to develop a more general application method for the isolation of nucleic acids from the bacteria *Mycobacterium tuberculosis* (Jones 1953). CTAB is a cationic detergent that can successfully disintegrate the cell wall and inner membranes (Jones 1953; Yang et al. 1998). An additional reagent known as 2-mercaptoethanol is also present to aid in the digestion of the cellular proteins by cleaving disulfide bonds. When this reaction is complete, the digested proteins and cellular debris are still present in solution with the released DNA, however, the properties of this detergent allows the precipitation of the nucleic acids when a salt at low concentration is added (Del Sal et al. 1989). A study performed by Ye *et al.* found this method to be a reliable, robust and efficient strategy when analyzing aged and burned bones (Ye et al. 2004). As previously mentioned, the use of CTAB on

human samples is still not well known, however, in studies that have been preformed on other types of DNA it has been concluded that the DNA is of high quality (Del Sal et al. 1989).

d.Pressure Cycling Technology

Pressure cycling technology (PCT) is a novel extraction method that utilizes a different form of biochemistry or technology to release nucleic acids. First developed in 2002, this technique was created by the commercial company, Boston Biomedica (Garrett 2002). Unlike the other methods previously discussed, this technique removes the necessity for the homogenization of the sample prior to the extraction. For this method, the sample and a lysis buffer are placed in a single-use processing container called a PULSE (Pressure Used to Lyse Samples for Extraction) tube which then undergoes rapid temperature-controlled pressure cycling between ambient and high pressures to induce cell lysis all contained within a specially designed apparatus called a Barocycler (Garrett 2002; Tao 2003). When pressure is applied to the PULSE tube, it results in the compression of the sample into the lysis buffer (Garrett 2002). As the pressure cycles repeat, the lysis disc causes further disruption of tissue and releases the molecular components into the buffer (Garrett 2002). It is important to note that the type of lysis buffer can vary from chaotropic reagents to detergents or even just water.

This method is beneficial because it allows the entire reaction to take place in a single tube, therefore preventing the possibility of cross-contamination. Since the sample only needs to be added to the PULSE tube, the sample preparation step is not required, resulting in a less time consuming analysis and potential for the introduction of contaminants (Tao 2003). Furthermore, having significant implications for aDNA

samples, this method is a nondestructive method, once the extraction is complete the sample is still intact (Garrett 2002; Tao 2003). However, in order to perform this extraction, the specialized PULSE tubes and the barocycler are required and therefore could be a costly method to use. Since it is still a novel technology, further research may be required to ensure its reliability and adaptability for different types of tissues.

iii.DNA Purification/ Separation/ Concentration

Once the nucleic acids have been released into solution during the extraction, they must be isolated from the remaining cellular debris that are also present in solution. This can be achieved through either a DNA purification or DNA separation technique. Although DNA separation is a form of purification, it uses centrifugation as a means to physically separate the DNA from the remaining debris. Separation can also refer to using reagents with different densities or polarities such that the DNA will reside within one fraction, or phase, separate from the cellular debris, which is in the other phase. This is often referred to as an organic separation, which commonly incorporates a phenol/chloroform (40:1 volume:volume) mixture. The DNA is more soluble in the aqueous phase, while the proteins are soluble in the organic phase, thus isolating the DNA (Butler 2001). An additional step to this purification technique, or may even be used in conjunction with other purification protocols is a series of washes using reagents that are ethanol based to remove any remaining proteins or substances that may interfere with subsequent stages of the analysis. This stage may also concentrate the DNA, as the final step in this method is typically a desiccation of the sample. Once the purification

step is complete, theoretically, all that remains is the DNA either desiccated or in solution, usually either sterile water or a buffered solution.

Sometimes, especially during aDNA studies, an additional purification step is required in order to remove remaining compounds that were co-purified with the DNA. An alternative purification method can be used or the use of a commercially available spin column can be incorporated. These columns employ a selective binding of the DNA to a resin or membrane, followed by a wash step to remove the remaining inhibitors and then finally the elution of the highly purified DNA (Herrmann and Hummel 1994). This form of purification prevents the loss of DNA, as well as preventing any damage to the DNA that can be incurred by some of the other isolation protocols such as the phenol/chloroform purification method (Herrmann and Hummel 1994).

Purification protocols are also used at other stages of DNA analysis, such as after the initial amplification or following the sequencing PCR. These purifications are performed to remove any unincorporated deoxynucleotide and dideoxynucleotide triphosphates (dNTPs and ddNTPs), any remaining primers, or magnesium ions, which can interfere with further amplifications or genetic analysis techniques.

iv.Polymerase Chain Reaction

The Polymerase Chain Reaction (PCR) allows the *in vitro* amplification of a specific DNA sequence of interest without limit (Mullis 1990). Kary Mullis first described this method in 1983 and was first published in 1986 (Mullis et al. 1986; Mullis 1990). PCR is considered a standard technique in molecular biology and has

revolutionized our ability to study a large range of genes. Additionally, it allows the amplification of DNA that may be degraded or only present in small quantities.

PCR utilizes the basic elements of the natural DNA replication process to exponentially amplify a specific segment of DNA in one single reaction (McPherson and Moller 2000). There are three steps involved in the amplification process; DNA strand denaturation, primer annealing, and primer extension (daughter strand synthesis). In the first step, the two strands of the template DNA are first separated by heat; this causes the hydrogen bonds formed between the base pairs to break. During the annealing step, oligonucleotide primers find their complementary sequence on the template strands and bind to the template at a specific location. Finally, a DNA polymerase begins to build the daughter strands by adding nucleotides to the 3' OH group of the previous nucleotide (McPherson and Moller 2000). This results in two copies of the original template DNA. This process is then repeated, however, during the second cycle there are now four template stands that the primers can bind to. Thus, with each additional cycle of PCR, the template DNA is amplified exponentially. It is because of this exponential amplification that millions of copies of target DNA can be generated from as little as one DNA molecule.

The original method first developed by Mullis used a DNA polymerase extracted from *Escherichia coli* since it was a commonly used tool for many other molecular biology applications, including the development of new sequencing technologies (to be discussed later) (Mullis et al. 1986; Bartlett 2003). However, when the reaction is heated to denature the double stranded DNA at the beginning of each cycle, it also inactivates the enzymatic activity of the *E. coli* polymerase irreversibly (Bartlett 2003). Therefore,

new polymerase had to be added at the beginning of each cycle. This resulted in a time consuming method, therefore an alternative DNA polymerase that would remain stable during the denaturation was required. In 1988, a bacterium, *Thermophilus aquaticus* was first isolated from hot springs by Saiki *et al.* (Saiki et al. 1988). Since these bacteria are able to survive in extremely high temperatures, its DNA polymerase, often referred to as *Taq* polymerase, would not be inactivated when exposed to the high temperatures used in PCR (Bartlett 2003). This allowed the PCR to proceed without having to add additional polymerase and thus, less time to complete the reaction. Also, because the *Taq* polymerase functions optimally at 72°C, DNA amplification could be conducted at higher temperatures and increase the fidelity of the PCR (Bartlett 2003). This technique was further improved with the advent of the thermocycling machine. The thermocycler automatically adjusts the temperature for each step of the PCR, alleviating the need to transfer the tubes from different water baths set at specific temperatures (Bartlett 2003).

a. Standard PCR

A standard PCR is the initial amplification of the region of interest to ensure the success of the extraction method in yielding DNA. It is also useful for increasing the quantity of DNA for additional analyses such as DNA sequencing. The conditions for this reaction to occur successfully are highly specific. The primary components within the PCR are a buffer solution, magnesium, deoxynucleotide triphosphates (dNTPs), oligonucleotide primers and DNA polymerase.

The purpose of the buffer solution is to maintain the optimal pH required for the DNA polymerase to operate with high fidelity. These buffers are commonly made with Tris and KCl. The Tris buffer is a dipolar ionic buffer whose pH varies based on

temperature and KCl is present to aid the annealing of the primers and the template (McPherson and Moller 2000). During the PCR the pH tends to vary between 6.8 and 8.3. Typically, DNA polymerase has a higher fidelity when the pH is at the lower values, which occur at higher temperatures (McPherson and Moller 2000).

The second component, magnesium, is considered to be the most significant component in the reaction. The concentration of magnesium will affect the specificity and efficiency of the reaction (McPherson and Moller 2000; Bartlett and Stirling 2003). It can also influence the fidelity of the DNA polymerase. When there is an excess of magnesium, the *Taq* DNA polymerase is more prone to error than when the magnesium ion concentrations are lower (McPherson and Moller 2000). The concentration of the magnesium required in each reaction is specific to each reaction and can be influenced by the template size and sequence and the primer size and sequence. In some cases, magnesium is included in the buffer solution, as magnesium chloride or ammonium chloride. However, it is important to determine the concentration of magnesium for each PCR and this is best done experimentally.

In order for extension of the new DNA strand to occur, the DNA polymerase requires dNTPs. The four dNTPs; dATP, dGTP, dCTP, dTTP must be present in equimolar concentrations in order to ensure high fidelity of the PCR. The typical range of concentration is between 50 – 200 μM (Butler 2001). If the concentration is higher, it may cause the polymerase to misincorporate dNTPs more often than normal, however if it is lower, the success of the PCR may be affected (McPherson and Moller 2000). In order for the new daughter strand to be synthesized, oligonucleotide primers are required to provide a starting point for the addition of dNTPs. This is because the DNA

polymerase is incapable of *de novo* generation of the DNA strand (Bartlett and Stirling 2003). Primers are also required because they specify the region of interest for amplification. The DNA polymerase is responsible for selecting the correct dNTPs that are to be incorporated into the growing DNA strand. The incorporation of dNTPs is highly specific and is based on conventional Watson and Crick base pairing rules (Watson and Crick 1953a). Polymerase synthesizes the new DNA strand in the 5' to 3' direction and there are many different types of polymerases that can be used. Some have a 3' to 5' proofreading activity which verifies the newly synthesized DNA has the correct bases inserted (McPherson and Moller 2000). There are two important factors to consider when selecting and using a DNA polymerase; the efficiency and the fidelity of the polymerase in synthesizing the new DNA strand. The efficiency of the polymerase is influenced by the strength of the interaction between itself and the template strand (McPherson and Moller 2000). The stronger the interaction, the longer the synthesis of the new DNA strand before dissociating from the template. Fidelity refers to the ability of the polymerase to incorporate the correct dNTPs (McPherson and Moller 2000). The concentration of the DNA polymerase in the reaction ranges from 0.5 to 5 units and depends on the number of cycles being performed during the PCR along with the initial concentration of the DNA template. If there is a high concentration of starting template, more polymerase is required to ensure exponential amplification occurs. Also, as the number of cycles increases during the PCR, a greater concentration of polymerase is required. This is because the increased fluctuation in temperature results in the loss of integrity of the polymerase (Bartlett and Stirling 2003). Reducing the integrity of the polymerase then results in lowering the efficiency of the overall reaction.

There are two other important components of the PCR; sterilized water and the DNA template. The volume of water is dependent upon the volumes of the reagents previously mentioned, whereas the volume of DNA template is dependent upon the suspected quantity of DNA isolated. The volume of the reaction can range from 25 to 100 μL. Typically, the reaction volume is 25 or 50 μL, and the previously mentioned components comprise only 5.2 or 10.4 μL of the total volume. Therefore, the remaining volume is made up with a combination of the template and sterile water. A desirable starting template concentration for PCR is in the order of $3.0X10^5$ DNA molecules. Therefore, when amplifying ancient or degraded DNA extracts higher volumes of the template is added, whereas PCRs using modern DNA extracts require only a fraction of the DNA template volume. In both cases, the remaining reaction volume is achieved with sterile water.

The cycling parameters in which these reactions undergo are specific to the type of DNA being amplified. These cycling parameters will vary whether modern DNA, or ancient or degraded DNA is being analyzed, as well as the sequence of the oligonucleotide primers that are being used. The denaturation step typically occurs between 90 to 95°C for 1 to 5 minutes, however, this will vary depending on the length of the amplified fragment (Hummel 2003; Hyndman 2003). The annealing step also occurs for up to 1 minute with a temperature in the range of 50 to 70°C (Hummel 2003; Hyndman 2003). This temperature is dependent upon the annealing temperature of the primers being used and the specificity required for the reaction. The temperature for the third phase, the extension phase, can range from 60 to 76 °C and typically lasts for up to 1 minute although the length of this phase can vary depending on the length and the

sequence of the amplicon being amplified, as well as the processivity of the polymerase (Hummel 2003). For example, if the sequence contains a large number guanine or cytosine nucleotides, the extension temperature will need to be higher, since three hydrogen bonds occur between these nucleotides. The number of cycles the PCR will undergo is dependent on the initial quantity of the DNA template. Modern DNA extracts have a higher copy number of the DNA template than do ancient or degraded DNA extracts. Therefore the modern DNA extracts typically undergo 25 to 35 cycles, where as the ancient or degraded DNA extract undergo upwards of 45 cycles (and may even be higher).

b. Primer Design and Primer Selection

The success of the PCR can often be dependent upon the primers that were selected. Since the primers are responsible for locating the target region for amplification, it is important that proper primers are designed or selected. There are numerous factors that must be considered in the design of the primers. Typically, two primers are used during a PCR and are identified as either a forward or reverse primer depending upon which parent DNA strand they anneal (McPherson and Moller 2000). They must be at least partially complementary (primarily at their 3' end) to the template in order for annealing to occur. The primer should also be 20 to 30 nucleotides long (McPherson and Moller 2000), which would provide a high level of specificity, especially for a unique target sequence. Primers should contain a relatively equal ratio of each of the nucleotides (McPherson and Moller 2000), however, if this ratio cannot be achieved, the guanine and cytosine nucleotides may exist in a slightly greater number than adenine and thymine. Since guanine and cytosine bind to one another with three hydrogen bonds, it allows a

stronger interaction between the primer and the template, than if there were a greater number of adenine and thymine residues. It is also important to avoid any repetitive sequences or stretches of the same nucleotide as this can cause slipping of the primer on the DNA template, resulting in the primer or template shifting or even dissociation (McPherson and Moller 2000). These stretches should be avoided especially with three or more guanine or cytosine nucleotides at the 3' end as this can cause mispriming of the primer to the template in GC rich regions (McPherson and Moller 2000). When designing primers it is important to ensure that no secondary structure or internal complementarity will occur within the primer itself. Also, the sequence of the primer at the 3'end should not be capable of base pairing with itself or with any of the other primers present within the reaction. Internal complementarity of the primer can lead to the formation of primer-dimers causing premature depletion of other reagents within the reaction (Hyndman 2003). If reagent depletion does occur during the reaction, it will result in reduced success of the overall amplification process.

c. Nested and Hemi-Nested PCR

The nested and hemi-nested PCR are variations of the standard PCR. A nested PCR is performed following the detection or standard PCR and as is seen in Figure 5, a new pair of primers amplifies a region internal to the region initially amplified during the detection PCR. The hemi-nested PCR is the same as the nested PCR, however, only one primer anneals internally to the amplicon from the detection PCR and the second primer is the same as was used in the initial PCR. It is important to note that between the detection PCR and the nested PCR, the samples are purified to remove any possible interference that can be caused from remaining reagents from the first PCR. These

techniques are used to increase the sensitivity of the PCR, especially for samples that contain low copies of template DNA. Although, the initial concentration of the template DNA may be very low, it should theoretically increase following the detection PCR. By having the newly amplified product undergo a nested or hemi-nested PCR, the second set of primers will preferentially amplify the region of interest rather than non-specific products, since the probability of these non-specific products having the primer sequence to anneal to is unlikely (McPherson and Moller 2000). It has been estimated that nested or hemi-nested PCRs can result in a 10^4-fold increase in sensitivity of detection of the amplified region (McPherson and Moller 2000).

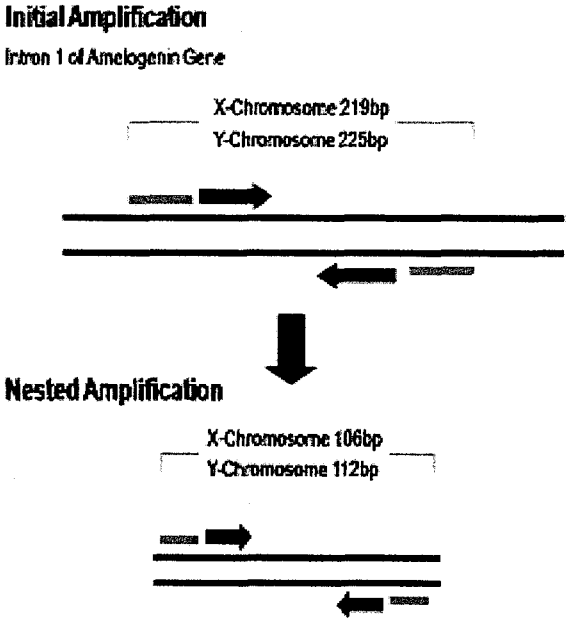

Figure 5: Schematic Diagram of a Nested PCR.

The first PCR amplifies a specific target amplicon and the second PCR amplifies a smaller amplicon located within the initial target region, resulting in a shorter amplified product

d. PCR Inhibition

Even if the proper concentrations of the reagents are used and the amplification parameters are optimized, amplification of DNA may still fail especially when dealing with aDNA specimens. This failure may be due to inhibitors present within the DNA solution. The presence of inhibitors could be the result of either co-purification with the template DNA or due to an ineffective extraction. When present during the PCR, these inhibitors may interfere with the DNA polymerase activity by either inhibiting the enzymatic activity or by preventing the polymerase to gain access and bind to the template DNA. An indication of PCR failure is observed during detection PCR where the amplified target region of interest is not present; furthermore an indication of inhibition can be determined due to the lack of any primer dimers, smears or any other nonspecific amplified products (Hummel 2003).

There are many substances that can inhibit the successful amplification of DNA. These can vary from textile dyes from clothing, hemoglobin from red blood cells, collagen from bone samples, organic and phenolic compounds, urea or even substances within soil that contains humic acids, tannins or fulvic acids at various concentrations (Wilson 1997; Butler 2001; Hummel 2003). The presence of yellow to dark brown staining of sample is indicative of inhibitors, more specifically those found in soil (Hummel 2003).

In order to overcome or reduce the effects of inhibition and be able to salvage the already extracted sample, there are additional steps that can be employed. Possibly the simplest and what is typically used in forensic analyses, is diluting the extract by adding

water or buffer or using smaller aliquots of the amplification reaction (Butler 2001; Hummel 2003). Although diluting the extract dilutes the DNA template, it also dilutes the PCR inhibitor, therefore it may allow the template to be re-amplified with less inhibitor (Butler 2001). Alternatively, the volume of DNA polymerase added to the reaction can be increased. In doing so, this would cause a portion of the polymerase to bind with the inhibiting factor preventing it from interfering with the amplification reaction, while the remaining polymerase allows the reaction to proceed (Butler 2001). If it is suspected that the inhibiting factor is present in significantly larger amounts, it may be preferred to perform an additional purification. This can be done by using an appropriate size exclusion column or an alternate form of precipitation (Hummel 2003).

PCR inhibition does not only occur when compounds other than the template DNA are present, but can also occur when there is too much template DNA. This is not usually considered with aDNA studies since the DNA is present in low copy numbers, however, it is very common with modern DNA extracts. The mechanism behind this form of inhibition is not completely understood. Unlike the other forms of inhibition mentioned, this type may produce visible results following electrophoresis in the form of smears (Hummel 2003). If this is the cause of PCR failure, the reaction should be repeated using a smaller volume of template DNA.

v. DNA Detection and Profiling

a. Real-Time PCR

Real-Time PCR is a modification of the typical PCR that allows simultaneous amplification and detection of DNA amplicons to be observed while the reaction is in

progress (Eglinton and Logan 1991). This technique was first described by Higuchi *et al.* (Saiki et al. 1988) by adding ethidium bromide (EtBr) to the PCR mixture and observing the accumulation of the PCR product after each cycle. The EtBr binds to double stranded DNA and emits fluorescent light when excited by UV light. Therefore, an increase in fluorescence is indicative of a successful amplification (Saiki et al. 1988). From this, Higuchi *et al.* (Saiki et al. 1988) developed the term 'Kinetic' or 'Real-Time' PCR by measuring the increase in fluorescence of the EtBr with a charge-coupled device camera. By plotting the increasing fluorescence against the cycle number they were able to determine that the accumulation of fluorescence was related to the initial number of DNA copies (Saiki et al. 1988). Since this development, other reagents and fluorogenic probes have been developed to monitor amplification of PCR products after each cycle. These new modifications have increased the specificity and greater quantitative precision (Eglinton and Logan 1991). There are many advantages to the use of Real-Time PCR aside from integrating the amplification and detection into one procedure. The use of fluorescent dyes and probes allow continuous monitoring as the reaction proceeds. This technique allows multiple samples to be amplified simultaneously and the reaction is complete within 1 to 2 hours (Eglinton and Logan 1991; Schmittgen 2001).

The various fluorogenic probes and dyes developed for Real-Time PCR detection can be divided into two main groups based on their fluorescent chemistries; "generic" and "strand-specific" methods (Lee 2004). Those that are generic methods are dyes or probes that bind non-specifically to the DNA. Strand- specific methods will bind to a specific sequence of the DNA within the amplified region (Lee 2004).

Of the types of generic detection methods for real-time PCR the most commonly reported is the fluorescent dye SYBR® Green I (Lee 2004). Becker *et al.* (Becker 1996) first introduced this dye as an alternative to EtBr, by testing it with a series of dilution PCRs and kinetic PCRs. They determined this dye was effective in calculating small quantities of the initial DNA in a sample with high accuracy (Becker 1996). SYBR® Green I binds to the minor groove of double stranded DNA (Lekanne Deprez 2002; Lee 2004). In Figure 6, the SYBR® Green I binds to the dsDNA created when the primers anneal, and continues to bind as a new strand is being extended. It is during these steps that florescence is detected. The SYBR® Green I is then released when the fully extended strands are denatured. When it binds, its fluorescence increases 20 – 100 fold (Lekanne Deprez 2002; Lee 2004). Therefore, an increase in fluorescence intensity is indicative of an increase of PCR products being amplified.

This type of dye is commonly used because it is readily available from suppliers and it can be easily added to the PCR mixture with little experimental modification (Lee 2004). Also, its emission maxima is close to that of fluorescein allowing the optics in most Real-Time instruments to detect this wavelength range (approximately 520nm) (Lee 2004). The most optimal concentration of SYBR® Green I is typically in the range of 1:30 000 to 1:100 000, however, this is dependent on the type of tube used for analysis and the optical efficiency of the instrument being used (Lee 2004). Although the non-specificity of the binding dyes like SYBR® Green I can be easily incorporated into the methodology to provide quick results for both the identification and quanitification of PCR products, its non-specificity is also a limitation. Since the dye will bind to any dsDNA, it will not only bind to the DNA amplicon of interest but also any non-specific

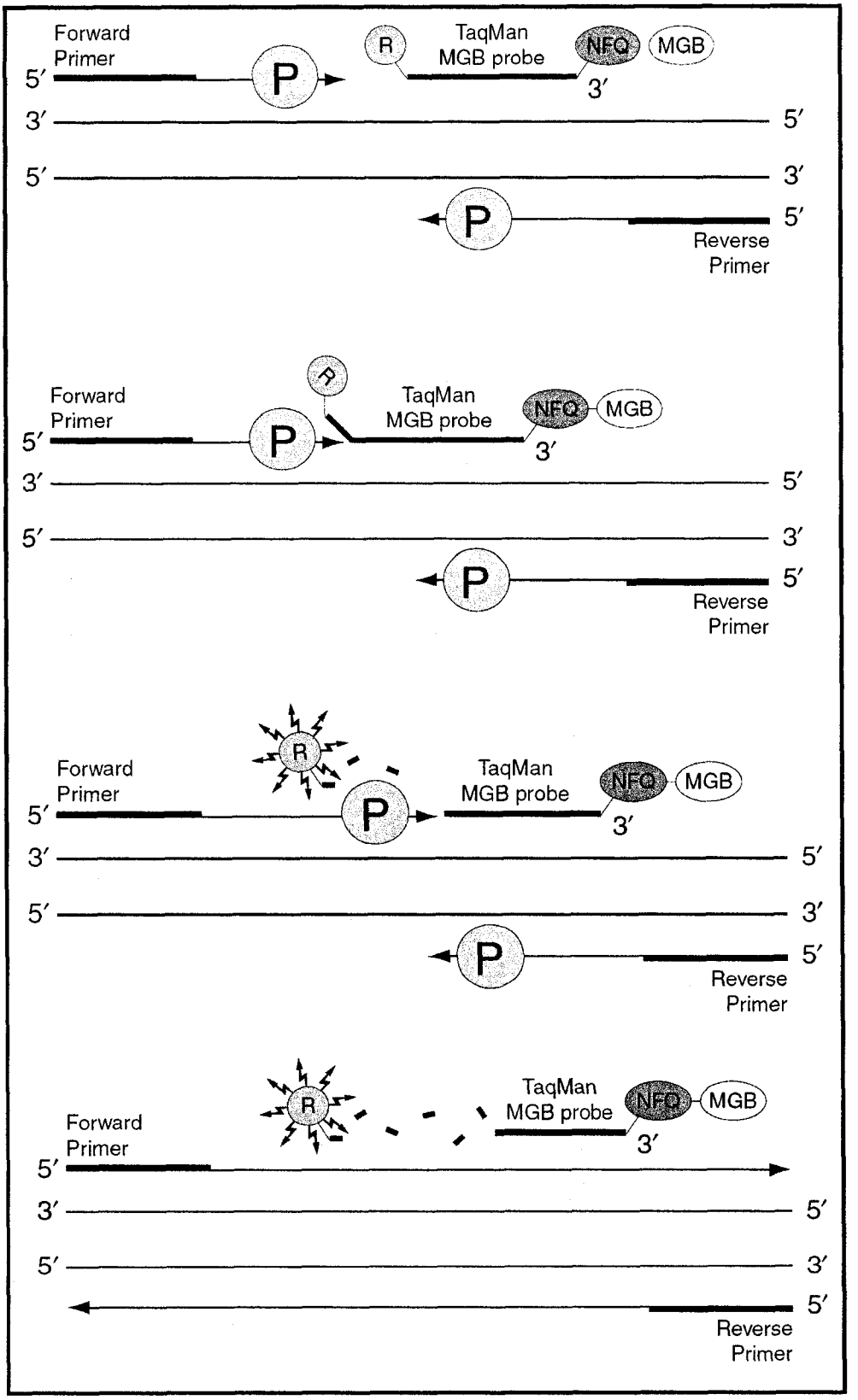

Figure 6: Real time PCR using the TaqMan probe assay.

(Image taken from: Applied Biosystems Inc User Manual: Quantifiler® Kits)

products that are amplified in a sub-optimal PCR (Klein 2002; Lekanne Deprez 2002; Lee 2004).

Strand specific methods for Real-Time fluorescence detection utilize nucleic acid sequences coupled with fluorophores (Lee 2004). These sequences probe the amplified PCR products for a specific target sequence in which it will anneal. Therefore, these types of probes offer greater specificity than generic methods. There are many different types of these probes that differ by their mode of action, however most are structured on the fluorescent resonance energy transfer (FRET) chemistry (Lee 2004).

FRET involves a non-radiative energy transfer between two fluorophores that are in close proximity. One fluorophore can be excited and emit light at a longer wavelength than that of the second fluorophore. This first fluorophore is termed the "donor" while the second is the "acceptor" (Cardullo et al. 1988; Lee 2004). When the two fluorophores are on the same molecule, emission energy of the donor is transferred to the acceptor (Cardullo et al. 1988; Lee 2004). This causes variations in the intensity of fluorescence that results from spatial changes during amplification, thus detecting a change in the quantity of amplified product (Cardullo et al. 1988). An alternative to having both dyes being fluorescent is having a non-fluorescent acceptor dye, known as a quencher (Lee 2004).

One variation of FRET using the quencher is the TaqMan® Probe (Applied Biosystems). This type of probe also incorporates another strand specific real-time chemistry, a 5'nuclease assay. The 5' nuclease assay incorporates the 5'to 3'exonuclease activity of *Taq* polymerase as first described by Gelfand in 1989 (Saiki et al. 1988). The 5' nuclease assay probe is developed to utilize the 5' to 3' exonuclease activity of the *Taq*

polymerase to cleave the probe as it is extending the 3' end of the newly developing DNA strand. This type of probe also has one dye of the FRET pair at the 5'end and is usually the high-energy donor, while the second dye, the acceptor, is at the 3'end. The optimal distance between the two dyes is dependent upon the dyes being used, however typically six nucleotides apart is used. If the dyes are too close, it may result in a sub optimal energy transfer (Lee 2004). With the TaqMan® Probe, the donor is referred to as the Reporter while the acceptor is called the Quencher. When the probe is intact and excited by a light source, the Reporter dye is not able to emit fluorescence as the Quencher molecule suppresses it (Applied Biosystems Manual). Then, when the probe is cleaved by the polymerase, the Reporter and the Quencher will separate, and as the distance increases the energy transfer will no longer occur. This process can be observed in Figure 6 This allows the Reporter to emit fluorescence. It is this fluorescence that is detected and analyzed by the software to create an amplification plot (Applied Biosystems Manual). Therefore, the increase signal from the Reporter dye is proportional to the quantity of product being amplified. Even though this technique is useful for many types of analysis, it is an expensive technique.

As shown, there are various chemistries and techniques that can be used for real-time PCR, however, the effectiveness of these methods is dependent upon the instrument and software that is being used in conjunction with it (Lee 2004).

b. DNA Sequencing

DNA sequencing enables the analysis of the order of nucleotide bases within an amplified DNA fragment (Hummel 2003). By being able to determine the sequence of nucleotides within a region of DNA, unique polymorphisms including insertions and

deletions are discovered and differences between individuals and populations can be compared (Hummel 2003). This technique, first described in 1975, has undergone many variations and employed different types of chemistries in order to make the reaction more sensitive and time efficient (Sanger and Coulson 1975).

As mentioned, the first described method was in 1975, where Sanger and Coulson developed a technique known as the 'plus and minus' sequencing method (Sanger and Coulson 1975). The method used *E. coli* polymerase I and polymerase from the bacteriophage T4, combined with limiting nucleotide triphosphates (Franca et al. 2002). Although the method was simple and fast, it was not completely accurate (Sanger and Coulson 1975; Sanger et al. 1977). Therefore in 1977, Sanger *et al.*developed a second technique using enzymatic polymerization (Sanger et al. 1977). This method is essentially an enzymatic primer extension reaction that incorporates polymerase along with both deoxynucleotide triphosphates (dNTPs) and didexoynucleotide triphosphates (ddNTPs) (Franca et al. 2002; Hummel 2003; Stirling 2003). This modified PCR differs from that of a standard PCR because it is asymmetric where only one primer is used, either a forward or a reverse primer; therefore resulting in linear amplification of a single stranded DNA product (Hummel 2003). A ^{32}P-labelled primer anneals to a specific region on the DNA strand, allowing DNA synthesis to begin on the 3'end. The presence of the polymerase allows the new DNA strand to extend with dNTPs, until a ddNTP is incorporated; terminating the DNA synthesis since hydroxyl group on the 3' oxygen on the dideoxyribose sugar is absent (Sanger et al. 1977; Franca et al. 2002; Stirling 2003). This results in all the linear DNA fragments produced having the same 5' end, but they differ in length and the 3' end residue depending on when and which ddNTP is

incorporated into the reaction (Franca et al. 2002). The reaction occurs in four separate tubes, each containing the ddNTP form of one of the four nucleotides (Franca et al. 2002; Stirling 2003). Therefore, when combining all four reactions, this random incorporation of ddNTPs produces fragments of every possible length within the amplicon of interest. Once the four reactions are complete, they are electrophoresed with a denaturing polyacrylamide gel in four parallel lanes. The fragments are separated by length and the bands produced indicate the exact sequence of the DNA using autoradiography (Franca et al. 2002; Hummel 2003). In 1986, a modification was made to the method by adding fluorescent labeled and multi-coloured ddNTPs rather than using the ^{32}P-labelled primer, thus permitting the reaction to occur within one tube rather than four (Smith et al. 1986).

As technology and instrumentation evolved the technique of capillary electrophoresis allowed the visualization of the sequence to become less time consuming, more efficient, and automated. This technique runs the reaction in one lane separating the fragments by size and distinguishing the end terminating nucleotide by the wavelength of the fluorescence emitted (Stirling 2003). Therefore the automated capillary electrophoresis sequencers can process multiple samples simultaneously.

c. Short Tandem Repeats (STR)

Short Tandem Repeat PCRs amplify DNA regions that contain repeat units 2-6 base pairs in length. The number of repeats at each loci can be highly variable between individuals making this method highly effective for identification purposes (Butler 2005). The location and size of these repeats units vary throughout the 22 chromosomes of the nuclear genome. The amplification of these loci can be performed in either a singleplex PCR, where only one locus is analyzed or in a multiplex PCR where many loci are

analyzed simultaneously. More often, STR amplifications are performed in multiplex reactions as it contains a higher discriminatory power for determining differences between individuals. The multiplex STR method is routinely used in forensic investigations because it is able to individualize the results with great efficiency.

When analyzing aDNA samples, the highly fragmented DNA still allows STR analysis to be performed because of the short length of the tandemly repeated structure (Hummel 2003). The type of analysis for aDNA studies permits individuation results that are less susceptible to the issue of contamination because more than one locus is being analyzed; therefore the likelihood of the data being from random contamination is very limited. However, it may also be advantageous to perform singleplex amplifications for aDNA since there is a limited quantity of template DNA, it would prevent any competition between the multiple primer sets. Other alternatives would be to apply nested or hemi-nested PCRs to increase the probability of achieving informative results.

Over the past decade, the technology has developed to create more sensitive and rapid measurements of STR alleles. Initially, three or four STRs were simultaneously amplified; now, systems have been developed where over ten STRs using fluorescent labels can be analyzed at the same time (Gusmao et al. 2005). STR analyses are typically amplified using kits, which contain fluorescently labeled primer sets for the multiple loci, a reaction buffer and DNA polymerase. The primers used are fluorescently labeled for rapid detection of the STRs using capillary electrophoresis. Since these kits are developed by commercial companies the contents of the reagents and primer sequences are patented and remain unknown. However, these kits are easier to perform since they

have been previously optimized, therefore all that is required is mixing the reagents and performing the PCR under given conditions.

Although the reagent contents are not known for the kits, the type of polymerase used can be determined based on the parameters of the PCR. The DNA polymerase typically used is a hotstart DNA polymerase, for example AmpliTaq Gold™. This type of polymerase is a chemically modified enzyme that is rendered inactive until heated (Birch 1996). Therefore when first starting the PCR, an extended pre-incubation known as a hotstart, usually around 95 °C for 10 to 11 minutes is used in order to activate the polymerase (Butler 2001) The benefit of using a hotstart DNA polymerase is that it prevents the reaction from beginning prior to starting the PCR. If another type of DNA polymerase is used, and the reaction is not prepared on ice or quickly, non-specific products may be generated.

The denaturation, annealing and extension phases of the PCR are very similar to that of a standard PCR, however there is an additional final extension step once the cycling has completed. This step is usually conducted around 60 °C for anywhere between 30 to 60 minutes. This step is essential since the size of the amplicons is extremely important and since *Taq* polymerase has the potential to add an additional adenine residue onto the end of each newly synthesized DNA strand. Therefore this final extension ensures each strand is completely adenylated to ensure all strands are the same length.

Once the cycling of the STR reaction is complete, the detection of the STRs is achieved using capillary electrophoresis. Since the primers are fluorescently labeled it

allows the detection of the multiple loci simultaneously because each loci will be a different colour dye even if they overlap in size.

There are currently two commercial companies that are leading in the development of STR kits, the Promega Corporation and Applied Biosystems. These two companies continue to develop new systems by increasing the number of loci analyzed while maintaining reliability and sensitivity. As mentioned, this method is often used in forensics and in fact has increased the credibility of DNA for identification purposes in the judicial system. This method also has many possibilities for aDNA as well since it is amplifying much shorter fragments, which may be able to accommodate the fragmented nature of aDNA.

d. Sex Determination

The ability to determine the genotypic sex of unknown remains or a sample can be very useful in both forensic investigations and archaeological studies. Circumstances in which this type of analysis would be essential include sexual assault cases where the victim can be distinguished from the perpetrator, also missing persons or mass disasters investigations would benefit from sex determination analysis. There have been many sex determination analyses developed, most of which utilize PCR (Seno 1977; Akane et al. 1991; Nakahori et al. 1991; Parsons et al. 1997; Hellmann et al. 2001).

Although the analysis of the sex chromosomes, X and Y, are the loci most commonly used for analysis, there are different assays that can be used to determine the genotypic sex. These could provide positive results for both chromosomes or preferentially detect only one of the chromosomes. However, the disadvantage of detecting only one chromosome is the inability to determine if the test provided negative

results or was a failed reaction. One of the most common assays used focuses on the X-Y homologous amelogenin gene. The amelogenin gene is a single copy gene that encodes for a protein within teeth enamel (Hummel 2003). The basis of this technique is the presence of a six base pair deletion on intron I on the X-chromosome (Parsons et al. 1997). Therefore, this allows amplification using a single primer pair that would generate a single 106 base pair fragment for females and two amplicons for males, a 106 base pair product representing the X chromosome and a 112 base pair product representing the Y chromosome (Parsons et al. 1997; Hummel 2003). Since the use of a single primer pair amplifies both chromosomes, the amplification of the X chromosome acts as a positive control (Butler 2001; Hummel 2003). This method has also been found to be very sensitive, where results were detected from only 20 picograms (pg) of DNA (Mannucci 1994). Another advantage of this technique is that it has been incorporated into the STR analysis system (Butler 2001). Even though this method has been proven to be accurate and sensitive, there are rare cases in which the 6 base pair deletion can exist within the Y chromosome and thus result in an incorrect genotypic sex determination (Santos 1998). The amplification of the amelogenin gene is carried out under the same conditions as a standard PCR and does not require any additional steps within the analysis.

Other sex determination assays may focus on only one of the sex chromosomes, one such example of these assays focuses on the alphoid repeat region which is a repetitive region of DNA located in the pericentromeric region of all chromosomes (Willard 1987). Within the Y chromosome these alphoid repeat regions have a region of higher organization than other repeat regions and therefore can be used for the

determination of sex (Venter et al. 2001). Since this form of assay is specific to the Y chromosome, a positive test would indicate the sample is male and the absence of any DNA would indicate female. Even though this method is considered highly sensitive and accurate (Hellmann et al. 2001), it is not possible to differentiate a between a failed reaction and a female result since both would produce negative amplification.

III.Project

i.Question

How do the pretreatment and the extraction methodology used in DNA analysis influence the quality and quantity of isolated DNA, depending on the type of biological sample.

ii.Purpose

The focus of this research is to analyze and compare the success of DNA isolation based on the pretreatment and extraction methods employed. These stages occur at the beginning of the analysis and thus, play a significant role in obtaining any DNA from a sample. In order to determine which technique is most effective at each stage, a variety of techniques that utilize different mechanisms will be tested. Furthermore, in some instances certain techniques at any one stage may complement a method used at another stage depending on the biochemical principles of the method. This study will compare these methods in attempt to determine the most versatile and reliable techniques depending on the type of sample available.

Not only does the biochemical makeup of the technique have a significant impact on the analysis, but also and possibly more importantly, the type of sample to be analyzed may influence the approach taken. Different types of samples may contain components that can cause inhibition or a decreased efficiency in the method. Therefore, this project will further determine if there is a relationship between the type of sample and the method to be used to produce high quality isolated DNA.

iii. Methodology

In order to be able to compare the success of each of the methods tested; four genetic detection techniques will be used. The quality of DNA isolated will be determined by assessing the presence of inhibition, the extent of fragmentation, and reproducibility. These characteristics will be analyzed using polyacrylamide and agarose gel electrophoresis while assessing multiple mtDNA amplicons and sex determination, as well as mtDNA sequencing and autosomal STRs using capillary electrophoresis. If the presence of contamination or inhibition is observed the source and stage at which they occur will be determined and additional analyses will be employed to achieve higher quality DNA. The quantity of DNA will be assessed using real-time PCR, as well as comparing band intensities from polyacrylamide and agarose gel electrophoresis.

iv. Samples

The types of samples tested within this research will be from three main sources; bone, teeth and tissue. The ancient samples will be taken from five collections that vary in their depositional environment, age, and extent of degradation. These are critical factors to consider as they may influence the extent of fragmentation or the presence of possible inhibitors. The collections and the type of sample used from each, as well as details about the environment in which the samples were collected from can be found in Table 1. In total 45 teeth extractions from 18 individuals, including both ancient and modern samples, 50 ancient bone extractions from 18 individuals and 10 ancient soft tissue extractions from 3 individuals were performed.

Table 1: Summary of the ancient samples to be tested.

Collection	Number of Individuals	Age	Type of Sample	Depositional Environment
Southwestern USA	6	900-1200 B.P.	Bone and Teeth	Arid, Dry, Sandy
Western Europe	3	90 B.P.	Teeth	Moist, Cool
Southern Mexico	8	1200-1500 B.P.	Bone and Teeth	Moist
Southern USA	5	900-1100 B.P.	Bone and Teeth	Fluctuating, Sandy
South American	3	1000-2000 B.P.	Tissue	Arid, Dry

IV.Methods & Procedures

i. Decontamination

When first receiving a sample for DNA analysis, the handling of the sample prior to its arrival is not always known. Also, the environment in which the sample was originally discovered may contain substances that could inhibit the analysis. To prevent any contamination or inhibition, a decontamination method can be used. Depending on the type of tissue, there are many procedures that can be performed. There are three main categories of decontamination techniques that will be used for this study; wash/soak solutions, ultraviolet (UV) irradiation and sonication. These procedures will be tested on bone and teeth samples.

a. Solution Decontamination

Three types of solutions will be tested: a 10% Tergazyme detergent solution, a 6% (w/v) sodium hypochlorite solution, and a 70% ethanol solution. These solutions will be used independently for some samples, and will be used in conjunction with each other on other samples. In each case, the concentrations of the solutions will be kept consistent and each wash will last for 5 minutes.

b. UV Irradiation

To maintain consistency, the same wavelength of UV light, 254 nm, will be used, as well the distance from the sample to the UV light source will remain the same at 30 cm. For this study, this method will be applied to bone and teeth samples.

c. Sonication

The sample is placed inside the sonicator and submerged in one of many possible solutions. In this study, each sample was sonicated for 20 minutes. There are

four solutions that will be tested; 10% Tergazyme detergent solution, 6% (w/v) sodium hypochlorite, 70% ethanol, and sterile water.

ii.Sample Preparation

Sample preparation techniques will be used on bone, teeth and tissue. Since both bone and teeth are hard surfaces, drilling and pulverization will be the two techniques tested. This will result in the formation of a powder. For tissue, there will be two methods tested as well, tearing and grinding in a plastic mortar and pestle.

a. Drilling

A dremmel drill will be used to collect powder from both bone and teeth, as well as for cleaning dental caries present in some tooth samples.

Required Equipment

Dremmel drill

1.5mL Microcentrifuge tubes

Protocol:

1. Select a smooth surface free of any damage on the sample being prepared.
2. Using a dremmel drill, apply firm pressure to the surface of the sample and begin to drill on low speed. It is important to drill slowly with pressure to prevent heating up of the drill or burning the sample.
3. Once reaching the inner portion of the sample, stop drilling and gently tap the sample to remove any of the powder already formed. This removes contaminated powder.
4. Proceed to drill at low speed and move the drill in a circular motion while in the hole created.

5. Periodically, cease drilling and tap the powder in the drilled hole into a 1.5mL microcentrifuge tube.

6. Once all the powder is collected, store the sample dry, at room temperature until further analysis.

b.Pulverization

Pulverization of bone and teeth samples will be performed using a mixer mill and steel chamber. The resulting powder will then be aliquoted into 1.5mL microcentrifuge tubes and weighed.

Required Equipment

Steel chamber and ball

Mixer mill

1.5mL Microcentrifuge tubes

Protocol:

1. Place either the entire or a fragment of the sample (this is dependent upon the size of the sample) into the steel chamber with the steel ball.

2. Seal the chamber with tape and place the chamber horizontally into the mixer mill. Tighten the holder using the bolt at one end. It is important to ensure the chamber is securely fastened in the mixer mill before operating otherwise the chamber may fall out.

3. Turn the mixer mill on for 20 seconds.

4. Remove the chamber from the mixer mill and remove the seal.

5. Aliquot the powder formed into 1.5mL microcentrifuge tubes and store dry, at room temperature until further analysis.

c. Tearing

Prior to extraction, the tissue samples will be torn into smaller pieces to increase the surface area in attempts to increase the success of DNA retrieval.

Required Equipment

Forceps

1.5mL Microcentrifuge tube

Protocol:

1. Place a piece of tissue into a 1.5mL microcentrifuge tube.
2. Using two pairs of forceps begin to tear the piece of tissue into smaller pieces until they are 2-3mm in width or length.
3. Store the tissue sample dry at room temperature until further analysis.

d. Mortar and Pestle

Another option in preparing softtissue samples other than tearing would be to grind the sample, forming a paste. This can be achieved using a mortar pestle. This technique operates under the same principle as tearing, where it increases the surface area of the sample for DNA extraction to improve the yield of DNA.

Required Reagents

Plastic Mortar

1.5mL Microcentrifuge tubes

Protocol:

1. Place a piece of tissue into a 1.5mL microcentrifuge tube.
2. Place the plastic mortar inside the tube and begin to grind the tissue sample by moving the mortar in a circular motion with the tissue between the microcentrifuge tube

and the mortar.

It is advisable to perform this preparation technique just prior to preparing the extraction.

iv.Extraction Protocols

In order to successfully retrieve DNA from any type of sample, there are a variety of different methods that can be employed. The techniques that will be used in this research include Guanidinium Thiocyante and Cetyl-trimethylammonium Bromide, both chemical extractions methods, Proteinase K, an enzyamtic extraction method and a novel technique the Pressure Cycling Technology (PCT), where the sample is subjected to an extraction buffer and high pressure allowing the release of DNA.

a. Proteinase K

Proteinase K (PK) is an enzymatic extraction method that is usually used as the standard extraction method in forensic laboratories. A modified Hansen (Hansen 1974) PK extraction will be used to extract DNA from bone, teeth and tissue.

Required Reagents:

Proteinase K (20mg/mL)

TNE Buffer (1M Tris pH 8.0, 0.5M NaCl, 0.5M EDTA)

20% SDS

0.39M DTT

ddH_2O

Protocol:

1. Prepare 400µL 1X Extraction Buffer as follows; 290µL TNE Buffer, 40µL 20% SDS, 40µL 0.39M DTT, 2µL Proteinase K, and 28µL of water, and add to sample.

2. Incubate at 37°C in a fume hood overnight with gentle agitation.

b. Guanidinium Thiocyanate

This modified (Boom et al. 1990) extraction method will be conducted on modern, ancient bone, teeth and tissue samples.

Required Reagents:

4M GuSCN Extraction Buffer

Protocol:

1. Add 500µL of 4M GuSCN extraction buffer (0.1M Tris-HCl pH 6.4, 0.02M EDTA pH 8.0, 1.3% Triton X-100) directly to the sample.

2. Incubate at 56°C overnight with gentle agitation.

c. Cetyl-trimethylammonium Bromide

The efficacy of the Cetyl-trimethylammonium Bromide (CTAB) extraction buffer will be tested on bone and teeth samples. The preparation of this extraction method will follow that of (Ye et al. 2004).

Reagents Required:

CTAB Extraction Buffer

Protocol:

1. Add 1mL of CTAB Extraction Buffer (2%CTAB, 100moL/L Tris-HCl at pH 8.0, 20mmol/L EDTA, 1.4mmol/L NaCl and 0.2% 2-mercaptoethanol) to the sample.

2. Vortex the sample and incubate at room temperature overnight.

3. Heat the solution at 65°C for 1 hour, while vortexing the sample every 15 minutes to release the DNA.

4. Centrifuge the sample at 5 000rpm for 25 minutes.

5. Collect the supernatant.

d. Pressure Extraction

The pressure extraction method tested in this research project uses Pressure Cycling Technology (PCT) developed by Pressure Biosciences Inc. (West Bridgewater, U.S.A.). This method will be tested using two extraction buffers; GuSCN and a proprietary buffer provided by the company. Both variations will be used to extract DNA from bone and teeth samples.

Required Reagents:

PULSE Tubes

Extraction Solution of Choice

Protocol:

1. Assemble the PULSE Tube by inserting the cap into broader end of column (bottom of column) using the specially designed screwdriver.
2. Add sample to the column, ensuring it is resting on the lysis disk.
3. Add the extraction solution directly to the column.
4. Insert the ram into the narrower end of the column (this is considered the top of the column), again using the specially designed screwdriver.
5. The columns containing the samples then undergo Pressure Cycling Technology (PCT), in which they are subjected to alternating cycles of high and ambient pressures in a pressure-generating instrument.
6. The ram is then removed and the extraction solution containing non-purified DNA is removed.

v. Purification Protocols

Following the extraction procedure, the remaining cellular debris and impurities that are also present in the solution must be removed. For this research, the purification methods will be performed in conjunction with the extraction method it is typically described to be paired with.

a. Phenol Chloroform Separation

The Phenol/Chloroform separation technique allows the physical separation of the DNA from denatured proteins and other cellular debris that still remain in the extraction solution. This method will be performed with the Proteinase K extraction methods.

Required Reagents:

Phenol

Chloroform: Isoamyl Alcohol (24:1 v/v)

Protocol:

1. In a set of clean 1.5mL tubes, add 200µL of Phenol and 200µL of Chloroform: Isoamyl alcohol (24:1) to each tube.
2. In a second set of clean 1.5mL tubes, add 400µL of Chloroform: Isoamyl alcohol (24:1) to each tube.
3. Once the extraction solution completed its incubation period, 200µL of Phenol and 200µL of Chloroform: Isoamyl alcohol (24:1) was added directly to the extraction solution.
4. The solutions were then centrifuged for 5 minutes at 13 000rpm.

5. The aqueous layer was removed and placed in the previously prepared tube with 200μL of Phenol and 200μL of Chloroform: Isoamyl alcohol (24:1). It is important to note that none of the interphase containing denatured protein was removed.

6. The solutions were vortexed and then centrifuged for 5 minutes at 13 000rpm.

7. The aqueous layer was once again removed and placed into the previously prepared tube with 400μL of Chloroform: Isoamyl alcohol (24:1). This tube was vortexed and centrifuged for 5 minutes at 13 000rpm.

8. The aqueous layer was removed and placed into a clean 1.5mL tube.

9. If the sample was not subjected to an additional purification, the solutions were placed in a heating block at 65°C with the caps open to allow any remaining chloroform to evaporate.

b.Ethanol Precipitation

The ethanol precipitation purification in this project will be performed as an additional purification step following the phenol/chloroform separation or to further concentrate the DNA. This method is to be attempted on teeth, bone and soft tissue.

Required Reagents:

3M Sodium Acetate

100% Ethanol

95% Ethanol

ddH_2O

Protocol:

1. Add 10% volume of 3M Sodium Acetate to the entire volume of the extract in clean sterile 2.0mL tube.

2. Vortex the tube 1 minute.

3. Add 2.5 times the volume of COLD 95% Ethanol to the tube containing the extraction solution.

4. Place the tube on ice for 30 minutes.

5. Centrifuge the tube for 5 minutes at 13 000rpm.

6. Discard the supernatant liquid.

7. Add 500µL ice cold 100% Ethanol to the tube.

8. Vortex the tube for 1 minute, then centrifuge for 10 minutes at 13 000rpm.

9. Discard the supernatant and let dry for 30 minutes.

10. To resuspend add water and incubate at 37°C for 15 minutes.

c. Silica Bead Purification

The silica bead purification method is routinely used in conjunction with the GuSCN extraction method. In fact, GuSCN is used in this protocol again as a chaotropic agent to expose the negatively charged DNA to the positively charged silica beads. When chilled on ice, the DNA and the silica beads form a tight bond. The series of washes that follow assist in removing any remaining cellular debris. For this research project, a modified (Boom et al. 1990) method will be used for purification following the GuSCN extraction method.

Required Reagents:

4 M GuSCN solution (0.1M Tris-HCl pH 6.4, 0.02M EDTA pH 8.0, 1.3% Triton X-100)

Silica Beads Resin

Working Wash Buffer (0.01M Tris-HCl pH 7.5, 0.05M NaCl, 1mM EDTA, 50% EtOH)

100% Ethanol

ddH_2O

Protocol:

1. Boil the GuSCN extract at 94°C for 10 minutes and centrifuge at 13000 rpm for 1 minute.
2. Remove the supernatant and place in a sterile 1.5 mL tube.
3. Add 900 µL of GuSCN solution and 20 µL of silica to the sample and vortex briefly.
4. Place the sample on ice for 60 minutes and invert the sample was inverted every 15 minutes to resuspend the silica.
5. Spin the sample and discard the supernatant.
6. Add 500 µL of Working Wash Buffer to the sample and vortex for 1 minute to resuspend the silica beads.
7. Zip spin the samples, remove and discard the supernatant.
8. Repeat the Working Wash Buffer wash step.
9. Add 150 µL of 100% ethanol to the sample, vortex for 1 minute and then zip spin.
10. Remove and discard the supernatant.
10. Air dry the silica pellet for 30 minutes.
11. Add 100 µL of ddH_2O to the dry pellet and vortex to resuspend the DNA in the silica.
12. Incubate at 56°C for 1 hour.
13. Prior to the preparation of a PCR, centrifuge the sample at 13000rpm for 1 minute.

d. Commercial Spin Columns

There are a variety of commercial spin columns available for purification. In this research project, columns will be used for the purification of sample extracts or for post PCR purification. Two types of columns will be incorporated into this project; size

exclusion chromatography columns and bind/elute columns. Size exclusion chromatography columns quickly purify nucleic acids from other chemical elements or macromolecules, as would be the case for sample extracts. They are also effective in purifying PCR products by separating the amplified DNA template from any remaining PCR reagents that may inhibit or interfere in subsequent PCRs or sequencing. The size exclusion chromatography columns used in this research include; Bio-Rad Micro Bio-Spin® P-30 Microcentrifuge Chromatography Columns, Dyex Purification Columns, and E.Z.N.A.® Dye Terminator Removal Columns. The bind/elute columns will be used for post PCR purification. The sample is mixed with a high salt binding buffer, which is then put through the column. When centrifuged the DNA will bind to the column, while the impurities pass through. The DNA can then be diluted with a low salt elution buffer or ddH_2O. The bind/elute columns used in this project include the QIAquick PCR Purification Columns, and E.Z.N.A.® Cycle Pure Columns.

Bio-Rad Micro Bio Spin® P-30 Microcentrifuge Chromatography Columns

P-30 Microcentrifuge chromatography columns are used to separate macromolecules based on the molecular weight of the components. The matrix within the column is comprised of porous polymeric beads, allowing larger molecules to pass through the column, while the porous beads trap the smaller molecules. These columns will be used as an additional purification step to remove any inhibitors that may have co-purified during the extraction and initial purification procedures.

Required Reagents:

Micro Bio-Spin® P30 Chromatography Columns

Protocol:

1.Invert the column several times to resuspend the gel in the column and remove any bubbles.

2.Snap the tip off and place column in a 2mL microcentrifuge tube and remove top cap.

3.Centrifuge the column for 2 minutes at 3400rpm to remove the packing buffer. Discard the buffer.

4. Place the column into a clean 1.5mL tube. Apply the sample (20 – 75μL volume) directly to the center of the column. It is important to not that by applying more or less than the recommended volume it may decrease the efficiency of the column.

5. Centrifuge the column for 4 minutes at 3400rpm. The purified DNA is now in solution with TRIS buffer.

6. Discard the column.

E.Z.N.A.® Dye Terminator Removal Size Exclusion Columns

This size exclusion chromatography column will be used to purify post PCR and post sequencing PCR products just prior to capillary electrophoresis. It is effective in removing primers, dNTPs, unincorporated dye terminators or any other low molecular weight components that remain in the PCR product.

Required Reagents:

E.Z.N.A.® Dye Terminator Removal Columns

Protocol:

1. Gently vortex the spin column to resuspend the resin.

2. Loosen the cap of the column a quarter turn, allowing the formation of a vacuum inside the column.

3. Snap off the bottom tip of the spin column and place the column into a 2mL collection tube.

4. Centrifuge the column for 3 minutes at 3400rpm.

5. Transfer the spin column to a sterile 1.5mL microcentrifuge tube and slowly apply the sample to the surface of the center of the gel matrix.

6. Centrifuge the column for 3 minutes at 3400rpm.

7. Remove the spin column from the tube. The eluate contains purified DNA.

Dye Ex Dye Terminator Removal Purification Columns

The Dye Ex dye terminator removal purification columns (Qiagen, Mississauga, Canada) use the same principles as mentioned in the E.Z.N.A.® dye terminator removal columns and were also used for post PCR purification.

Required Reagents:

Dye Ex Dye Terminator Removal Columns

Protocol:

1. Gently vortex the spin column to resuspend the resin.

2. Loosen the cap of the spin column and place into a 2mL collection tube.

3. Centrifuge the column for 2 minutes at 3400rpm. Transfer the spin column to a sterile 1.5mL microcentrifuge tube and slowly apply the sample to the surface of the center of the gel matrix.

4. Centrifuge the column for 3 minutes at 3400rpm.

5. Remove the spin column from the tube. The eluate contains purified DNA.

QIAquick PCR Purification Bind/Elute Columns

The QIAquick bind/elute columns (Qiagen, Mississauga, Canada) will be used for the purification of modern DNA extracts and for pre-sequencing PCR purification. When purchasing these columns the buffer solutions are also provided to ensure optimal conditions.

Required Reagents:

QIAquick Bind Elute Columns

PB Buffer

PE Buffer

ddH_2O

Protocol:

1. Add 5X volume of Buffer PB to 1X volume of the Sample and mix.

2. Place a QIAquick spin column into a 2mL collection tube.

3. Apply the Buffer PB and Sample mixture to the QIAquick spin column and centrifuge at 13 000rpm for 1 minute.

4. Discard the liquid.

5. Wash the spin column with 750µL of Buffer PE and centrifuge at 13 000rpm for 1 minute.

6. Discard the liquid and centrifuge the empty column for an additional 1 minute at 13 000rpm.

7. Transfer the spin column to a sterile 1.5mL microcentrifuge tube. Add 30-50µL of ddH_2O water to the center of the column matrix. Allow the column to sit for 1 minute and then centrifuge the spin column for 1 minute at 13 000rpm to elute the DNA.

E.Z.N.A.® Cycle Pure Purification Bind/Elute Columns

The E.Z.N.A.® bind/elute columns will be used only for the pre-sequencing PCR purification of samples. As with the QIAquick columns, the buffer solutions for binding the DNA to the column are provided.

Required Reagents:

E.Z.N.A.® Cycle Pure Bind Elute Column

ddH_2O

Protocol:

1. Add an equal volume of Buffer XP1 to the Sample.

2. Insert a Hi Bind DNA Spin Column into a 2mL collection tube and apply the sample to the column.

3. Centrifuge the column at 13 000rpm for 1 minute and discard the liquid.

4. Wash the column by adding 700μL of SPW Buffer diluted with absolute ethanol.

5. Centrifuge the column at 13 000rpm for 1 minute and discard the liquid.

6. Repeat steps 4 and 5 with another 700μL of SPW Buffer.

7. Centrifuge the empty column at 13 000rpm for 1 minute to dry the column matrix.

8. Transfer the column to a sterile 1.5mL microcentrifuge tube. Add 30-50μL of ddH_2O water directly to the center of the column matrix and centrifuge at 13 000rpm for 1 minute to elute the DNA.

vi. Amplification

In order to conduct further analyses with the purified template DNA, regions of interest are usually amplified to increase the sensitivity and specificity of the detection methods. There are many different types of amplification protocols and they depend on

the concentration, age and integrity of the template DNA, as well as the type of analysis that will be conducted following amplification. The following protocols listed are those that will be used in this research project.

a. Primer Design

The primers that are selected for the amplification of DNA are dependent upon the type of DNA being amplified.

Table 2: The mitochondrial primers used in this research.

Mitochondrial Primer and Sequence	Primer Length (bp)	Melting Temperature, Tm (°C)	GC Content (%)
mt15971F TTA ACT CCA CCA TTA GCA CC	20	58.4	45.0
mt16071F CCC ATC AAC AAC CGC TAT GT	20	60.4	50.0
mt16210F CCC ATG CCT ACA AGC AAG TA	20	56.0	45.0
mt16301F CAG TAC ATA GTA CAT AAA GCC A	22	57.8	36.4
mt1F GAT CAC AGG TCT ATC ACC C	19	60.5	52.6
mt15F CAC CCT ATT AAC CAC TCA CG	20	60.4	50.0
mt155F TAT TTA TCG CAC CTA CGT TC	20	56.3	40.0
mt247F GAA TGT CTG CAC AGC CAC	18	60.7	55.6
mt187R CGC CTG TAA TAT TGA ACG TAG G	22	61.5	45.5
mt279R GAT GTC TGT GTG GAA AGT GG	20	60.4	50.0
mt389R CTG GTT AGG CTG GTG TTA GG	20	62.5	55.0
mt429R CTG TTA AAA GTG CAT ACC GCC A	22	61.5	45.5
mt16322R TGG CTT TAT GTA CTA TGT ACT G	22	57.8	36.4
mt16420R TGA TTT CAC GGA GGA TGG TG	20	60.4	50.0

Primers are available for both nuclear and mitochondrial DNA. In this research for mitochondrial DNA, the primers that will be used target various fragment lengths of the mitochondrial D-Loop. These mitochondrial DNA primers with their optimized annealing temperatures are listed in Table 2.

Nuclear DNA primers were designed for a target region located on the Amelogenin gene on the sex chromosomes. These primers were designed to flank the previously published Amelogenin forward and reverse primers that are commonly used to aid in sex determination. Both the published amelogenin primers and the designed primers can be found in the following table (Table 3). These primers were used to conduct a nested PCR, which will be discussed in a later section.

Table 3: The nuclear DNA primers used in this research.

Primer and Sequence	Primer Length (bp)	Melting Temperature, Tm (°C)	GC Content (%)
Amel-A* CCC TGG GCT CTG TAA AGA ATA GTG	24	65.4	50.0
Amel-B* ATC AGA GCT TAA ACT GGG AAG CTG	24	63.7	45.8
AmF GCT ACC ACC TCA TCC TGG GCA CCC	24	64.0	67.0
AmR ACA GGC TTG AGG CCA ACC ATC AGA GC	26	63.0	58.0

*These primers were previously designed by Sullivan et al. 1993.

b.Detection PCR

Following the extraction and purification of the DNA sample, a target region of interest on the DNA template is amplified to detect the presence of viable DNA and the degree of fragmentation of the template. This is referred to as detection or standard PCR.

All detection PCRs performed in this project will be comprised of the following reagents and their corresponding final concentrations for each sample (Table 4).

Table 4: The conditions used for the polymerase chain reaction

Reagent	Final Concentration
10X Buffer, minus Mg	1X
50 mM $MgCl_2$	200 μM
dNTP Mixture	2 mM
10 μM Forward Primer	0.2 μM
10 μM Reverse Primer	0.2 μM
Platinum Taq DNA Polymerase (5U/μL)	1.0 U
ddH_2O	--*
Template	--*

*The volume of ddH_2O added is dependent upon the volume of template added DNA that is added.

The cycling parameters for the detection PCR will be maintained and provided below in Table 5. However, the number of cycles will differ depending on the age performed will be 35 and for ancient and degraded samples, the number of cycles will be 45.

Table 5: Cycling conditions for the polymerase chain reaction

Temperature (°C)	Time	Number of Cycles
94.0	2:00	1
94.0	1:00	30 – 45**
60.0*	1:00	
72.0	2:00	
4.0	Hold	1

*This annealing temperature may vary based on the sequence of the primer. The mitochondrial primers that are used in this project use the above annealing primer. However, for the nuclear primers the annealing temperature will be different and will be stated when discussing these procedures.

**The number of cycles varies depending on the type and nature of the sample being tested. For modern samples, 30-35 cycles will be sufficient, where as 40-45 cycles will be used for ancient or degraded samples.

c. Nested and Hemi-nested PCR

Nested and hemi-nested PCR amplification is performed following the detection PCR. The PCR products from the detection PCR will be purified using one of the commercial columns mentioned in the purification protocols sections. These purified products will then undergo a second PCR with the same conditions as the detection PCR, except a different pair of primers will beused.

For this research project, hemi-nested PCRs will be conducted for mitochondrial DNA samples that were either highly degraded or considered ancient samples. The nested PCR will be performed on nuclear DNA for the amelogenin gene again for samples that were considered highly degraded or ancient.

d. Mitochondrial DNA Sequencing

The mitochondrial DNA sequencing reaction that will be preformed during this project employs the chain terminating chemistry. More specifically, the ABI Big Dye® Terminator v3.1 Cycle Sequencing kit will be used to prepare the sequencing reactions, however the reagent volumes have been altered from those listed in the manufacturer's protocol.

Required Reagents

Big Dye® Terminator v3.1

Protocol

1. For each DNA sequencing reaction, add the following reagents into a 0.2mL tube; 3.0 μL of Big Dye® Terminator v3.1, 0.3μL of either the forward or reverse primer used in the detection PCR and 7.0μL of the purified PCR product.

2. The DNA sequencing reaction is then amplified using the following PCR cycling parameters (Table 6).

Table 6: The cycling parameters for the DNA sequencing reactions.

Temperature (°C)	Time (minutes)	Number of Cycles
94.0	0:30	35*
50.0	0:15	
72.0	4:00	
4.0	Hold	1

*Please Note that for ancient or degraded samples the cycle number was sometimes increased to 40 cycles.

3. Purify the sequencing PCR product using either the Dyex or E.Z.N.A size exclusion DTR columns mentioned in the purification protocols section.

4. Desiccate the purified sequencing product.

5. Store the sample at 4 °C until sequencing detection with the ABI PRISM 3100 Genetic Analyzer.

e. Multiplex Autosomal Short Tandem Repeats

Autosomal Short Tandem Repeats (STRs) will be amplified using a commercially available kit developed by Applied Biosystems. The AMPF*l* STR® Identifiler® PCR Amplification kit is able to amplify 15 loci from the autosomal chromosomes and the amelogenin locus on sex chromosomes simultaneously in one reaction. Since it is a commercial kit, the reagents used in the reaction are proprietary. However, it is known that the kit uses 5-dye chemistry to allow differentiation of different groups of loci, when being run on an ABI PRISM® 3100 Genetic Analyzer.

Required Reagents

AmpF*l* STR Identifiler Mastermix

AmpF*l* STR Identifiler Primer Mix

AmpliTaq Gold Polymerase (5U/μL)

ddH_2O

Protocol

1. For each AmpF*l* STR® Identifiler® PCR amplification sample, the following reagents are combined in a 0.2 mL tube.

Table 7: The reaction volumes for the multiplex PCR

Reagent	Volume Per Reaction (μL)
AmpFl STR Identifiler Mastermix	10.5
AmpFl STR Identifiler Primer Mix	5.5
AmpliTaq Gold Polymerase (5U/μL)	0.5
ddH_2O	--*
Template DNA	--*

*The total volume of the reaction is 25μL. However, only 15 μL of the mastermix, primer and AmpliTaq cocktail will be added to the reaction, while the extra volume is allowed for pipetting error. The volume of ddH_2O added is dependent on the volume of template added.

2. The AmpF*l* STR® Identifiler® reaction mix is amplified using the following PCR cycling parameters (Table 8).

Table 8: The cycling conditions for the multiplex PCR

Temperature (°C)	Time (minutes)	Number of Cycles
95.0	11:00	1
94.0	1:00	28
59.0	1:00	
72.0	1:00	
60.0	1:00:00	1
4.0	Hold	1

3. Following the amplification, cover the products in aluminum foil. Due to the fluorescently labeled primers the reactions are light sensitive.

4. Store the samples at 4 °C until fragment analysis is conducted on the ABI PRISM® 3100 Genetic Analyzer.

vi. Quantification

a. Real-Time PCR

In theory, there is a quantitative relationship that exists between the concentration of the template DNA before amplification and the final concentration of the amplified target DNA sequence at any cycle during PCR. Therefore, real-time PCR detects the increase in the amount of target DNA as the cycle number increases. There are different methods that can be used to detect the progress of the reaction. This research project assessed the Applied Biosystems Quantifiler™ Human DNA Quantification Kit.

Applied Biosystems Quantifiler™ Human DNA Quantification Kit

The Applied Biosystems Quantifiler™ Human DNA Quantification kit is a commercial kit that incorporates the 5'nuclease activity of AmpliTaq Gold® DNA Polymerase to cleave a TaqMan Probe that has annealed to the single stranded DNA downstream. When the probe is cleaved it will emit fluorescence. Therefore an increase in fluorescence indicates an increase in the number of single strands of DNA being amplified. This technique will be used to quantify extracted DNA samples from bone, teeth and tissues.

The reaction for each sample is prepared using the volumes listed in the table below (Table 9).

Table 9: Reaction conditions for the Quantifiler reaction

Reagent	Volume per Reaction (µL)
Quantifiler™ PCR Reaction Mix	12.5
Quantifiler™ Human Primer Mix	10.5
DNA Template	2

Since this is a commercial kit, specific cycling parameters have been developed to allow optimal amplification and the detection of fluorescence. These parameters are provided in Table 10.

Table 10: The cycling conditions for the Quantifiler reaction

Temperature (°C)	Time	Number of Cycles
95.0	10:00	1
95.0	0:15	40
60.0	1:00	
4.0	Hold	1

vii.Detection

Following the amplification of the DNA template, it must be determined if the DNA target of interest was successfully amplified. There many different methods that may be used and each have advantages and disadvantages in regards to their sensitivity and consistency. In this research project, electrophoresis using three different matrices will be used for detection. These three matrices are agarose gel, polyacrylamide gel and capillary.

a. Agarose Gel Electrophoresis

Agarose gel electrophoresis is a detection method that will be used to detect the absence or presence of DNA, as well as for the comparison of different sizes of amplified

DNA fragments throughout this project. Agarose gels however, have a lower resolution than some of the other detection techniques and therefore are not useful for differentiating fragments that differ by only a few base pairs in length.

Required Reagents

1X TBE Buffer

Agarose

10mg/mL Ethidium Bromide

Gel Making Protocol

*The following procedure will make a 1-1.5% agarose gel.

1. Weigh 0.375g of agarose and pour into a 125mL Pyrex flask.
2. Measure 25mL of 1X TBE Buffer and slowly add it to the agarose, while swirling the flask continuously.
3. Cover the top of the flask with aluminum foil.
4. Heat the solution while swirling every 1-2 minutes until agarose is completelydissolved.
5. Allow the solution to cool for 5 minutes.
6. Add 2.0μL of 10mg/mL ethidium bromide stain.
7. Slowly pour solution into an agarose plate and insert the gel comb.
8. Allow the gel to settle for 30-45 minutes at room temperature. The gel may also be placed in the fridge for less time.

Loading and Running the Gel

1. Once the gel has settled, slowly remove the comb from the gel and carefully place the gel into the gel apparatus.

2. Fill the gel apparatus with 1X TBE Buffer until the gel is completely submerged. There is usually a fill line indicator on the side of the apparatus.

3. Purge the wells in the gel with a Pasteur pipette to ensure no air bubbles are present.

4. Mix 7.0μL of the PCR product with 3.0μL of 6X Loading Dye and a sample to each well of the gel (Be sure to leave one well empty)

5. Load 3.0μL of a DNA size standard in the one remaining well.

6. Set the voltage to 110 volts and the time for 25 minutes on the electrophoretic power pack, and plug the gel apparatus into the power pack.

7. Once the gel has run, place the gel on the transilluminator (set at wavelength UV B) and photograph.

b. Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide gel electrophoresis is the second technique that will be used for detection of amplified DNA. This detection technique is advantageous as it is highly sensitive to low concentrations of PCR products. As well, it is capable of resolving fragments of DNA that differ only by only a few base pairs in length. PAGE gels will be used for analysis of the amelogenin gene, nested and hemi-nested PCRs and for fragmentation analysis.

Required Reagents

1X TBE Buffer

5X TBE Buffer

Acrylamide

TEMED

10% APS

ddH_2O

Gel Making Protocol

*This protocol will result in 12 – 6% PAGE gels

1. The 10% APS solution is prepared by mixing 0.1g APS to 1.0mL ddH_2O.

2. Chill a 125mL Pyrex flask on ice.

3. Set out 12 gel cassettes and combs prior to making the solution.

4. Combine 20mL 5X TBE Buffer, 12.5mL Acrylamide, 66.5mL ddH_2O, 900µL of 10% APS, and 90.0µL of TEMED into the Pyrex flask.

5. After mixing, keep the solution on ice and continue to swirl every 2 minutes.

6. Fill the gel cassettes with the solution using a transfer pipette. It is important to tilt the cassette and fill one corner at a time to avoid the formation of bubbles.

7. Place the comb into the top of the filled cassette.

8. Allow the gel to settle standing up for 1 hour at room temperature.

9. Store the gels at 4°C until ready to use.

Loading and Running the Gel

1. Remove the white tape on the bottom of the gel and the gel comb from the gel cartridge.

2. Slide the gel cartridge into the gel apparatus. Each apparatus may hold up to two gels, if only 1 gel is being loaded place an empty cassette or a spacer in the other slot.

3. Fill the gel apparatus with 1X TBE Buffer until the wells of the gel are completely submerged in the centre of the apparatus and the slot at the bottom of the gel cassette is also submerged.

4. Purge the wells with a Pasteur pipette ensuring no air bubbles are present.

5. Combine 7μL of the PCR product with 3 μL of 6X Loading Dye.

6. Load 3μL of the DNA size standard into 1 well, followed by the samples into the remaining wells.

7. Connect the gel apparatus to the electrophoretic power pack and adjust the voltage to 125 volts and the time to 40 minutes.

8. Once the run is completed, open the gel cassette and carefully remove the PAGE gel.

9. Stain the PAGE gel for 15 minutes with Ethidium Bromide.

10. Place the gel on the transilluminator (set at wavelength UV B) and photograph.

c. Capillary Electrophoresis

The detection of autosomal STRs and mitochondrial DNA sequencing will be achieved using an ABI PRISM® 3100 Genetic Analyzer. This technique has the ability to not only to differentiate DNA fragments by a single base pair, but can also analyze multiple fragments of the same length with the use of fluorescently labeled primers during amplification.

Genescan Fragment Analysis Detection

Required Reagents

Hi- Di Formamide

Size Standard (usually 500-LIZ)

Preparation and Loading of Sample

1. Resuspend the desiccated sample to be sequenced in 15μL of Hi-Di Formamide.

2. Vortex the sample for 1 minute and zip spin the tube.

3. Heat the sample at 95°C for 3 minutes and then immediately place on ice for 2 minutes.

4. Vortex and zip spin the sample.

5. Hold the sample on ice until ready to load for capillary electrophoresis.

6. Transfer the entire 15μL volume sample into a 96 well ABI Plate.

7. Be sure that the 16 wells that correspond to a capillary during analysis contain either 15μL of sample or Hi-Di Formamide.

8. Centrifuge the ABI Plate to remove any air bubbles prior to loading the plate in the ABI PRISM® 3100 Genetic Analyzer.

Fragmentation Analysis using the ABI AMPFlSTR® Identifiler Kit

Required Reagents

Hi Di Formamide

500-LIZ Size Standard

Preparation and Loading of Sample

1. Label a 0.5mL tube for each sample, and an additional tube for the Allelic Ladder sample.

2. Add 10μL of the sample to the appropriately labeled tube and 10μL of the Allelic Ladder to its labeled tube.

3. Add 0.3μL of the 500-LIZ Size Standard and 9.0μL of Hi-Di Formamide to each tube.

4. Vortex the sample and then zip spin.

5. Heat the sample to 95°C for 3 minutes, and then immediately place on ice for 2 minutes.

6. Briefly vortex and zip spin the sample.

7. Hold the sample on ice until ready to load for capillary electrophoresis.

8. Transfer the entire volume of the sample (10.3μL) into a well on a 96 well ABI Plate.

9. Be sure that the 16 wells that correspond to a capillary during analysis contain either 15μL of sample or Hi-Di Formamide.

10. Centrifuge the ABI Plate to remove any air bubbles prior to loading the plate in the ABI PRISM® 3100 Genetic Analyzer.

IV.Results

All three types of specimens tested incorporated the use of different decontamination, sample preparation and extraction methods for the comparison of success for different types of genetic profiling techniques including sex determination, mtDNA sequencing and autosomal STRs.

i. Decontamination and Sample Preparation

The hard tissues, bone and teeth, underwent three decontamination techniques; solution washes, UV irradiation and sonication. In some instances, two of the techniques would be used together. These samples were prepared using one of two methods, fragmentation or complete homogenization / pulverization (refer to Table 12). For the soft tissue samples, only UV irradiation was applicable for decontamination and they were prepared prior to extraction either by tearing or grinding in a mortar and pestle. Positive amplification and sequencing of mtDNA without the presence of inhibition or contamination indicate success.

All three decontamination techniques were tested on bone and teeth samples. The solution washes consisted of one wash with distilled water, one wash of 10% (w/v) sodium hypochlorite solution and one wash with 70% Ethanol. A summary of these results is provided in Table 11. The solution washes were tested on eight bone samples and 10 teeth samples. Three of the bone samples and one tooth sample were amplified with further analysis. Thirteen bone samples and 14 teeth samples were tested with both the solution rinse and the UV irradiation methods together. These samples were exposed to UV for 20 minutes on each side at 254nm. Five bone and six teeth samples, using both

the solution washes, and UV irradiation together were successfully detected. Sonication was conducted using 1L of a water and Tergazyme detergent solution for 20 minutes. This method was applied to eight bone samples and four teeth samples. Only two bone and one tooth sample were successfully detected.

Table 11: Summary of the results for the decontamination techniques applied to ancient bone and teeth

Decontamination Method	Type of Sample	Number of Samples	Successful Detection
Solutions Washes	Bone	8	3
	Teeth	10	1
Solution Rinse and UV Irradiation	Bone	13	5
	Teeth	14	6
Sonication	Bone	8	2
	Teeth	4	1

Of the 50 bone extractions tested, 11 were fragmented and 39 were pulverized. As seen in Table 12, none of the fragmented bone samples were successfully detected, while 15 of the pulverized bone extractions were successful. The 49 teeth extractions were divided into 14 fragmented and 35 pulverized. Six of the fragmented teeth samples were from modern medically archived specimens. Seven of the fragmented teeth were detected, six of which were modern samples, and 23 of the ancient teeth samples that were pulverized were amplified successfully. These results are observed in Table 12. For the tissue samples, amplification success was low for all samples, however, of the six samples that used the tearing preparation, none amplified. Four samples were tested with the mortar and pestle preparation method and all four amplified (see Table 12).

Table 12: The results of the sample preparation methods applied to bone, teeth and tissue.

Type of Sample	Sample Preparation	Number of Sample Tested	Successful Detection
Bone	Fragmented	11	0
	Pulverized	39	15
Teeth	Fragmented	14 (6 modern)	7 (6 modern)
	Pulverized	37	23
Tissue	Tearing	6	0
	Mortar and Pestle	4	4

ii. Extraction

Four extraction methods were tested for bone and teeth, PK, GuSCN, CTAB and PCT technology using both the PCT buffer and the GuSCN extraction buffer. The tissue samples were tested using two extraction methods, PK and GuSCN. These methods were evaluated using mtDNA fragmentation analysis, mtDNA sequencing and Real-Time PCR for quantification.

a. Bone

The success of the extraction techniques tested on ancient bone samples was determined using mtDNA fragmentation analysis and sequencing.

Proteinase K

The PK extraction was tested on 21 ancient bone samples from 3 collections, Southern USA, Southwest USA and Southern Mexico. The success of PK on bone was assessed with mtDNA amplification with three initial amplicons, 112bp, 210bp and 259bp. Alternatively, for poor success samples hemi-nested PCR was tested using the three aforementioned amplicons. These amplified results are summarized in Appendix

1A. Two individuals from the Southern USA collection successfully amplified an 112bp amplicon. Five individuals from the Southwest USA were successfully amplified with one, IND6 2:1, amplifying a 259bp amplicon, while the remaining four individuals amplified multiple hemi-nested amplicons from all three of the original amplicons tested. The third collection, from South Mexico, only one sample was successfully amplified with the 112bp amplicon. Those samples, which amplified successfully, were further analyzed with mtDNA sequencing.

Guanidinium Thiocyanate

Three collections, Southwest USA, Southern USA and Southern Mexico, were also used for mtDNA amplification fragmentation analysis to determine the success of GuSCN as an extraction method. Fourteen samples were tested using the 112bp, 210bp and 259bp amplicons and many hemi-nested PCR amplicons from the three amplicons previously mentioned. A summary of these results is provided in Appendix 1A. Seven samples were successfully amplified. Two of these samples were from the Southern Mexico collection, while the remaining five were from the Southern USA collection. The Southern Mexico collection amplified one sample with an 112bp amplicon (CAL20 1:2), while the second sample amplified a 210bp amplicon (XV2 1:8). From the four samples that were successful from the Southern USA collection, two amplified the 112bp amplicon (B20 amd B37). The fifth sample, B41, amplified a hemi-nested amplicon from the 112bp amplicon. The samples that amplified successfully were further analyzed with mtDNA sequencing.

Cetyltrimethylammonium Bromide

Four bone samples from two individuals belonging to the Southwest USA collection were tested using CTAB. Mitochondrial DNA amplification was tested with a 210bp amplicon that failed for every sample, therefore a shorter fragment, approximately 112bp in length was tested however, these all failed as well.

Pressure Cycling Technology

The ancient bone samples used to test the PCT technology using both the unknown PCT extraction buffer and using a GuSCN extraction buffer were from one collection, the Southern USA collection. A total of 11 samples were tested from five individuals, five were analyzed using the unknown PCT extraction buffer and the remaining six were analyzed with the GuSCN extraction buffer.

To determine the presence of mtDNA, a 131bp amplicon was tested, however no positive results were observed. The samples underwent a second p30 size exclusion chromatography column, but a second PCR using the same 131bp amplicon was also negative.

b. Teeth

The successful extraction of the 39 ancient teeth samples and 6 modern teeth samples tested, was assessed with mtDNA fragmentation analysis, two sex determination assays and mtDNA sequencing. Additionally for the modern samples, quantification using real-time PCR and autosomal STR profiling was performed.

Proteinase K

For all teeth samples extracted using PK, mtDNA amplification was attempted to determine the presence of DNA and the degree of fragmentation. Three amplicons

ranging from 112bp to 259bp in length were initially tested. Further amplification using hemi-nested PCR was tested, if required, to determine the presence of fragmentation. The results of these amplifications are tabulated in Appendix 1B. Of the 25 extractions conducted from 12 different individuals, 9 individuals amplified successfully for the 210bp amplicon, the 259bp amplicon, or at least one of the hemi-nested amplicons. The degree of fragmentation varied depending on the collection being analyzed. Three individuals failed for every amplicon size, even when duplicate extractions were performed. The samples that amplified successfully were further analyzed with mtDNA sequencing

Determination of the genotypic sex of teeth specimens was tested using six samples from the South American collection and detected with both Genescan Fragment Analysis and PAGE. This type of analysis required the amplification of the sex chromosomes using two types of assays. The results of these analyses can be observed in Table 13 below. For the first two samples listed in Table 13, initial detection using both the Amelogenin gene and the ARY assays were negative. When the nested and booster PCRs were performed using the two assays, results that indicate male were observed. However, these results could not be duplicated for either type of booster analysis. The amelogenin booster PCR produced two nonspecific bands in the first trial, while the second trial although faint, indicated male with the presence of two bands located at approximately 106 and 112 bp. The second assay the ARY primer set detected positive results indicating male, but smearing was observed throughout the migration of the sample during the first trial. The second trial indicated male. For the remaining samples,

only the amelogenin assay was tested. Negative results were observed for all 4 samples, even when the nested Amelogenin PCR was attempted.

Table 13: Sex determination of ancient teeth samples extracted using Proteinase K

Sample	Amelogenin PCR	ARY PCR	Nested Amelogenin PCR	Booster ARY PCR
IRI 1:1	2 trials both negative	2 trials both negative	NT	NT
IRI 1:2	negative	negative	non-specific bands* indicate male**	indicate male but smearing present* indicate male**
IND2 1:3	negative	NT	negative	NT
IND7 1:3	negative	NT	negative	NT
IND8 1:1	negative	NT	negative	NT
IND7 1:5	negative	NT	negative	NT

*No purification procedure was performed between the two PCRs.

**Purification procedure performed between the two PCRs.

NT refers to Not Tested, these samples were not subjected to the testing listed in the given column heading.

Guanidinium Thiocyanate

The GuSCN extraction method was tested on five aDNA teeth samples from two collections, Southern USA and Western Europe. As can be observed in Table 14, the Western Europe sample amplified for all amplicons tested, while the Southern USA samples amplified a 210bp amplicon. Positively amplified mtDNA samples were subsequently sequenced.

Table 14: mtDNA amplification of ancient teeth samples extracted using GuSCN targetting different size amplicons and hemi-nest PCR.

Sample	Amplicons				Hemi-net PCR Amplicons				
	1 (112bp)	2 (210bp)	3 (259bp)	4 (351bp)	1	2	3	4	5
MIA3 1:3	+	+	+	+	N/A*	N/A*	N/A*	N/A*	N/A*
B37 2:1	-	+	-	-	-	-	-	-	-
B37 2:2	-	+	-	-	-	-	-	-	-
B40 2:1	-	+	-	-	-	-	-	-	-
B40 2:2	-	+	-	-	-	-	-	-	-

*Hemi-nested PCRs were not necessary since initial amplification was successful.
+ indicate the sample successfully amplified, while – indicates no result was observed.

Cetyltrimethylammonium Bromide

The extraction of DNA using CTAB on teeth samples was tested using four ancient teeth samples from one collection, Southern USA. mtDNA fragmentation analysis was then used to detect the presence of DNA. Two amplicon sizes were attempted, however no amplification was detected for either size even when performed in duplicate.

Pressure Cycling Technology

The PCT extraction method was tested with eight ancient teeth sample fragments from the Southern USA collection, and six modern medically archived teeth samples. The aDNA teeth samples both extracted with the PCT extraction buffer and the GuSCN extraction buffers were amplified using an 112bp amplicon and different volumes of template mtDNA. The volumes tested were 5μL, 10μL and 20μL in a 25μL reaction. Of the four samples extracted with the PCT extraction buffer, samples S8 through S11, none of the samples successfully amplified using any of the three volumes. It is important to note as well, that no primer dimer or any other indicator to ensure the reaction was carried out successfully was observed. Only one sample from the four aDNA teeth

samples using the GuSCN extraction buffer amplified successfully using all three volumes (sample S14). S12 was detected when 10μL of template mtDNA was used, however S13 and S16 were unsuccessful using any of the three volumes. A second trial was attempted using a 50μL reaction however, only S12 was detected again when using 10μL of template mtDNA. The remaining samples resulted in smearing regardless of the volume of template DNA used. A second mtDNA region of 140bp in length was amplified, however none of the samples extracted using the PCT extraction buffer or the GuSCN extraction buffer were detected.

The 6 modern teeth samples utilized the PCT technology with two possible extraction buffers. Three samples were extracted with the unknown PCT extraction buffer while the other three were extracted with the GuSCN extraction buffer. Following extraction, the PCT extraction buffer samples underwent a p30 chromatography column and the GuSCN extraction buffer samples were subjected to silica bead purification. The quantification of DNA was tested using two methods, band intensity during gel electrophoresis and real-time PCR. Initially, the quantity of DNA present was tested with different volumes of DNA added for the amplification of an 113bp amplicon. Three volumes were tested 1μL, 5μL, and 10μL in a 25μL reaction.

Figure 7 illustrates the difference in intensity based on the volume of mtDNA present, which is indicative of the quantity of template DNA that was extracted. Only two samples, S35 and S30 amplified with all three volumes. All samples except for S32 amplified with 1μL of template mtDNA added. Five of the six samples were successfully amplified with 5μL of template mtDNA and only four samples successfully amplified with 10μL of template mtDNA.

The fragmentation analysis of mtDNA for all 6 samples was amplified using 5μL template DNA for each amplicon. Of the three modern teeth samples tested using the unknown PCT buffer, only one amplified successfully for all 5 amplicons tested (S32). The other two samples, S29 and S30 amplified successfully for the 113bp, 229bp, and 414bp amplicons. S29 failed to amplify the 807bp amplicon, but amplified with the 1027bp amplicon. S30 successfully amplified the 807bp amplicon, however the intensity of the band was very low and failed to amplify Amplicon 5, the 1027bp fragment.

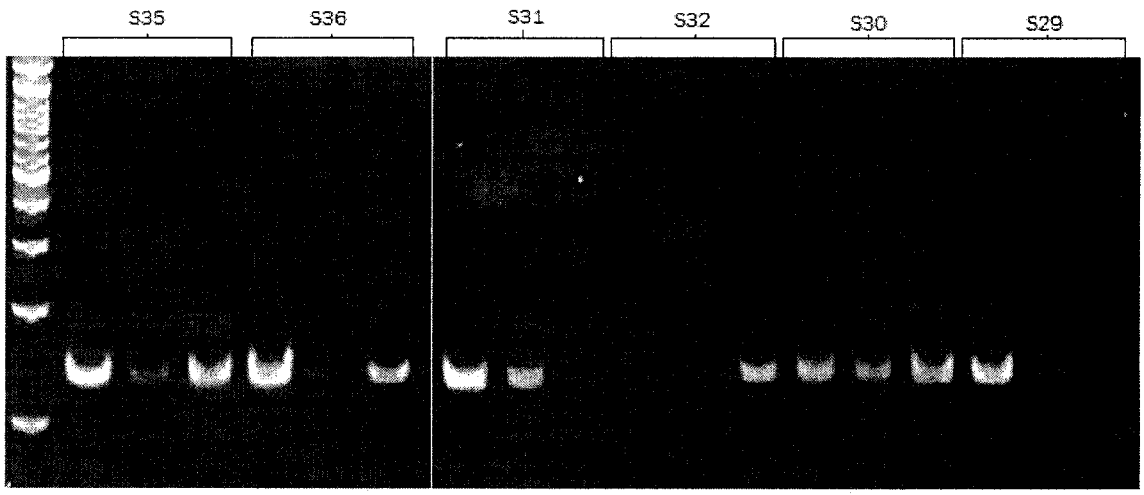

Figure 7: mtDNA amplification of different template volumes to determine the relative quantity of DNA. Utilizing a 100 bp standard ladder (increments of 100bp) the amplification of the 112bp amplicon is detected where the sample volumes are presented in the order 1, 10 and 5μL respectively.

The three modern teeth samples extracted using PCT but with the GuSCN extraction buffer all amplified successfully with all five amplicons tested. The fragmentation analysis of S32 and S35 to compare the two extraction buffers can be observed in Figure 8. The fragmentation analysis of all mtDNA amplicons tested using both the PCT and GuSCN buffers for extraction can be observed in Table 15.

Table 15: Degradation analysis of mtDNA from modern teeth samples extracted with both PCT buffer and GuSCN buffer.

Modern Tooth Sample	Extraction Method	Amplicon 1 (112bp)	Amplicon 2 (229 bp)	Amplicon 3 (414 bp)	Amplicon 4 (807 bp)	Amplicon 5 (1027 bp)
29	PCT Buffer	+	+	+	-	+
30	PCT Buffer	+	+	+	Faint	-
31	GuSCN	+	+	+	+	+
32	PCT Buffer	+	+	+	+	+
35	GuSCN	+	+	+	+	+
36	GuSCN	+	+	+	+	+

+ symbol indicates positive amplification was observed, while – indicates a negative result.

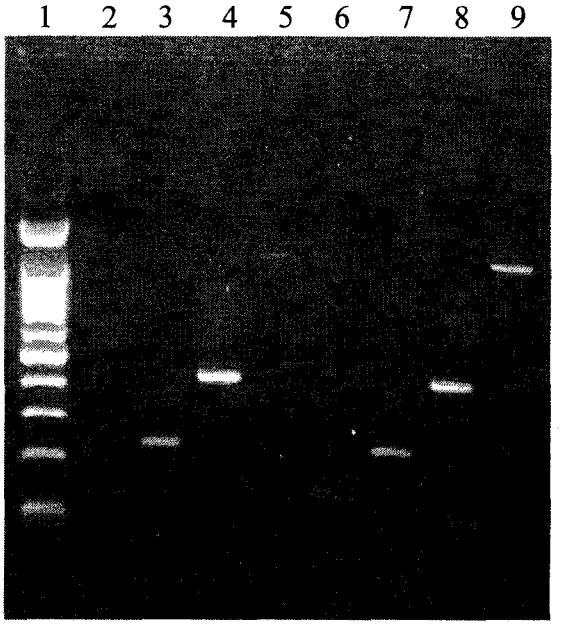

Figure 8: mtDNA fragmentation analysis of modern teeth samples.

S32 extracted with PCT (left) and the unknown buffer and S35 extracted with PCT and a GuSCN extraction buffer (right) using gel electrophoresis. Lanes 2 and 6 are the 113bp fragment, Lanes 3 and 7 are the 229bp fragment, Lanes 4 and 8 are the 807bp fragments and Lanes 5 and 9 are the 1027bp fragments.

The use of real-time PCR was a second method of quantification used for this study. Using the Applied Biosystems Quantifiler™ Human DNA Quantification Kit, each of the six samples was relatively quantified. The following table, , provides the

Cycle Threshold (Ct) value for each sample and the corresponding calculated quantity of DNA determined by using the slope of the standard curve.

Table 16: Quantification of modern teeth samples extracted using the PCT buffer and the GuSCN buffer using Applied Biosystems Quantifiler™ Human DNA Quantification kit

Sample	Cycle Threshold Value (Ct)	Quantity (ng/ µL)
S29	30.61	0.52
S30	25.66	1.23
S31	32.35	0.16
S32	29.96	0.81
S35	30.30	0.64
S36	35.51	0.02

Although amplification of nDNA was attempted for all six modern teeth samples, no amplicons amplified successfully. Therefore, another profiling technique used to detect nDNA was autosomal STRs. Of the six samples tested, one sample, S30, amplified a complete STR profile, S35 amplified 15 of 16 loci while 11 loci were amplified for S32. The remaining three samples, S29, S31 and S36, also produced partial profiles with 6, 9 and 4 loci being amplified respectively. The corresponding STR profiles for each of the samples can be observed in Appendix 2A.

c. Tissue

These tissue types were sampled from different locations of the body including muscle, lung, heart and tissue remaining from a vertebrate. The detection of mtDNA was attempted using two amplicons, one 112bp long and the other 210bp long. Hemi-nested PCR was also attempted if no DNA was detected in the previous two amplicons. Two extraction methods; GuSCN and PK were tested using the soft tissue.

Proteinase K

Six of the ten samples were extracted with PK, four of which underwent the tearing sample preparation method while the remaining two used the mortar and pestle method for sample preparation. The results of the mtDNA amplification can be observed in Table 17. When the 210bp amplicon was tested, none of the samples successfully amplified. The second amplicon tested, the 112bp amplicon, resulted in only one sample, T-54 1:1 amplifying successfully. A hemi-nested PCR with the inner target region being 112bp in length was also tested. Sample T54 1:1 amplified successfully along with T-32. These successfully amplified samples were further analyzed with mtDNA sequencing.

Table 17: Summary of mtDNA amplification of tissue samples extracted with either PK or GuSCN

Sample	Sample Preparation	Extraction Method	Amplicon 1 (112bp)	Amplicon 2 (210bp)	Hemi-nested (Inner Region 112bp)
NAZ1 1:1	Tear	PK	-	-	-
NAZ2 1:1	Tear	PK	-	-	-
T-3	Tear	PK	-	-	-
T-54 1:1	Tear	PK	-	-	-
T-32	Mortar	PK	-	-	+
T-54 1:1	Mortar	PK	+	-	+
T-2	Tear	GuSCN	-	-	-
T-34 1:1	Tear	GuSCN	-	-	-
T-21	Mortar	GuSCN	+	-	-
T-34 1:2	Mortar	GuSCN	+	-	-

+ symbol indicates positive amplification was observed, while – indicates a negative result.

Guanidinium Thiocyanate

Of the four tissue samples tested using the GuSCN extraction method, two were prepared by tearing and the other two were prepared using a mortar and pestle. Similar to the tissue samples extracted with PK. A summary of these results can be observed in the previous table, Table 17. None of the samples were detected using the 210bp amplicon,

and only two samples were successfully detected with the 112bp amplicon but had the presence of smearing. When the samples were tested using a hemi-nested PCR with the inner target region of 112bp, two of the samples extracted with PK successfully amplified.

Table 18: Summary of the most optimal methods for each sample type

Type of Sample	Decontamination	Sample Preparation	Extraction
Bone	Solution Washes	Pulverization	GuSCN more versatile, but PK more efficient
Teeth	Solution Rinse & UV Irradiation	Pulverization	
Tissue	NT	Grinding with Mortar and Pestle	GuSCN

The overall assessment of the most optimal method for decontamination, sample preparation and extraction are summarized in Table 18.

iii.mtDNA Sequencing

Each of the successfully amplified mtDNA fragments detected were sequenced. There was a total of 112 amplicons submitted, with an additional 24 samples submitted for a second trial after initially failing. Of all the amplicons submitted, 45 produced clean readable sequences.

V. Discussion

The results of this study further support and prove how the type of extraction method employed during analysis that is most optimal is dependent on the type and depositional environment of the sample to be analyzed. The samples used for this research were collected from five different sites, as listed in Table 1. These collection sites vary in temperature, humidity and soil composition. The depositional environments bone and teeth samples were collected from were, located in the Southern USA, Southwest USA, Southern Mexico and Western Europe. The Southern USA site is a sandy environment with fluctuating temperatures, while the Southwest USA site is also a sandy environment, but is arid and dry. Therefore, these environments would be more susceptible to oxidative damage (Lindahl 1993). The samples from Southern Mexico were collected from a moist environment, similar to the final collection site, Western Europe, which is a moist environment with cool temperatures. The samples from these last two collections would be more susceptible to hydrolytic damage (Lindahl 1993). The tissue samples were collected from an arid environment in South America. Much like the Southwest USA collection, this type of environment may assist in the rapid desiccation of the tissues to help slow down natural degradative processes.

i. Decontamination and Sample Preparation

Prior to receiving samples in the laboratory, the manner in which specimens have been collected, handled or stored is unknown (Herrmann and Hummel 1994). Therefore it is essential to decontaminate to the surface to remove any possible contaminants, such as soil, plant material or bacteria, all of which may interfere with further analyses. This

stage of analysis also removes exogenous DNA from either other organisms in the environment, or more significantly, exogenous human DNA from improper handling.

We applied three decontamination techniques to bone and teeth; a series of solution washes with water, sodium hypochlorite and ethanol, UV irradiation and sonication. The washes are a simple procedure that physically cleans the surface of the sample. The sodium hypochlorite and ethanol solutions sterilize the surface from any bacteria or fungal growth. Additionally, the sodium hypochlorite destroys DNA by oxidative damage, resulting in base modifications and the formation of chlorinated base derivatives (Kemp and Smith 2005), which induce strand breaks to any remaining exogenous DNA. The use of UV irradiation causes covalent bonding between opposite thymine dimers, preventing the denaturation of double stranded DNA and thus any amplification (Sarkar and Sommer 1991; Herrmann and Hummel 1994). The third method tested, sonication, applies high frequency sound waves through a solution causing disruption of the cell membrane and thus degrading any contaminants present. Also, the presence of the solution acts as a wash step.

Less than half of the samples from each type of treatment were positively amplified (refer to Table 11). Furthermore, less than half of the samples submitted for mtDNA sequencing were successful, therefore the presence of contamination could not be thoroughly addressed. Further research incorporating fewer variables and applying more stringent conditions is required to accurately determine the effect these methods have on the overall success of amplification.

The necessity for proper sample preparation however, provided expected results. The primary purpose of sample preparation is to increase the available surface area of the

sample to further facilitate extraction (O'Rourke 2000). The samples that were broken into fragments of bone or teeth exhibited very low success, while those that were pulverized had a much higher success in the amplification of DNA (see Table 12 and Table 18). These results were expected since pulverization results in the formation of powder, resulting in a larger surface area and greater accessibility for extraction. When a sample is extracted while still partially intact, the tissue retained inside is not as accessible to the extraction buffer, therefore less cells will be lysed, meaning a smaller quantity of DNA being released.

Similarly, with soft tissue, negative results were observed for tissue torn into small pieces, where as tissue samples ground with a mortar and pestle to form a paste had complete success. The paste created a larger surface area and would mix easily with the extraction buffer.

Although these results indicate the sample preparation stage of analysis has positive implications for increasing the yield of DNA, it does requires further manipulation of the sampled and thus creates the possibility of introducing exogenous DNA molecules (O'Rourke 2000). Furthermore, these added steps could lead to loss of sample due to transferring the sample between the mixer mill chamber and tubes.

ii. Extraction

a. Bone

The ancient bone samples used were used to test four extraction methods. mtDNA fragmentation analysis and mtDNA sequencing was used to evaluate each extraction technique. The two most commonly used extraction methods in aDNA

analysis; PK and GuSCN were tested on 21 and 14 bone samples respectively (refer to Appendix 1A). Fragmentation analysis of mtDNA resulted in eight positive GuSCN samples and five positive PK samples. Four of the successfully amplified GuSCN samples were from the Southern Mexico collection, while the remaining four were from the Southern USA collection. Proteinase K was successful in amplifying samples from the Southern USA and the Southwest USA collection, however no samples from the Southern Mexico collection amplified using this extraction method. The success of the two USA collections, although highly fragmented, can most likely be attributed to the dry environment of these locations. Since it was known that the samples from the Southern Mexico collection had been exposed to cinnabar (mercury sulfides) in the depositional environment, the failed PK samples were presumably inhibited due to the presence of mercury . It has been previously reported that mercury distorts the stereochemistry of the protein structure of PK by binding to the Cys-73 residue located near the active site (Gourinath et al. 2001). This can result in a loss of up to 85% of enzymatic activity (Saxena et al. 1996; Gourinath et al. 2001). These collection samples were also extracted using GuSCN, which is not an enzymatic extraction and resulted in four positive samples.

A third extraction method, although not commonly used for human DNA analysis was evaluated as an alternative to the standard extraction methods previously discussed. This extraction technique is CTAB. It has been more commonly used for other biological sources including plants and bacteria (Jones 1953; Murray and Thompson 1980), but more recently applied to burned human bone (Ye et al. 2004). In our study, CTAB was applied to four bone samples from the Southern USA collection, however, none of them successfully amplified. The exact cause of failure is not known, as there was no PCR

inhibition detected. It could be the salt concentration present within the CTAB extraction method was too low and thus caused the DNA to form a complex with the CTAB and precipitate. An alternative cause could be thatthe DNA within the samples was highly fragmented or only present in low copy number. However, other bone samples from the same collection amplified successfully when another extraction method was used indicating that this is not the cause of the failure.

A novel technology assessed in this research, the Pressure Cycling Technology developed by Pressure Biosciences Inc. was tested with ancient bone and teeth samples, as well as modern medically archived teeth specimens to determine its ability in extracting nucleic acids from human samples. This technique subjects the sample, while in a specially designed tube, to cycles of changing hydrostatic pressures (ambient to high levels) resulting in the lysis of tissue and release of nucleic acids into solution (Tao 2003).

We tested using two extraction buffers, a GuSCN extraction buffer and an unknown extraction buffer provided by Pressure Biosciences Inc. The success of these two approaches was evaluated using 11 ancient bone samples. For all samples, mtDNA fragmentation analysis was performed to determine the presence of DNA, as well as the quality of DNA. Unfortunately, none of the ancient bone samples amplified. In the event that template inhibition was present, the samples underwent an additional purification using a size exclusion chromatography column to remove any possible inhibitors, however no amplification was still observed.

b. Teeth

Teeth are a commonly encountered tissue typedue to their hard enamel surface, which is able to withstand harsh environmental conditions. The enamel protects the DNA-rich pulp cavity from contamination and exogenous DNA. All samples were well intact with only a few exhibiting broken root tips; one had the presence of a dental carie and two of the samples, both molars, had dental restorations. One restoration was removed prior to the analysis, however the restoration in the other sample could not be removed.

The mtDNA amplification was successful in 17 of 24 teeth extracted with PK (Appendix 1B). The Southern USA teeth samples all successfully amplified a 200bp amplicon, where as the Southwest USA teeth samples had only three samples amplify the standard PCR, while multiple hemi-nest PCR amplicons were detected. Only one of the four Western Europe samples did not amplify, while the others amplified up to 259bp. These samples are the most recent samples, being less than a century old; therefore the DNA may still be somewhat intact. The one failed sample from this collection was the tooth in which the dental restoration could not be removed. This may indicate that inhibitors within the restoration are causing either the extraction or PCR to fail. STRs were attempted for the Western Europe samples, but profiles could not be generated due to low copy numbers of nDNA.

Five of the samples that successfully amplified mtDNA were used for testing sex determination assays. Initially, the Amelogenin gene was tested, in which a 6bp deletion differentiates between the X and Y chromosome (Hummel 2003). However, as listed in

Table 13, no amplified results were observed A second assay, Alphoid Repeat region of the Y chromosome, was tested. A positive result would indicate male, while a negative result would indicate female. The disadvantage of this type of assay is that a negative result could mean female or be a failed amplification. All five samples tested produced negative results (Table 13). These types of assays attempt to amplify nDNA, which in aDNA samples is often difficult to retrieve. Therefore, the incorporation of a nested PCR with the Amelogenin gene was designed. Two primers that flank the 106 or 112bp region of interest were developed and amplify a 225bp fragment. The advantages of subjecting the samples to a nested PCR allow an increase in copy number of the DNA, while increasing the specificity of the fragment to be amplified. This technique was applied to all five samples, with one sample indicating male. The Alphoid Repeats were also tested using a booster PCR to also increase the copy number of nDNA. The same sample that amplified with the nested Amelogenin test amplified and produced a positive result, indicating male as well (refer to Table 13). Since only one sample provided readable results, while other samples produced non-specific bands, the nested Amelogenin PCR requires further testing on a larger number of samples to further optimize its efficacy.

GuSCN extraction was conducted on five ancient tooth samples from two collections, the failed PK sample from Western Europe and four samples from the Southwest USA. As listed in Table 14, five samples were amplified, with the four Southwest USA samples amplifying a 200bp amplicon, and the Western Europe sample amplified for all fragments tested, including a 259bp amplicon.

Since the Western Europe sample amplified when extracted with GuSCN, it would suggest that the PK extraction was inhibited as opposed to inhibition during the

PCR. In order to determine the cause of inhibition, a partial fragment of the dental restoration was retained from the other tooth sample and analyzed with Scanning Electron Microscopy (SEM) to determine the elemental composition of the amalgam. This type of analysis is a rapid and simple technique that provides the elemental composition of a sample with statistically valid data having detection limits to the order of 1000 ppm (Smith 1990). Previously, this technique has been used to detect restorative material located on the facets of teeth (Smith 1990). In our study, the Scanning Electron Microscopy and Energy Dispersive Spectrometer (SEM-EDS) elemental analysis of the dental amalgam fragment concluded the primary elements were silver and mercury. Based on the spectrum in Appendix 1D, each of the major peaks produced could be identified and participated in the formation of one of two possible X-ray line patterns, representing either mercury or silver. Therefore, similar to the Southern Mexico bone samples, the mercury present in the amalgam was inhibiting the enzymatic activity of the PK.

The use of CTAB on the Southern USA ancient teeth samples did not result in any positive amplification. These results are similar to those observed with the ancient bone samples. The explanation of these failures again is unknown, as teeth samples from the Southern USA collection were successfully amplified using other extraction methods. Since this extraction method failed for both the bone and teeth samples, further modification to buffer concentrations or incubation times may be required to improve the retrieval of DNA.

As previously discussed in the Bone section, the PCT technology for extraction utilizes changes in pressure to lyse tissue and release nucleic acids (Tao 2003). This

technique was also tested on eight ancient teeth samples and six modern medically archived teeth samples.

Like the ancient bone samples, the PCT system was tested using two extraction buffers, a GuSCN extraction buffer and an unknown extraction buffer provided by Pressure Biosciences Inc. mtDNA fragmentation analysis was also performed to determine the presence and quality of DNA. Furthermore, the modern samples underwent real-time PCR quantification and STR analysis. Of the ancient samples tested, DNA was detected from only one sample that was extracted using the GuSCN extraction buffer. These were tested using varying volumes of template DNA since mtDNA copy number would have an influence on the success. For the failed ancient teeth samples they were further purified using a size exclusion chromatography column to remove any possible inhibitors, yet failed results were still observed.

The analysis of the modern teeth samples however was successful. Of the six teeth tested, three samples were tested for each extraction buffer, GuSCN and the unknown buffer, all amplified mtDNA. The fragmentation analysis of mtDNA (Figure 8) also proved intact DNA was recovered from both extraction buffers up to 1000 bp in length. Furthermore, as listed in Table 16, only one sample had less than 0.2ng/uL detected using real-time PCR and the ABI Quantifiler™ kit. STRs also determined nDNA was present, with at least partial profiles being detected for all, and one sample producing a full profile (see Appendix 1C).

Our results indicate that although this method may not be applicable for ancient samples, it is very successful in retrieving high quality DNA from modern samples. This technique is advantageous because it is non-destructive to the sample and allows the

entire reaction to occur within one tube. Furthermore, as will be discussed later is that this method was proven successful with other types of biological tissue. This entire extraction process can be completed in minutes, depending on the type of sample being analyzed. As well, the parameters including the number of cycles, the temperature and the pressure levels can all be adjusted to optimize conditions for different types of samples. The technique alleviates the sample preparation step of analysis, while the specialized PULSE tube allows the entire reaction to take place within one tube, which helps in preventing any cross contamination or loss of sample (Tao 2003). Also, even though the PULSE tube can accommodate 1.5mL of solution, the volume of lysis solution can vary. In our research, the entire volume was added and thus could have made the ancient samples too dilute to detect DNA.

The versatility of this method is supported by the positive results observed for both types of extraction buffers when used in conjunction with the PCT technology. Although not tested in this research, these results would indicate that alternative extraction buffers utilizing different chemistries may also be applicable to use with this technique.

c. Tissue

The soft tissue samples available for this research project were mummified tissues from lung, heart and muscle. These three types of soft tissue, in theory, should contain high copy numbers of mtDNA since each of these organs require a large amount of energy to function properly while the individual is still alive. Also, the depositional environment in which these samples were collected is an arid and dry climate, which

typically facilitates rapid desiccation of the tissue (O'Rourke 2000). The lack of humidity would slow down degradation caused by enzymatic autolysis and physical or chemical factors (Herrmann and Hummel 1994). However, these samples are estimated to be 1000 to 2000 years old and the storage and handling of the samples prior to our analysis is unknown. Of the 10 soft tissue samples analyzed, two extraction methods were tested. The success of these techniques was assessed using mtDNA fragmentation analysis. Both extraction methods were tested with both sample preparation methods, and the results proved the sample preparation appeared to play a more significant role in detecting mtDNA (Table 12). As previously mentioned, six samples were prepared with tearing, while the remaining four were ground with a mortar and pestle. Once extracted, none of the samples prepared with the tearing method successfully amplified, while all four of the samples ground with the mortar and pestle amplified. As listed in Table 17, two samples amplified with the detection PCR, while the remaining two samples amplified through the use of hemi-nested PCR. The successfully amplified samples were extracted with both PK and GuSCN. This is most likely because the mortar and pestle created a paste once completed and when the extraction buffer was added, it allowed the buffer to mix with the tissue more easily. Furthermore, the mortar and pestle preparation technique may have created more access to the nucleic acids by releasing tannins, humic and fulvic acids (compounds that can inhibit amplification (O'Rourke 2000). Also, if these inhibiting compounds are released into solution, they may be easier to remove during purification.

In comparing all four of the extraction methods used, regardless of the type of tissue tested, the PK and GuSCN extraction buffers were determined to be the most

reliable with 45% and 36% success, respectively (refer to Table 18). These results were expected since these methods are often used in forensic protocols (Ivanov et al. 1996). One advantage of GuSCN is that it does not require a fume hood or the handling of toxic compounds, as it is typically coupled with the silica bead purification. Conversely, as PK is coupled with the phenol/chloroform purification, which produces toxic vapors (O'Rourke 2000). One disadvantage of GuSCN however, is that the silica particles used in purification can inhibit PCR if they are not completely removed during the wash steps. Even with this caveat, this method has been found to have fewer PCR inhibitors detected than that of PK (Cattaneo 1997). In this study, GuSCN was more reliable when comparing the five collections tested, whereas inhibition was observed when using PK from two separate collections. It was also found that the CTAB extraction was not a reliable or robust method in retrieving human DNA, contrary to the results found by Ye *et al.* (Ye et al. 2004). The difference in results could be due to variations in the methodological approach or the samples being tested.

The novel extraction method tested, the PCT technology, proved to have promising results for modern samples, however did not seem applicable to aDNA samples. The advantages of this method however would suggest further analysis of this technique be pursued for its use in aDNA analysis. The ability to complete the extraction in minutes, while maintaining the integrity of the sample can have significant implications. However, the purchasing of the equipment can be costly and only one sample can be processed at a time.

iii. mtDNA Sequencing

The low success for the detection of mtDNA sequences obtained for the samples tested can attributed to many factors commonly in encountered in aDNA analysis. Even though all precautions are taken to prevent contamination, it may still be present and therefore produce false positives. Many of the published results of mtDNA sequences from the early 1990s were later determined to be due to contamination (Hummel 2003). Although exogenous DNA sequences matching that of the analyst were not observed, many sequences indicated mixtures. It cannot be concluded whether modern exogenous DNA was present, since the mtDNA sequences of individuals who may have handled the samples prior to arriving at the laboratory are unknown. Another pattern observed in some sequences was the presence of primer concatomer sequences. Primers were added in excess concentrations as a PCR control, since the presence of primer dimers remove the suspicion of PCR inhibition when negative results are observed. Therefore, rather than amplifying the template DNA, the primers would concatenate and extend, producing a false positive result. This occurrence could be due to low copy number of template DNA or template inhibition. Furthermore, many sequences simply just failed, either due to low copy number or the poor quality of template DNA.

For those sequences that were successful, attempts to repeat the reactions to obtain the results a second time were made, however only one collection, the Western Europe collection was successful. This repetition was performed in attempts to authenticate our results.

VI.Conclusions

The results of this research demonstrate that the efficacy of the extraction method used significantly influences the success of isolating high quality and quantity DNA. Furthermore, the decontamination and sample preparation techniques employed prior to extraction also impact the yield of DNA, especially in removing the presence of inhibitors or exogenous DNA. Since the primary purpose of sample preparation is to increase the surface area of the sample to make the DNA more accessible, the sample preparation techniques that did in fact increase the surface area, being either pulverization or grinding, were more successful in achieving a higher yield of DNA.

Although the type of analysis employed significantly influences the success of retrieving DNA, it was also determined that the integrity of the sample rather than the type of sample had a greater impact on the success of DNA extraction. The sample collections were each exposed to unique environmental conditions and thus were at different stages of degradation. In some instances inhibitors were introduced and had the presence of different artifacts (ie. dental restorations). These factors were observed to play role in the efficacy of the extraction.

Therefore, the type, the preservation and the depositional environment of the sample must all be considered in determining which methodological approach should be taken to ensure high quality results are obtained.

VII.Future Considerations

The results of this research have shown the significance of the initial stages of analysis in detecting aDNA. This project evaluated three types of tissues commonly encountered in archaeological and forensic investigations. However, there are other types of samples that are discovered, including biological fluids or hair. Therefore the findings of this research can be applied to determine methodological approaches that can be taken to test these other types of samples.

This research attempted to incorporate commonly used decontamination, sample preparation and extraction techniques, as well as novel technologies to assess the success of extraction on different DNA sources. Since these three stages of analysis can all have a significant impact on the success of isolating endogenous DNA, more detailed experimentation could be developed to isolate and assess each stage of analysis. Future research can extend to include other extraction methodologies, as well as, begin to manipulate the already established extraction methods to further increase the yield of DNA. As well, since inhibition was a significant factor in the success of the extraction method used, other scientific techniques could be applied to determine the source of inhibition. One example, which was performed in this research was SEM, other types of analyses that could be used include chromatography or spectrophotometry to determine the presence of organic or inorganic compounds present in the sample prior to analysis.

Further research is necessary to optimize the nested Amelogenin PCR for sex determination. The number of cycles and PCR parameters could be further optimized to prevent nonspecific product from amplifying. The booster PCR for the ARY region of

the Y-chromosome could also be subjected to further optimization to ensure negative results are reliable. These sex determination tests will need to be further validated with a greater sample size, including both modern and ancient samples.

VIII.References

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IX.Appendix 1A

mtDNA amplification ancient bone samples extracted with PK or GuSCN targeting different sized amplicons and hemi-nested PCR.

Sample	Extraction Method	Amplicon 1 (112bp)	Amplicon 2 (210bp)	Amplicon 3 (259bp)	Hemi-nest PCR Amplicons*					
					Amplicon 1		Amplicon 2		Amplicon 3	
CAL1 1:6	PK	-	-	-	-	-	-	-	-	-
CAL1 1:1	GuSCN	-	-	-	-	-	-	-	-	-
CAL2 1:2	PK	-	-	-	-	-	-	-	-	-
CAL2 1:1	GuSCN	-	-	-	-	-	-	-	-	-
CAL2 1:7	PK	-	-	-	-	-	-	-	-	-
CAL9 1:1	PK	-	-	-	-	-	-	-	-	-
CAL9 1:2	GuSCN	-	-	-	-	-	-	-	-	-
CAL20 1:1	PK	-	-	-	-	-	-	-	-	-
CAL20 1:2	GuSCN	+	-	-	-	-	-	-	-	-
XV2 1:2	PK	-	-	-	-	-	-	-	-	-
XV2 1:8	GuSCN	-	+	-	-	-	-	-	-	-
XV3 1:2	PK	-	-	-	-	-	-	-	-	-
XV3 1:1	GuSCN	+	-	-	-	-	-	-	-	-
B20 2:1	GuSCN	+	-	-	-	-	-	-	-	-
B20 2:2	GuSCN	-	-	-	+	-	-	-	-	-
B20 1:1	PK	-	-	-	-	-	-	-	-	-
B20 1:2	PK	-	-	-	-	-	-	-	-	-
B20 3:1	PK	-	-	-	-	-	-	-	-	-
B20 3:2	PK	-	-	-	-	-	-	-	-	-
B41 2:1	GuSCN	-	-	-	+		-	-	-	-
B41 2:2	GuSCN	-	-	-	-	-	-	-	-	-
B41 1:1	GuSCN	+	-	-	-	-	-	-	-	-
B41 1:2	GuSCN	+	-	-	-	-	-	-	-	-
B6 1:1	PK	+	-	-	-	-	-	-	-	-
B6 1:2	PK	+	-	-	-	-	-	-	-	-
B6 2:1	PK	-	-	-	-	-	-	-	-	-
B6 2:2	PK	-	-	-	-	-	-	-	-	-
B37 1:1	GuSCN	-	-	-	-	-	-	-	-	-
B37 1:2	GuSCN	-	-	-	+	-	-	-	-	-

IND1 1:1	PK	-	-	-	-	-	-	-	-	-
IND3 1:2	PK	-	-	-	-	-	-	-	-	-
IND3 1:3	PK	-	-	-	-	-	-	-	+	-
IND4 2:1	PK	-	-	-	-	-	-	-	-	-
IND4 2:2	PK	-	-	-	-	-	+	+	-	-
IND5 1:1	PK	-	-	-	-	-	+	+	-	+
IND6 2:1	PK	-	-	+	-	-	-	-	-	-
IND8 2:1	PK	-	-	-	-	-	+	+	-	+

*The hemi-nested PCR amplicons correspond to the three amplicons initially tested. Each hemi-nest incorporated two overlapping amplicons to ensure the entire fragment is amplified.
Successful amplification is indicated by + and negative amplfication is indicated by -.

X. Appendix 1B

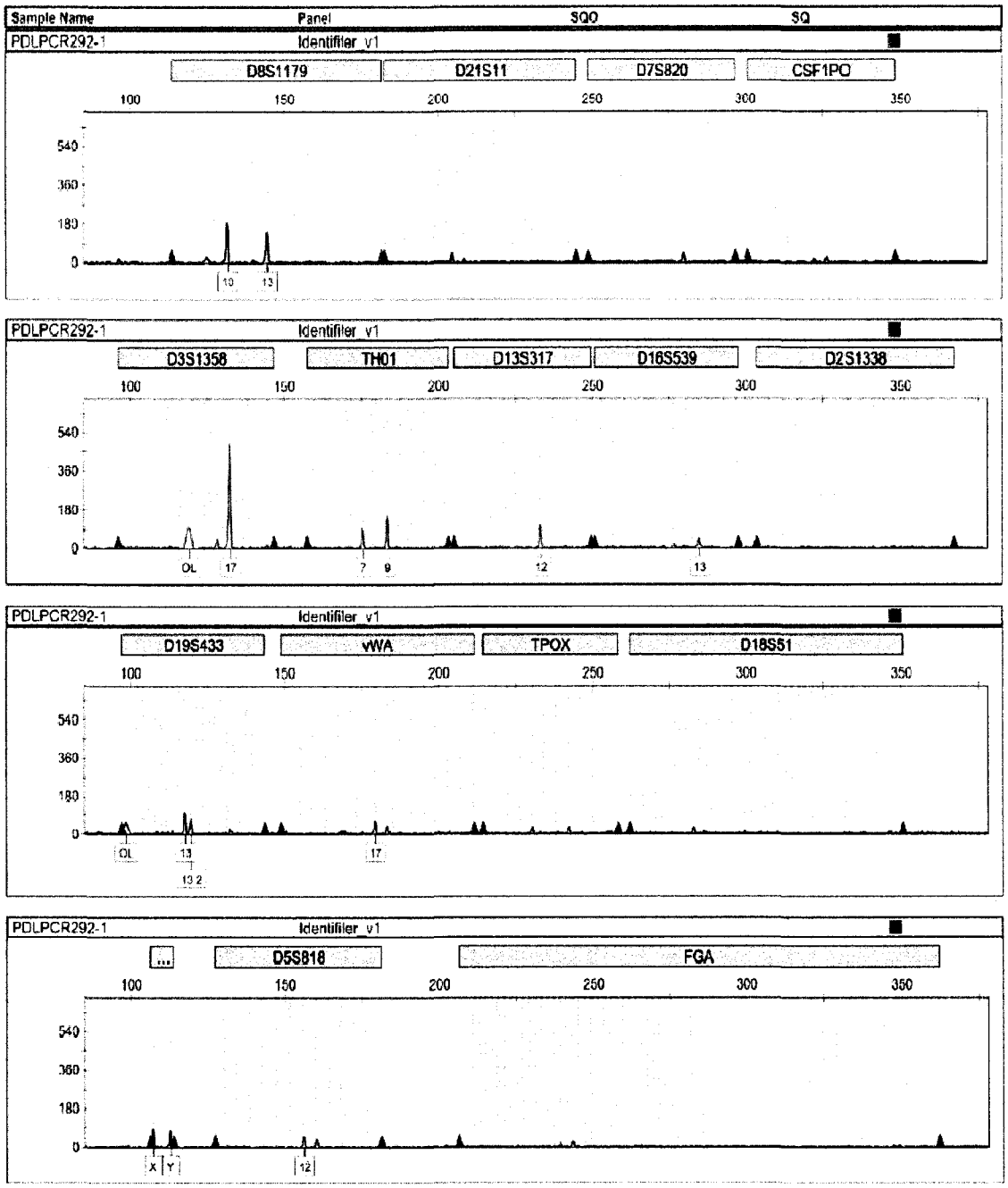
mtDNA amplification of ancient teeth samples extracted using PK targeting different size amplicons and using hemi-nested PCR.

Sample	Amplicon 1 (112bp)	Amplicon 2 (210bp)	Amplicon 3 (259bp)	Hemi-nest PCR Amplicons*					
				Amplicon 1		Amplicon 2		Amplicon 3	
IND3 1:1	-	-	-	-	-	-	-	-	-
IND4 1:1	-	-	-	-	-	-	-	-	-
IND4 1:2	-	-	-	-	-	-	-	-	-
IND2 1:2	-	-	-	-	-	-	-	-	-
IND2 1:3	-	-	+	-	-	-	-	-	-
IND7 1:1	-	-	-	-	-	-	-	-	-
IND7 1:2	-	-	-	-	-	-	-	-	-
IND7 1:5	-	-	-	-	-	-	+	+	+
IND7 1:3	-	-	-	+	-	-	-	-	-
IND8 1:2	-	-	-	+			+	+	+
IND8 1:1	-	-	+	-	-	-	-	-	-
IRI1 1:1	-	+	-	-	-	-	-	+	+
IRI1 1:2	-	-	-	-	-	-	-	+	+
MIA1 1:1	-	-	+	-	-	-	-	NT	NT
MIA1 2:4	-	-	+	-	-	-	-	NT	NT
MIA2 1:1	-	-	+	-	-	-	-	NT	NT
MIA3 1:1	-	-	-	-	-	-	-	NT	NT
MIA1 1:2	-	+	+	-	-	NT	NT	NT	NT
MIA1 2:10	-	+		-	-	NT	NT	NT	NT
MIA2 1:2	-	+	+	-	-	NT	NT	NT	NT
MIA3 1:10	-	-	-	-	-	-	-	-	-
B20 1:1	-	+	-	-	-	-	-	-	-
B20 1:2	-	+	-	-	-	-	-	-	-
B41 3:1	-	+	-	-	-	-	-	-	-
B41 3:2	-	+	-	-	-	-	-	-	-

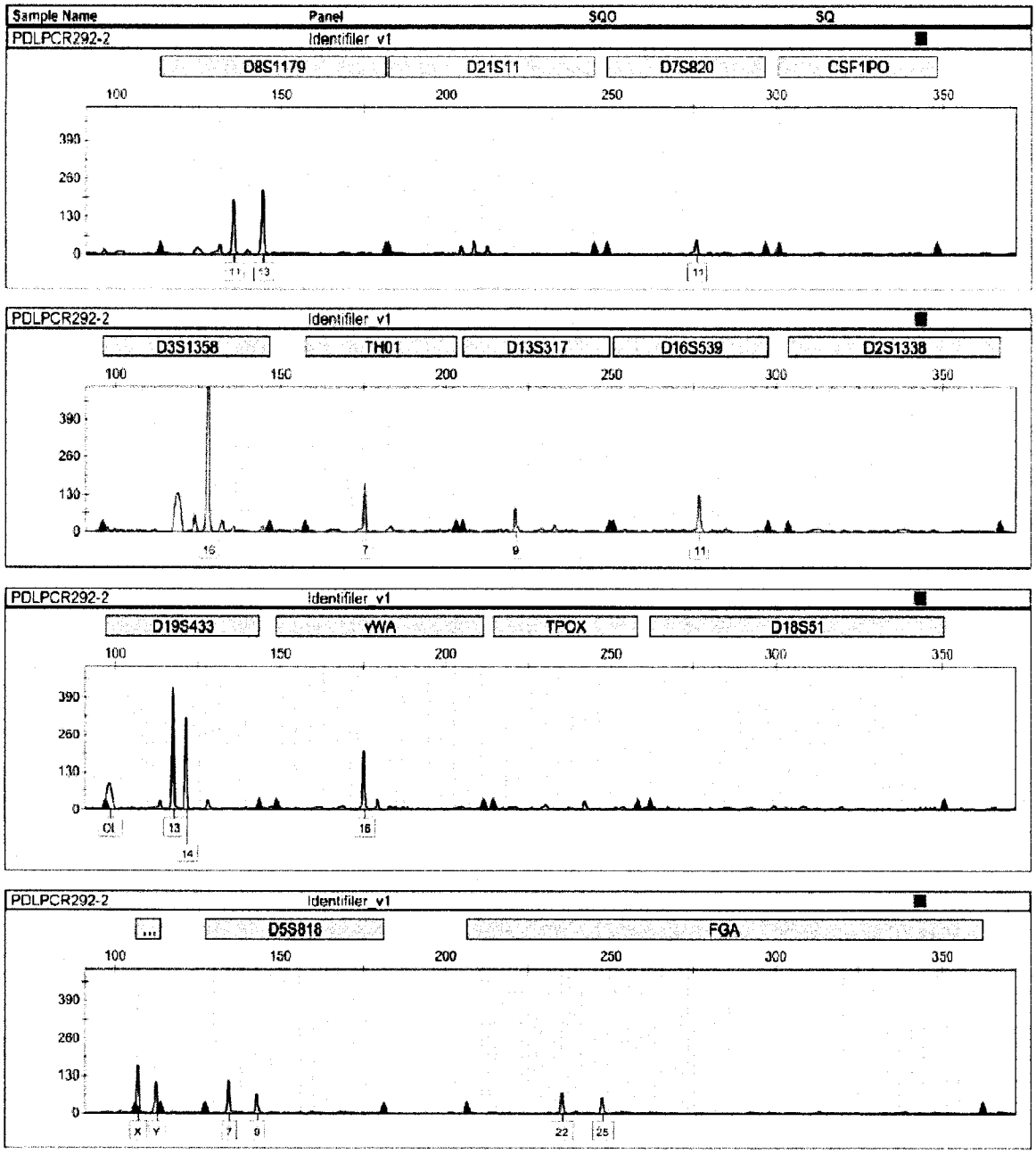
*The hemi-nested PCR amplicons correspond to the three amplicons initially tested. Each hemi-nest incorporated two overlapping amplicons to ensure the entire fragment is amplified.
Successful amplification is indicated by +, negative amplfication is indicated by – and NT indicates the amplification was not performed for the indicated amplicon.

XI.Appendix 1C

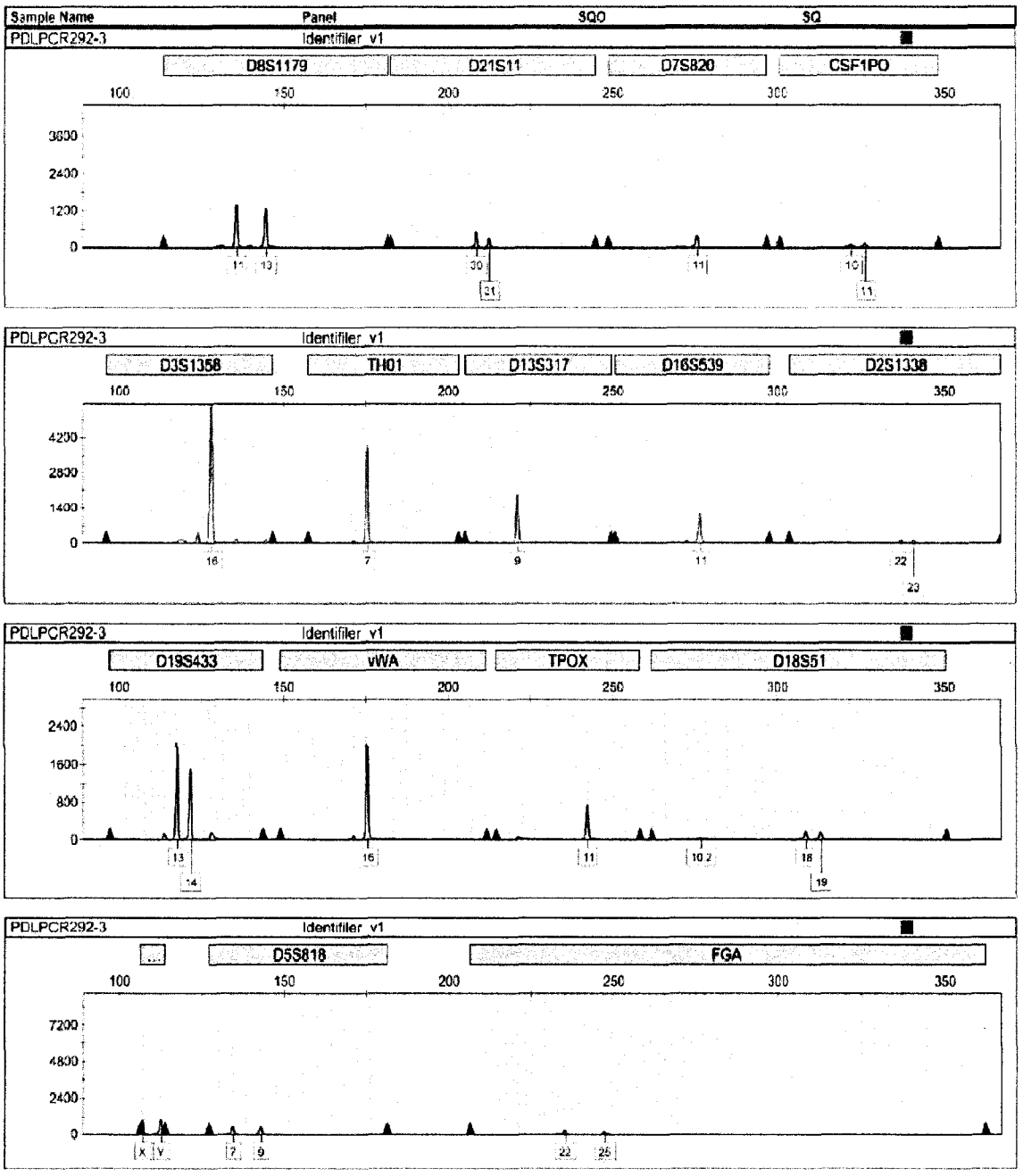
Electropherogram of Autosomal STRs for S31 extracted using PCT technology and the GuSCN extraction buffer.



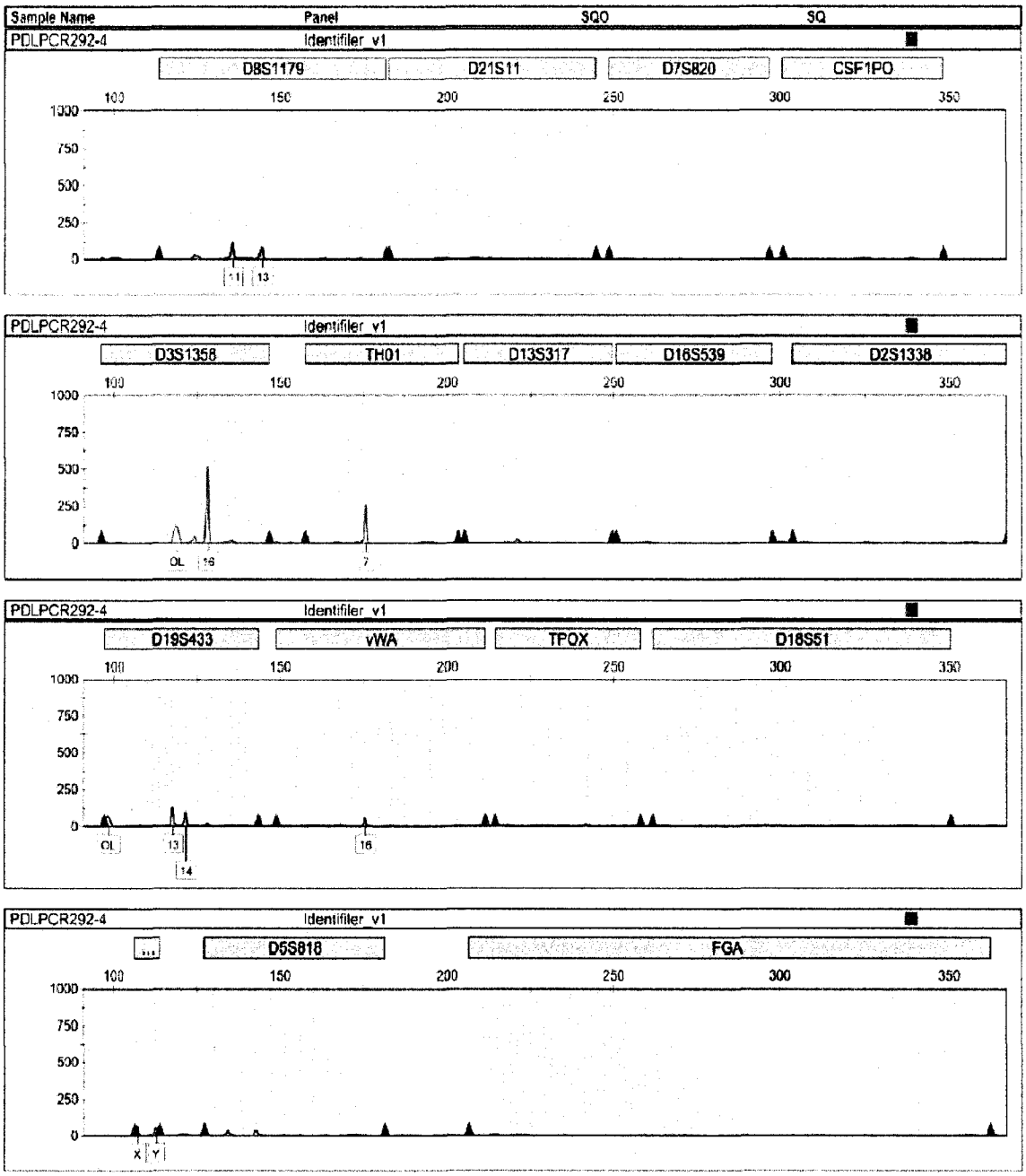
Electropherogram of Autosomal STRs for S32 extracted with PCT technology and the unknown PCT extraction buffer.



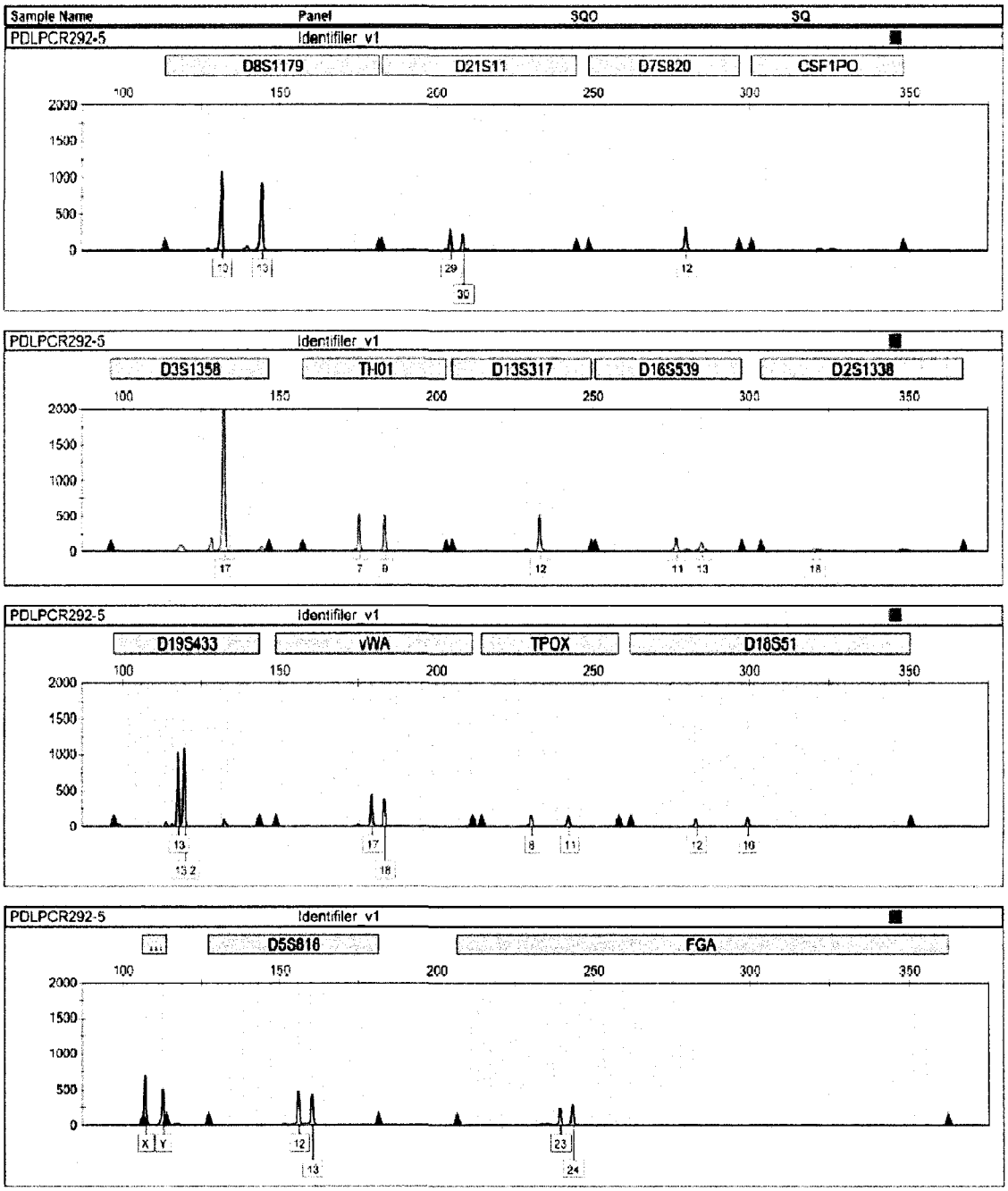
Electropherogram of Autosomal STRs for S30 extracted with PCT technology and the unknown PCT extraction buffer.



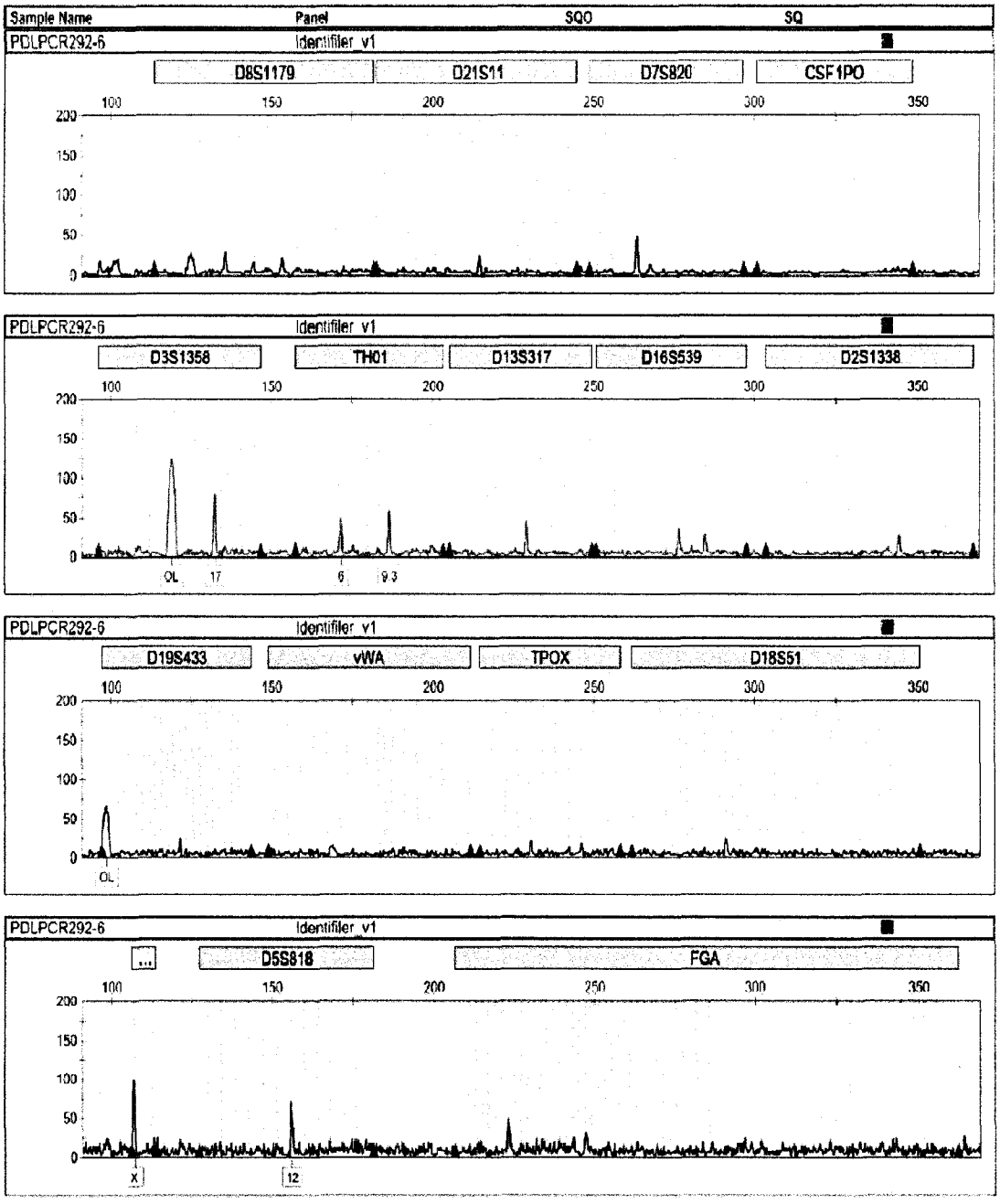
Electropherogram of Autosomal STRs for S29 extracted with PCT technology and the unknown PCT extraction buffer.



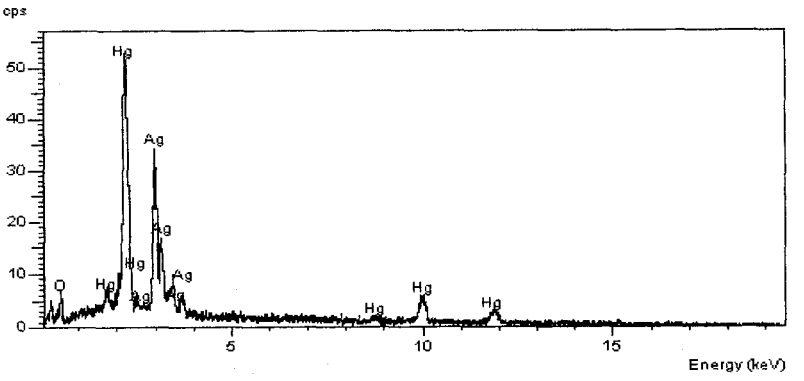
Electropherogram of Autosomal STRs for S35 extracted with PCT technology and the GuSCN extraction buffer.



Electropherogram of Autosomal STRs for the S36 tooth sample extracted with PCT technology and the GuSCN extraction buffer.



XII.Appendix 1D



Elemental analysis spectrum of the silver dental amalgam Alloy produce using Scanning Electron Microscopy (SEM) and a Energy Dispersive X-Ray Analyzer (EDXA) spectrometer. The spectrum shows the primary two components detected are silver and mercury.