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Nature and structure of DNA through the process of degradation

Beaulne, Christine

The Nature and Structure of DNA Through the Process of Degradation

By

Christine Beaulne

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Declaration

I declare that the work presented in this thesis is original, except where otherwise acknowledged and submitted to fulfill the requirements of a Masters of Biology at Lakehead University.

Christine Beaulne

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DNA	Deoxyribonucleic acid	EB	Elution Buffer
nDNA	Nuclear DNA	dATP	deoxyadenine triphosphate
mtDNA	Mitochondrial DNA	dCTP	deoxycytosine triphosphate
A	Adenine	dGTP	deoxyguanine triphosphate
T	Thymine	dTTP	deoxythymine triphosphate
C	Cytosine	dNTP	deoxynucleotide
G	Guanine	DA	Dalton
bp	Base pair	mRNA	Messenger RNA
rRNA	Ribosomal RNA	cDNA	Complementary DNA
tRNA	Transfer RNA	UV	Ultraviolet
HVR	Hypervariable region	STR	Single tandem repeat
aDNA	Ancient DNA	FRET	Fluorescent resonant energy transfer
PCR	Polymerase chain reaction		
HPLC	High performance liquid chromatography	PAGE	Polyacrylamide gel electrophoresis
ROS	Reactive oxygen species	ddNTP	dideoxynucleotide
e_{aq}^-	Solvated electrons	ET	Energy Transfer
AP site	Apyrimidinic/Apurinic site	CE	Capillary electrophoresis
h	critical distance	dsDNA	Double stranded DNA
DMSO	Dimethyl Sulfoxide	ssDNA	Single stranded DNA
Tyr	Tyrosine	PPi	Pysophosphate
GuSCN	Guanidinium thiocyanate	RFU	Relative fluorescence units
TE	Tris EDTA	BSA	Bovine Serum Albumin
gp32	T4 gene 32 protein	EDTA	Ethylene-diamine-tetra-acetic-acid
NADPH	Nicotinamide adenine nucleotide phosphate		

VI. ABSTRACT

The identification and characterisation of DNA damage is of great importance to molecular research and has significant implications for clinical and forensic fields. From the moment of cell death, DNA incurs many forms of damage, chemical and physical modifications, and fragmentation. This occurs primarily during the biological decay of a sample, but can then stabilise depending on the depositional environment. The damage and degradation continues if there is disruption or excavation of the sample through to the molecular analyses and storage in the laboratory. Fluorescent labelled multiplex PCR coupled with real time PCR has been developed to characterise secondary DNA damage incurred during laboratory analyses. This has been investigated by analysis of the stability and degradation of DNA placed under different storage conditions. Parameters, including storage buffer, storage temperature, and DNA exposure to repeated freeze/thaw cycles, were examined and analysed using a multiplex fragmentation analysis system. It was shown that finding the optimal storage conditions of DNA dramatically increased the stability and longevity of molecules for PCR analyses. Assessment of the quantity and quality of DNA from ancient or degraded samples allows for method improvements, provides indicators for further analysis, and can provide authentication of ancient or degraded DNA by quantity, quality and fragmentation. This assessment of DNA species in archaeological and forensic samples provides integral information for fast and resourceful use of limited and low-copy number DNA samples.

VII. INTRODUCTION

i. Background of the Nature and Structure of DNA

Within most cells of all organisms, an organic polymer, the building blocks of life deoxyribonucleic acid (DNA), is found. Animal cells are made up of various organelles of which, in most cases, only two contain DNA: the nucleus and mitochondrion (Hummel, 2003). DNA found in the nucleus is termed nuclear DNA (nDNA) and that found in the mitochondrion is referred to as mitochondrial DNA (mtDNA). Together, nDNA and mtDNA code for all genetically determined traits ranging from metabolism to immunogenic characteristics (Hummel, 2003). Although there are other forms of DNA within a cell, such as extra-chromosomal mini-circles, their activity and function are still unclear. These other forms of DNA have not yet been used for individual characterisation or identification (Pasion *et al.*, 1994). DNA is composed of three biochemically distinct components: phosphates, sugars and nitrogenous bases. The phosphates and sugars, which make up the backbone, remain constant in all forms of DNA in all organisms across the world's population. However, the order of the nucleobases varies, leading to different genes, alleles and generate genetic variation. Nucleobases are nitrogenous bases paired up with a nucleotide, either a purine, adenine (A) or thymine (T) or a pyrimidine, cytosine (C) or guanine (G). The combination of these four nucleobases determines the precise function and coding capacity of the DNA (Luftig, 2001). The nucleobases bind to each other in a complimentary pattern, G and C, the pyrimidines, bind to each other through the formation of three hydrogen bonds while A and T, the purines, bind to each other through the formation of two hydrogen bonds. This ultimately leads to the formation of a stable double stranded conformation of the DNA helix (Watson *et al.*, 1953). As seen in Figure I both strands of DNA

exhibit an anti-parallel directionality as determined by the positions of the carbon atoms in the sugar molecules (5' and 3') (Hummel, 2003). Nucleotide sequences are listed according to internationally agreed nomenclature from the 5'-end to the 3'-end.

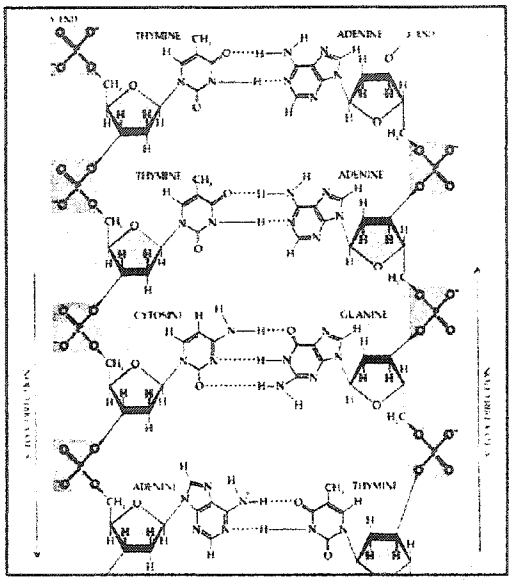


Figure I - DNA Structure
(Raven et al., 1992)

a. Nuclear DNA

Nuclear DNA is composed of a double stranded string of nucleotides, up to 3 billion nucleotides long in human nuclei, which is organised into 13 chromosomes (Luftig, 2001). However variations and small changes can occur in a sequence of the linear chain of DNA that makes up the chromosomes causing random variations (Luftig, 2001). This randomness means that no two individuals have the same sequence combination of bases within their nDNA sequence allowing us to easily differentiate between two individuals (with the exclusion of some twins).

The strands of nuclear DNA must form a compact DNA-protein complex, termed nucleosome, to suit the small nucleus of a cell. A nucleosome consists of double stranded DNA

(dsDNA) wrapped around an octamer of small basic proteins called histones; 146bp are wrapped around the core and the 50bp form a linker region to the next nucleosome causing negative supercoiling (Alberts *et al.*, 1994). See structural representation in Figure II. The supercoiling of multiple nucleosomes results in the formation of chromatin, ultimately leading to the commonly known chromosome structure (Alberts *et al.*, 1994). Nuclear genetics are inherited through Mendelian genetics or the transmission of a double set of 23 homologous chromosomes in the diploid status; each set inherited through the haploid gametes from either parent (Hummel, 2003).

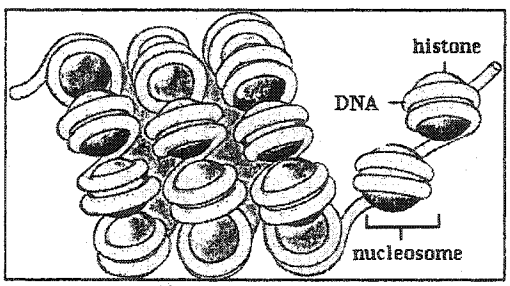


Figure II - Nucleosome

b. Mitochondrial DNA

The mitochondrion, a semi-autonomous organelle, exists as both a kidney-shaped organelle and a dynamic, reticular network within the cytoplasm (Poulton, 1995; Yaffe, 1999). The mitochondrion plays a vital role in cellular metabolism as it is the site of the oxidative phosphorylation pathway (Wallace *et al.*, 1995). A cell can contain anywhere from several hundred to ten thousand mitochondria depending on its tissue type, with more mitochondria found in tissues with high-energy demands. Human mitochondrial DNA, making up 1% of a cell's total DNA, is a double stranded, closed circular molecule made up of 16,569bp coding for a total of 37 genes (Berger *et al.*, 2001; Nass *et al.*, 1996). These 37 genes include 13 essential subunits of the oxidative phosphorylation enzymes as well as two ribosomal RNA (rRNA) and

22 transfer RNA (tRNA) necessary for intra-mitochondrial protein synthesis (Wallace, 1992). Within these thousands of base pairs there are several non-coding regions, referred to as control regions, showing a comparatively high rate of sequence variations or polymorphisms (Hummel, 2003). The control region, made up of about 900 bp, contains the hypervariable regions I, II and III (HVR I, HVR II and HVR III). HVR I and HVR II together consist of approximately 600 bp (Hummel, 2003). HVR I is commonly defined as the region between the nucleotide positions 16,024 and 16,365, HVR II is found between nucleotide positions 73 to 340 and HVR III is defined as bases 438 to 574 (Lutz *et al.*, 2000). HVR I has 88 polymorphic sites within a total length of 342 bp (26 %), HVR II has 65 polymorphic sites within a total length of 268 bp (24 %) while HVR III has 25 polymorphic sites within a total length of 137 bp (18 %) (Lutz *et al.*, 2000). Other control regions of the mitochondrial genome are less studied due to their lack of variability.

Mitochondrial DNA is present as a single copy in each somatic cell and is usually inherited through the female of the previous generation. This occurs because oocytes contain a full compliment of mitochondria while the sperm has very few mitochondria, all of which are located in the tail which does not penetrate the oocyte because it is lost at the moment of fertilisation (Hummel, 2003). This mechanism leads to all maternally related individuals or members of a maternal lineage having the same mtDNA sequence, revealing the same polymorphisms and defined as the same mitochondrial DNA type or haplotype (Hummel, 2003). However, a phenomenon known as heteroplasmy can occur. Heteroplasmy is where two or more mtDNA types are present within the one cell. There is believed to be a 2.5 % rate of heteroplasmy within the general populations (Gocke *et al.*, 1998). There are a number of mechanisms that can generate heteroplasmy; one of these mechanisms is the paternal inheritance

of mtDNA from the sperm. This can occur in the event that the sperm tail does penetrate the oocyte meaning that the developing individual displays two or more mitochondrial types or a mosaic in its mitochondria leading to a sequence polymorphism (Gill *et al.*, 1994). Additional sources of sequence heteroplasmy include inheritance from the mother or acquisition of a heteroplasmy by an individual though mutation or change. Other mutational events, such as a nuclear insertion may complicate mtDNA analysis, however these events are beyond the scope of this research.

The numerous nucleotide changes over time within a DNA sequence is the mechanism of mutation that results in the evolution of new species. DNA sequences may undergo numerous nucleotide changes over time resulting in the evolution of a species. Like all forms of DNA, the mitochondrial DNA can mutate and accumulate these mutations over time. The mtDNA has a distinctively rapid mutation rate and rate of sequence evolution. The mtDNA is said to be evolving 10 to 20 times faster than nuclear DNA (Wallace *et al.*, 1995). Through extensive research, the process by which mtDNA sequences accumulate differences over time has been deemed very complex and beyond the scope of this project. The mitochondrion also has varying mutation rates within its own genome. The control region is evolving 10 times faster than the coding regions and the HVR I and HVR II are sufficiently variable to distinguish between two non-maternally related individuals (Butler *et al.*, 1998; Howell *et al.*, 1996). A sequence variability of about 3 % between individuals allows for distinction between maternal lineages (Stoneking, 2000). These differences or polymorphic sites are not uniformly distributed throughout the mitochondrial genome but rather are thought to be found in clusters known as hot spots (Hummel, 2003).

ii. Low Copy Number DNA

Low copy number DNA also referred to as ancient DNA (aDNA) or degraded DNA is defined as having a minimal amount of starting DNA or low copy number of DNA template. The nature of these molecules of DNA is generally fragmented. Credible existence of analytical quantities of degraded or ancient DNA was first demonstrated in 1984 by Russell Higuchi, a member of Allan Wilson's group, through cloning of a fragment of DNA from a 140-year-old museum quagga skin, an extinct member of the horse family, to obtain two short mtDNA sequences (Higuchi R.G *et al.*, 1984). Subsequently, Svante Pääbo sequenced an *Alu* element and flanking DNA from the 2400-year old mummy of a young Egyptian boy (Paabo, 1985). These two findings generated much interest in general however they did not spark an interest in the field of aDNA. It was assumed that these were cases of exceptional preservation and that under normal circumstances DNA would degrade rapidly after death (Brown *et al.*, 1994). Most thought that such studies could be conducted only on specimens that retained a high degree of macroscopic biological integrity such as museum skins, natural and artificial mummies, frozen mammoths and some plant remains (Brown *et al.*, 1994). At the time, it was also thought that these samples would provide a limited amount of sequence data since the DNA molecules would have undergone extreme physical and chemical damage impeding the cloning process (Brown *et al.*, 1994).

The 1985 discovery of the polymerase chain reaction rekindled interest in the study of ancient DNA. PCR circumvents the difficulties involved in cloning aDNA by allowing direct, amplification of single DNA fragments (Brown *et al.*, 1994). PCR also specifically targets the DNA sequence of interest, avoiding the need for tedious screening of a clone library (Brown *et al.*, 1994). Collaboration between Pääbo and Wilson's research group, once again provided the

first meaningful outcomes from the application of PCR to the field of aDNA. Examples of such outcomes are that using a 7000-year-old human brain from a peat bog in Florida (Paabo *et al.*, 1988), followed with the 4000-year old liver of an Egyptian mummy (Paabo, 1989) and ultimately with museum skins of the extinct marsupial wolf (Thomas R.H *et al.*, 1989). Despite these findings, it was generally believed that ancient DNA could only be found in relatively young samples having undergone some type of preservation, be it natural or artificial, such as mummification (Brown T.A *et al.*, 1994). In 1990, two significant breakthroughs occurred that abolished these tenets. First, three groups simultaneously discovered that ancient DNA could be amplified from preserved hard tissues such as bones and teeth (Hagelberg E *et al.*, 1989; Hanni C *et al.*, 1990; Horai S *et al.*, 1989). This was a significant finding since these specimens were widespread throughout archaeological and palaeontological records. Secondly, a chloroplast DNA sequence from a 16-million-year-old *Magnolia* leaf preserved in anoxic deposits at Clarkia, Idaho was published shattering the assumed age limit for ancient DNA preservation (Golenberg E.M *et al.*, 1990). Despite its controversial nature, due to the difficulty in reconciling the existence of such old DNA fragments with the observed degradation rates for polynucleotides in aqueous solution (Lindahl, 1993), this report lead to an imperative reassessment of timescale for DNA preservation. This finding also sparked interest and competition among researchers to discover ancient DNA in other types of million-years-old material. The validity of much of this earlier work is still in contention however a great deal of verification and authentication has yet to be provided by the current field of ancient or degraded DNA research.

The age limits for ancient DNA have not yet been adequately defined for any type of biological tissue with the exception of amino acid racemisation models of degradation. However

since 1984, the list of possible ancient DNA sources have grown to include hair (Higuchi *et al.*, 1988), fingernails (Kaneshige *et al.*, 1992), faeces (Hoss *et al.*, 1992), and all other kinds of preserved organic material excluding fossilised material. Ancient DNA has also been isolated from biological residues having been in contact with non-biological material and preserved over time, including stones tools (Loy, 1992) and soil (Zhou *et al.*, 1996). Ancient DNA has now matured into a research tool relevant to disciplines as diverse as forensic science, archaeology, conservation biology, taxonomy, zoology and medical science.

The minimisation of DNA loss during extraction from ancient materials is vital in the analysis since it is suspected that only small amounts of aDNA are present (Brown *et al.*, 1994). This amount of DNA present in degraded samples has yet to be confirmed due to the difficulties in quantification of ancient or degraded DNA. These difficulties are due to the presence of possible fungal and bacterial DNA, which clouds the quantification results from methods including spectroscopy or estimations by densitometry (Brown *et al.*, 1994), when applied to ancient materials. Quantification performed using hybridisation analyses directed specifically at endogenous DNA have yielded amounts of 1-50 ng/g for archaeological bones and plant remains (Brown *et al.*, 1994). Nevertheless, these measurements relate to crude extracts in which the bulk of the hybridising material comprises short, insignificant oligonucleotides providing very little noteworthy data (Brown *et al.*, 1994). An established post extraction quantification protocol, perhaps using real time PCR, would be more accurate and may simplify the quantification of DNA in the analysis of aDNA.

Authenticity of results from aDNA analysis has been based on numerous features including the fragment length of recovered DNA. The fragmentation can vary between species of DNA, between archaeological samples and within the same sample. In nDNA the fragments

are usually found between 200-400bp as this is the length of DNA wrapped around each histone (Hewish *et al.*, 1973). Despite the detection of the 3.4 kb fragment of mummy DNA reported by Pääbo in one of the founding ancient DNA journal articles (Paabo, 1985), researchers profess that it is difficult to amplify authentic fragments longer than 200-300 bp from aDNA extracts (Handt *et al.*, 1994),(Hardy *et al.*, 1994; Krings *et al.*, 1997). These values relate to single stranded polynucleotides produced by denaturation of the ancient DNA and do not account for the possible presence of longer, partially double stranded molecules made of short overlapping single stranded polynucleotides present in pre-natured extracts (Brown *et al.*, 1994).

However in the case of mitochondrial DNA the fragmentation is caused by its inferior protection against degradation due to the lack of histone structures, which when present are thought to protect the 200 bp wrapped around them from fragmentation (Hummel, 2003). Ancient DNA research continues to focus on mtDNA due to its high copy number. Those making the above assumptions often explain amplification of long fragments of aDNA as non-target or exogenous DNA. However, these postulates do not account for the presence of fragmented modern DNA appearing as contamination (Hummel, 2003)

In 1993, Thomas Lindahl published an article in *Nature* seeding doubt that ancient DNA can really survive over long periods of time. Lindahl (1993) pointed out the discrepancy between the degradation rate of DNA in an aqueous solution and the existence of intact ancient DNA. Briefly, the accumulation of heat-induced DNA damage and other forms of chemical damage should cause a gradual degradation of aDNA, leaving only very short fragments after several thousand years at moderate temperatures (Lindahl, 1993). The extrapolation from a degradation rate measured in aqueous solution to the degradation of DNA in a complex depositional environment may not be an accurate correlation, but overall Lindahl proposes that

the existence of ancient DNA greater than 20,000 to 40,000 years old is not possible (Lindahl, 1994). The only possible evidence comes from HPLC and biochemical examination of extracts of dried skins and mummified remains (Paabo, 1989) suggesting that the aDNA in these materials had indeed undergone extensive chemical modification. Unfortunately, the degradation products were not identified prohibiting direct comparison with the degradation pathways predicted by Lindahl (Brown *et al.*, 1994). Confirmation or reassessment of these pathways would require chemical examination of ancient DNA by more sensitive and discriminatory methods (Brown *et al.*, 1994).

iii. DNA Damage

There are a multitude of biochemical pathways within a cell that can lead to DNA damage or degradation. These include natural enzymatic mechanisms causing severe DNA fragmentation, natural and induced oxidation reactions causing strand breakage and DNA base modifications, hydrolytic reactions leading to deamination and depurination, DNA-protein-cross-linking (DPC) and external physical force.

All cells possess natural enzymatic mechanisms within themselves with the sole purpose of protection and proliferation of the organism. These mechanisms mostly include the destruction of an intruding particle but can also include self-destruction of the cell. The latter is the case in apoptosis; morphologically distinct cell suicide is initiated when a cell within a multicellular organism is compromised beyond repair (Kerr *et al.*, 1972). This mechanism differs greatly from necrotic cell death since necrosis is a form of cell death resulting from acute tissue injury (Wikipedia, 2004).

In Apoptosis, the impaired cell is sacrificed for the greater good of the whole individual. An apoptotic cell shrinks, cytoskeleton collapses, nuclear envelope disassembles, chromatin condenses into apoptotic bodies, the DNA fragments and protein degrades (Lu *et al.*, 2004). Any threat to the genomic integrity of a cell triggers the release of apoptotic proteases, caspases, which specifically cleave all DNA leading to its orderly fragmentation (Didenko *et al.*, 2003). In most cells, fragmentation occurs in two stages: first, during the early stage of apoptosis, chromatin is cleaved into large molecular weight chromatin DNA fragments (about 50kb) (Lu *et al.*, 2004) and secondly the chromatin fragments are cleaved into smaller fragments by nuclease. The mechanism for this first stage of DNA remains a mystery (Lu *et al.*, 2004). In the late stage of apoptosis, the fragmented chromatin DNA is digested further into short oligonucleosomal DNA ladder-like fragments by the nucleases (Lu *et al.*, 2004). Over twenty nucleases have been identified and implicated in the late stage of DNA degradation (Lu *et al.*, 2004). The start command for programmed cell death may come from the cell itself or the surrounding tissue. Failure to initiate apoptosis in a damaged cell results in its uncontrollable division, developing into a tumour (Hengartner, 2000).

Necrosis is defined as an unprogrammed death of living tissue and is thus a consequence of damage rather than a defence mechanism. The most common beginnings of necrosis are due to injury, infection, cancer, infarction, and inflammation. Throughout the necrotic pathway, DNA is randomly cleaved through the activation of non-specific lysosomal nucleases caused by the disintegration of cellular membranes (Didenko *et al.*, 2003).

Recent studies have aimed to differentiate between apoptotic and early stage necrotic cell death using a combination of recognised chemical processes such as TUNEL, Klenow enzyme-based labelling and *in situ* ligation. This differentiation relies on the distinct structural DNA

damage cause by each apoptosis and necrosis (Didenko *et al.*, 2003). Apoptotic cells were found to have 5' overhangs caused by 3'→5' exonuclease activity, double-strand DNA breaks with single nucleotide 3' overhangs and blunt ended 5' phosphorylated DNA breaks both attributed to the presence of caspase-activated deoxyribonuclease or Dnase1-like endonucleases (Didenko *et al.*, 2003). On the other hand, early necrotic cells displayed only the 5' overhangs, no blunt ended breaks or 3' overhangs (Didenko *et al.*, 1996). During the necrotic process, no new enzymes are synthesised and thus the creation of these overhangs can only be attributed to pre-existing enzymatic activity, particularly 3' to 5' exonucleolytic activity (Didenko *et al.*, 2003). These studies revealed that both apoptotic and necrotic cell death are non-random DNA cleavage resulting in very specific, discernible DNA structure (Didenko *et al.*, 2003).

A second major source of DNA damage is oxidative stress, which can lead to DNA lesions, mostly modified bases, but can also lead to strand breaks and abasic sites. All aerobic cells produce oxygen-derived species, or Reactive Oxygen Species (ROS), a collective term used to include oxygen radicals [superoxide ($O_2^{\bullet-}$), hydroxyl ($OH^{\bullet}$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$)] and certain non-radicals that are either ionising agents and/or are easily converted into radicals, such as HOCl, ozone (O_3), peroxynitrite ($ONOO^-$), singlet oxygen and H_2O_2 (Halliwell *et al.*, 1989; Wiseman *et al.*, 1996). ROS are formed by several different mechanisms such as the interaction of ionising radiation with biological molecule, the Fenton reaction, fabrication by dedicated enzymes in phagocytic cells like neutrophils (NADPH oxidase) and macrophages (myeloperoxidase) and creation as unavoidable by products of cellular respiration (Kimball, 1994). Base modification is a hindrance in the study of DNA since these new bases are not recognised by DNA polymerase and thus are not amplified in a standard PCR.

Oxidative damage occurs through the formation of $OH^{\bullet}$ via two specific pathways: γ-radiolysis and the Fenton reaction. Oxidative damage caused by such free radicals in vivo has been implicated as a factor in the incidence of biological processes such as mutagenesis, carcinogenesis and ageing (Halliwell *et al.*, 1989). In vivo, free radicals are generated by endogenous and exogenous and cause damage to biological molecules including DNA (Halliwell *et al.*, 1991b). A free radical is a cluster of atoms which contains an unpaired election in its outermost shell of electrons (Kimball, 1994). This configuration is highly unstable causing radicals to react with other molecules or radicals in order to achieve 4 pairs of electrons in their outermost shell, a stable conformation (Kimball, 1994). Hydroxyl radicals produce a number of lesions in DNA and in nucleoproteins such as base lesions, sugar lesions, single-strand breaks, double-strand breaks, abasic sites and DNA-protein cross-links by a variety of mechanisms (Dizdaroglu, 1992). Free radicals attack DNA in highly specific locations, as seen in Figure III (Schafer, 1997). Ionising radiation-generated e_{aq}^{-} and H atoms can also modify DNA bases (Dizdaroglu, 1992).

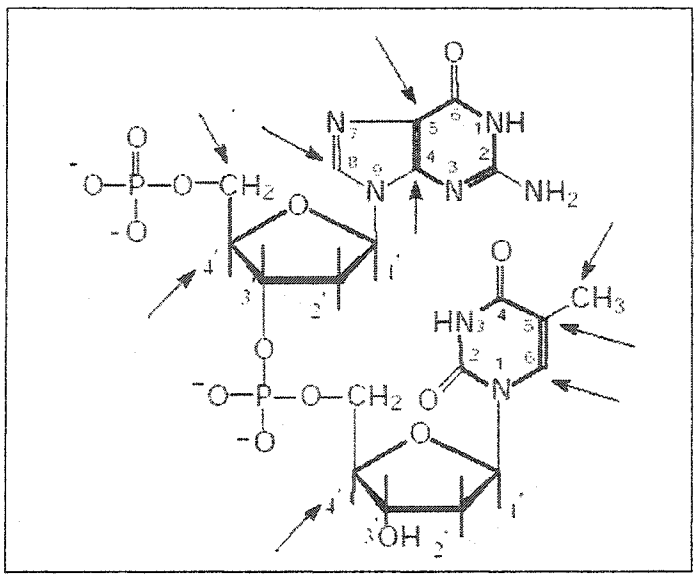


Figure III - Free Radical Hot Spots.
(Schafer, 1997)

When exposed to ionising radiation, water reacts to create hydroxyl radicals, solvated electrons (e_{aq}^-) and H atoms. A superoxide anion ($O_2^{\bullet-}$) is produced, at a diffusion-controlled rate, when e_{aq}^- and H atom react in the presence of oxygen (Dizdaroglu, 1992). Hydrogen peroxide is produced in all aerobic cells as a result of normal cellular metabolism and is ubiquitous in biological systems (Halliwell *et al.*, 1989). The superoxide reacts with cellular hydrogen peroxide in the Haber-Weiss reaction (Equation 1), leading to the production of hydroxyl radical ($^{\bullet}OH$) (Frelon *et al.*, 2003).

Equation 1

$$O_2^- + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH$$

The hydroxyl radical is known to cause about 70% of radiation-induced DNA damage in oxic cells (Roots *et al.*, 1972). The following modified bases were identified in a linear radiation dose-yield relationship: 5,6-dihydrothymine, 5-hydroxy-5-methylhydantoin, 5-hydroxyhydantoin, cytosine glycol, 5-hydroxy-6-hydrothymine, 5-hydroxy-6-hydrocytosine, 5-(hydroxymethyl) uracil, thymine glycol, 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine (FapyAde or FapyGua), 8-hydroadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine and 8-hydroxyguanine (8-OH-Gua) (Dizdaroglu, 1992). The structure of these modified bases, along with some of less common altered bases, is found in Figure IV. According to Lindahl (1993), the major damaging base lesion generated by hydroxyl radicals is 8-hydroguanine. This modified product base-pairs preferentially in solution with adenine rather than cytosine, thus generating a transversion mutation after DNA replication (Lindahl, 1993). Guanine-derived bases amount to approximately 45 % of the net total yield of modified bases suggesting a high reactivity of $^{\bullet}OH$ toward the guanine residues in cells (Dizdaroglu, 1992). *In vivo*, cells have natural repair mechanisms, involving specific DNA glycosylases, which excise the lesion caused by a modified base (Lindahl, 1993).

The metabolism of reactive oxygen is delegated to the mitochondria shielding the cell nucleus from severe oxidative damage. Consequently, mtDNA is highly sensitive to oxidative damage as proven by the presence of elevated levels of 8-hydroxyguanine compared to that isolated in nuclear DNA (Lindahl, 1993). This condition is further aggravated by the lack of histones in the mtDNA structure, which otherwise quench the generation of oxygen radical-induced lesions (Lindahl, 1993). The excessive oxygen radical-induced DNA damage in mitochondria is suggested to be responsible for the high mutation rate, rapid evolution and decline with age of this organelle, as well as contributing to several maternally inherited human degenerative diseases (Linnane *et al.*, 1989; Wallace *et al.*, 1988). Studies also show that the number of 8-OH-Gua lesions augment significantly when isolated mitochondria are exposed to oxidative stress (Richter *et al.*, 1988).

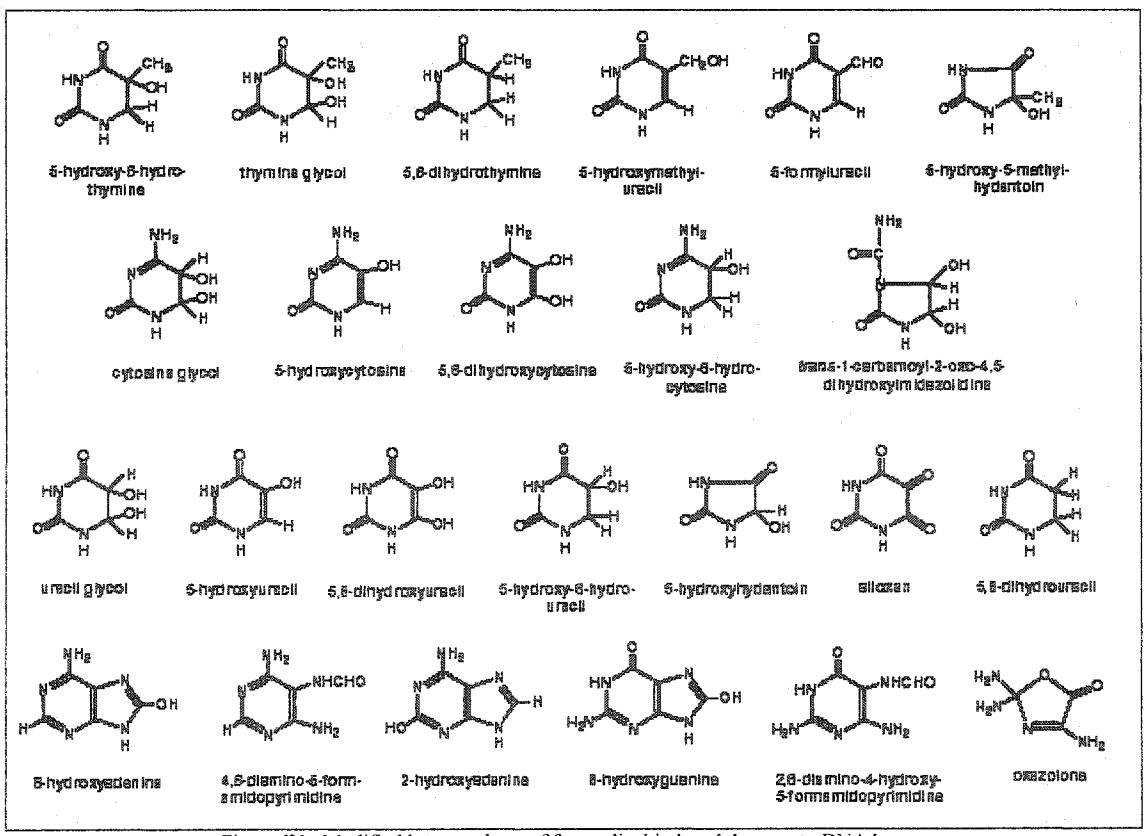


Figure IV - Modified base products of free radical induced damage to DNA bases.
(Dizdaroglu *et al.*, 2002)

The distribution of radical induced modified bases is known to be sensitive to a series of parameter involved in the degradation reaction (Frelon *et al.*, 2003). First the nature of the oxidising species is expected to play a major role. For example, hydroxyl radical reacts with all nucleobases at several positions of the purine and pyrimidine rings. This leads to the formation of at least 60 DNA lesions, including hydroperoxides and diastereoisomers of several products, identified in monomeric model systems (Frelon *et al.*, 2003). In contrast, singlet oxygen ($^1O_2$) specifically reacts with guanine, leading to the overwhelming formation of 8-oxo-dG (Frelon *et al.*, 2003). The highly specific attack of singlet oxygen is due to positive charge migration along the DNA to the component with the lowest oxidation potential, guanine (Frelon *et al.*, 2003). Other parameters involved in the degradation reaction involve oxygen, pH, reducing species and structural factors (Frelon *et al.*, 2003). These parameters are likely to modulate the chemical reaction of initially produced radicals (Frelon *et al.*, 2003).

Hydroxyl radicals may also be produced through the Fenton reaction (Equation 2), the cyclic reduction of hydrogen peroxide, the dismutation product of superoxide anion, by metal ions.

Equation 2

$$M^{nt} + H_2O_2 \rightarrow M^{n+1} + OH^- + {}^{\cdot}OH$$

Hydrogen peroxide is especially damaging since it can diffuse long distances in cells to reach DNA and can also cross biological membranes unlike $^{\bullet}OH$ which has a short diffusion distance in cells due to its high reactivity toward organic molecules and thus reacts at or close to its site of formation (Dizdaroglu, 1992). Transition metal ion-catalysed conversion of H_2O_2 is responsible for most of the $^{\bullet}OH$ generated in vivo, except during exposure to ionising radiation (Halliwell *et al.*, 1989). The effects of H_2O_2 in the presence of various transition metal ions such as Fe(III), Cu(II), Co(II) and Ni(II) on isolated chromatin were studied. The main focus being if transition

metal ions capable of converting H_2O_2 to $^{\bullet}OH$ are in close proximity to DNA *in vivo* it allows for the Fenton reaction to occur. Iron and copper, present in the nucleus, are carefully sequestered into proteins, such as ferritin, as a protective measure (Wiseman *et al.*, 1996). DNA is a powerful chelating agent for transition metal ions, however an oxidative stress may cause the release of intracellular iron and/or copper ions into forms that could then bind to DNA (Halliwell *et al.*, 1991a). The level of damage through the Fenton pathway was found to be directly dependent on the hydrogen peroxide concentration (Frelon *et al.*, 2002). The following modified bases were identified: 5-hydroxy-5-methylhydantion (5-OH-5-MeHyd), 5-hydroxyhydantoin, cytosine glycol, 5-(hydroxymethyl)uracil (HMU), thymine glycol, 5,6-dihydroxycytosine, 4,6-diamino-5-formamidopyrimidine (FapyAde), 8-hydroxyadenine, 2-hydroxyadenine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 8-hydroxyguanine (8-OH-Gua) (see Figure IV) (Dizdaroglu *et al.*, 1991b). These modified bases are typical products of site specific reactions of $^{\bullet}OH$ with DNA nucleotides (Dizdaroglu, 1992). Dizdaroglu, *et. al.* (1992) demonstrates that more damage occurred when H_2O_2 was in the presence of Cu(II) than in the presence of Fe(III). Further studies of modified base distribution by Frelon, *et. al.* (2003) suggest the Fenton reaction generates singlet oxygen as the predominant reactive species, as indicated by the overwhelming production of FapyGua and 8-oxodGuo. Hydrogen peroxide alone does not produce the herein identified base products in DNA (Aruoma *et al.*, 1989).

Once formed, modified DNA nucleotides follow a binding model significantly different than the Watson and Crick binding rules. These distinctive binding arrangements may lead to mutations or misreads when sequencing the target region if this segment includes modified bases (see Table 1). The DNA damage may lead to either a transversion mutation or a transition mutation within the DNA sequence. A transversion is defined as the replacement of a purine by

a pyrimidine or vice versa whereas a transition mutation is the substitution of a purine with another purine or a pyrimidine with another pyrimidine. For example, adenine to thymine is a transversion while adenine to guanine is a transition.

The heterocyclic bases of nucleic acids are significant sites of free radical-mediated modification, however the sugar-phosphate backbone is also highly vulnerable to such attacks. Although the majority of OH radicals react with DNA by adding to the double bonds of the bases, a small percentage will react with the sugar moiety by abstracting hydrogen atoms, forming sugar radicals (Von Sonntag, 1987).

Table 1 - Base substitutions produced by products of free radical induced DNA damage. (Dizdaroglu *et al.*, 2002)

	Pairs with	mutation	
 8-hydroxyguanine	A	G → T	
 2,6-diamino-4-hydroxy-5-formamidopyrimidine	A	G → T	
 8-hydroxyadenine	C, G	A → G	A → C
 2-hydroxyadenine	A, G	A → T	A → C
 4,6-diamino-5-formamidopyrimidine	A, G	A → T	A → C
 5-hydroxycytosine	A, C	C → T	C → G
 5-hydroxyuracil	A, C	C → T	C → G
 isodialuric acid	?		
 5-hydroxyhydantoin	?		
 5-hydroxy-5-methylhydantoin	?		

Extensive research in this area has established the formation of four distinct types of product (Von Sonntag, 1987): Type 1 - both phosphate links are broken and the unaltered base is lost to yield free deoxyribose-derived compounds; Type 2 - altered sugars bound by one phosphate

linkage to a damaged DNA strand nearly always causing the unaltered base to be lost; Type 3 - unaltered base is lost and the sugar is altered, either oxidised or fragmented and oxidised but both phosphate linkages are still intact with no strand break; Type 4 – or the base is altered, the glycosyl linkage is no longer stable leading to the elimination of the altered base and thus the formation of an apyrimidinic/apurinic site (AP site). The formation of product types 1 and 2 imply a direct strand break whereas types 3 and 4 constitute alkali-labile sites which change into strand breaks on alkali treatment (Von Sonntag, 1987).

These alterations can either be the result of a direct oxidant attack on the sugar backbone of the DNA or from a transfer of charges from the targeted base to a sugar ultimately leading to its oxidation. First, abstraction of a hydrogen atom from deoxyribose produces a carbon-based sugar radical that can rearrange, ending in scission of the nucleic acid strand (Pogozelski *et al.*, 1998). A deoxyribose residue has seven hydrogen atoms (see Figure V) each attached to a carbon, that are available for abstraction by an oxidising agent or free radical (Pogozelski *et al.*, 1998). The goal of recent research has been to identify which of these hydrogen atoms is abstracted in the formation of a DNA strand break. Pardo *et al.* (1992) confirmed, using quantum mechanical calculations, the theoretical probability of abstraction correlates with carbon-hydrogen bond strength. Research conducted on double stranded B-DNA found that hydrogen abstraction is governed by proximity to oxygen and the accessibility of the hydrogen atom to the oxidising species (Pogozelski *et al.*, 1998).

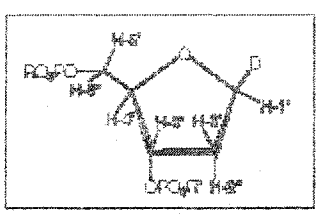


Figure V - Deoxyribose Residue.
(Pogozelski *et al.*, 1998)

Abstraction may occur at any of the seven hydrogen atoms however not all hydrogen removals result in immediate strand breaks. Loss of a certain hydrogen may weaken the DNA strand leaving it wounded and thus an easy target for other reagents or conditions to bring about strand breaks (Pogozelski *et al.*, 1998). All hydrogen abstraction result in production of DNA strands with phosphate termini that are easily detectable but do not indicate much about the chemistry of their formation (Pogozelski *et al.*, 1998). The reaction pathway can be revealed through examination of the characteristics of the modified sugar at the site of the strand break, which are unique to the specific hydrogen abstracted (See Table 2) (Pogozelski *et al.*, 1998).

Table 2 - Marker Product formed and Position of hydrogen abstraction.
(Pogozelski *et al.*, 1998)

marker product		position of hydrogen abstraction
	5-methylene-2-furanone	C-1'
	base propenoate	C-2'
	oligonucleotide 3'-phosphoglycaldehyde	C-3'
	oligonucleotide 3'-phosphoglycolate	C-4'
	base propenal	C-4'
	nucleotide 5'-aldehyde	C-5'
	furfural (FUR)	C-5'

The DNA backbone sugar may also undergo oxidation due to a transfer of charges from a targeted base. Upon oxidant attack of the 4'-hydrogen of the DNA base, the hydrogen is abstracted causing a hydrogen rearrangement within the ring and further breaking the 3'-carbon-

oxygen double bond (see Figure VI) (Schafer, 1997). A modified base, for example a uracil radical, may react with the ribose of the DNA backbone to yield a cationic sugar ultimately undergoing attack from the surrounding oxidants (Schafer, 1997). The backbone sugar then collapses leading to a single stranded break in the strand of DNA (see Figure VII).

Double-stranded DNA undergoes single stranded breaks linearly with the increase of irradiation in aqueous solutions, whereas double stranded breaks follow a linear relationship if plotted against the square root of the ultraviolet (UV) light dose (Von Sonntag, 1987). This quadratic dependence supposes that, in aqueous solutions, double-strand breaks are formed by the coincidence of two single-strand breaks on both strands of the double helix (Von Sonntag, 1987). These two strand breaks may either be directly opposite to each other or some nucleotides apart. The ionic strength of the aqueous medium determines the critical distance *h*, beyond which two single-stranded breaks on opposite strands would no longer lead to a double-stranded break (Von Sonntag, 1987). The lower the ionic strength of the medium the more readily the opening of the double-helical structure. Overall, double strand breaks are caused by multiple radical events within a short section of the dsDNA (Von Sonntag, 1987).

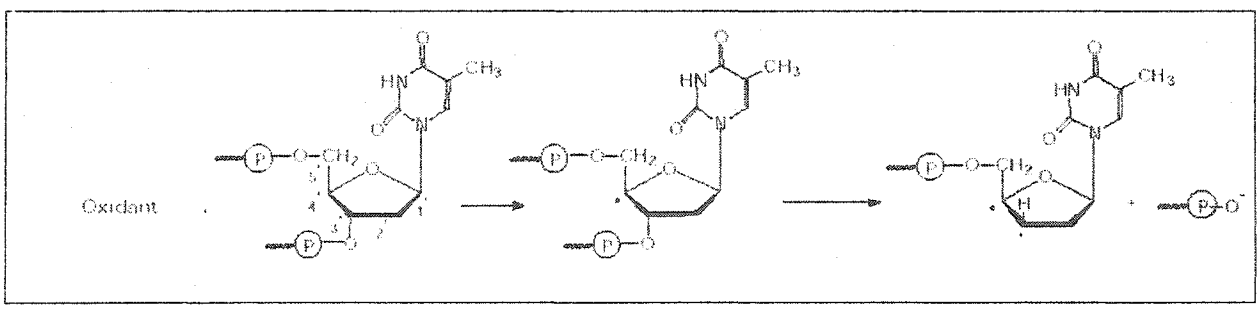


Figure VI - DNA oxidant attack.
(Schafer, 1997)

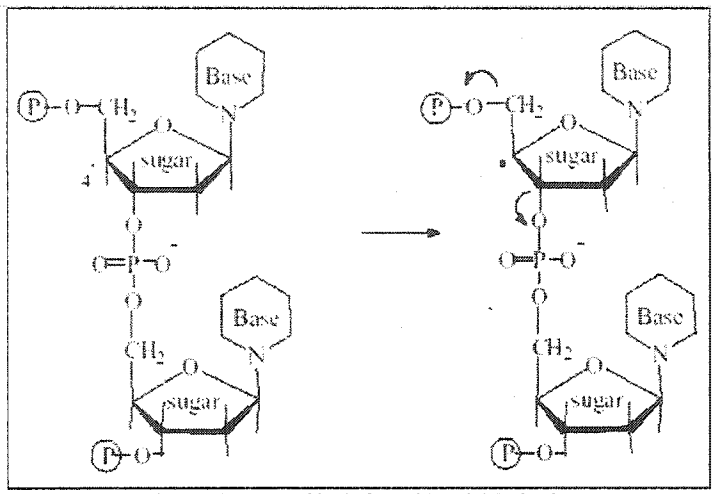


Figure VII - DNA Single Strand Break Mechanism
(Schafer, 1997)

Oxidative damage has also been suggested to lead to the formation of ring-saturated pyrimidine derivative (Lindahl, 1993). These lesions occur primarily as thymine and cytosine glycols and pyrimidine hydrates (Lindahl, 1993). These non-coding derivatives are characterised by the loss of the 5,6 double bond and in consequence, loss of standard planar ring structures (Breimer, 1990; Wallace, 1988) that ultimately decompose into fragments of bases (Lindahl, 1993). *In vitro*, ring-saturated pyrimidines are formed in DNA at rates marginally higher than that of 8-OH-Gua and are excised by a specific DNA glycosylase with an associated apyrimidinic lyase activity that promotes cleavage of the sugar-phosphate chain by β-elimination (Lindahl, 1993). Attempts to estimate the rate of conversion of DNA pyrimidines to ring-saturated forms *in vivo* were unsuccessful (Wagner *et al.*, 1992).

Another major type of DNA damage that occurs in a cell exposed to various agents is DNA-protein cross-linking. Hydroxyl radicals appear to be involved in the formation of covalent bonds between DNA and surround proteins induced by ionising radiation or hydrogen peroxide (H_2O_2) (Dizdaroglu, 1992). DNA-protein cross-links are detected through ratio analysis of the optical density at 260 nm and 280 nm, with an increased ratio with increase in

radiation dose (Von Sonntag, 1987). A number of DNA-protein cross-links were identified by Dizdaroglu (1992) in γ-irradiated and H_2O_2/metal ion treated chromatin samples (Dizdaroglu, 1992). These cross-links were found to involve the DNA bases thymine and cytosine, and the amino acids glycine, alanine, valine, leucine, isoleucine, threonine, lysine and tyrosine (Dizdaroglu, 1992). Chromatin samples irradiated in an oxic environment displayed no cross-linking with the exception of Thymine-Tyrosine (Thy-Tyr) cross-links (Dizdaroglu, 1992). Oxygen generally reacts with carbon-centred radicals at diffusion controlled rates and thus inhibits dimerization (cross-linking) reactions of radicals (Dizdaroglu, 1992; Von Sonntag, 1987). The Thy-Tyr cross-link is generated from addition of the $^{\bullet}OH$-generated allyl radical of thymine to carbon-3 of tyrosine followed by oxidation of the resulting adduct radical and/or from combination of the allyl radical of thymine with $^{\bullet}OH$-generated tyrosine radicals (see Figure VIII) (Dizdaroglu *et al.*, 1989). Oxygen may not be able to react with thymine or tyrosine radicals prior to cross-linking (Dizdaroglu, 1992). It has been suggested that oxygen may not be able to prevent cross-link formation between thymine and tyrosine due to the highly specific nature of the union (Dizdaroglu, 1992). Cross-linking is also partially inhibited by mannitol and dimethyl sulfoxide (DMSO), which is consistent with the site-specific generation of $^{\bullet}OH$ upon reaction of chromatin-bond metal ions with H_2O_2 (Goldstein *et al.*, 1986; Samuni *et al.*, 1983; Ward *et al.*, 1985).

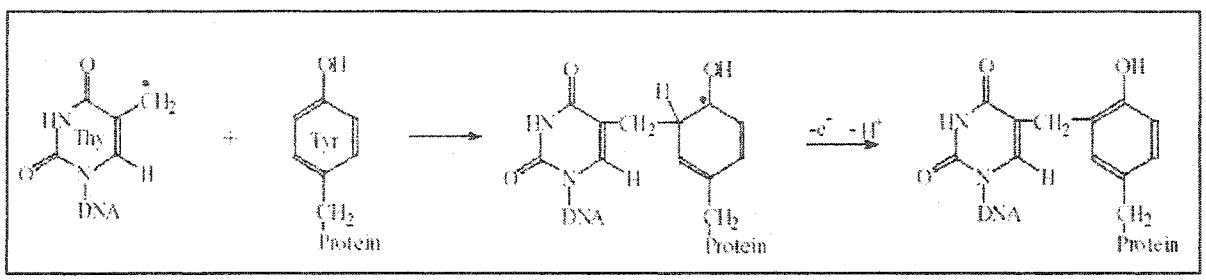


Figure VIII - DNA Protein Cross-Link.
(Schafer, 1997)

The DNA molecule is particularly prone to hydrolytic damage. Hydrolysis is defined as the breaking of bonds by the addition of water. DNA damage by hydrolysis can occur chemically or enzymatically. First of all, DNA sugar-base bond, N-glycosyl bond, is highly labile and thus highly susceptible to acid hydrolysis (Lindahl, 1993). Direct hydrolytic attack leads to base loss, predominately through depurination, and ultimately leads to the formation of a single stranded nick at the abasic site. Strong acids liberate pyrimidine bases while purine bases are selectively released in weak acid (Lindahl, 1979). There is a 4-fold difference in the rate of depurination between single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA) suggesting the double-stranded conformation confers relatively little protection against hydrolysis of the glycosyl bond (Lindahl, 1979). The two purines, guanine and adenine are lost at similar rates, with guanine being released slightly faster. Depurination involves protonation of the base followed by direct cleavage of the glycosyl bond (Lindahl *et al.*, 1972a; Zoltewicz *et al.*, 1970). DNA depyrimidination is analogous to depurination at neutral pH although it occurs 20 times slower. Cytosine and thymine are liberated at 5 % of the rate of purines (Lindahl, 1993).

In addition to the intrinsic lability of glycosyl bonds, DNA base residues are susceptible to hydrolytic deamination. Hydrolytic loss of amino groups by DNA bases cytosine, adenine, 5-methylcytosine and guanine, result in the formation of uracil, hypoxanthine, thymine and xanthine respectively (Poinar, 2003). The main targets of this reaction are cytosine and 5-methylcytosine as determined by following their conversions to uracil or thymine residues in radiolabeled DNA with the function of temperature, pH and secondary structure (Lindahl, 1993). In a mammalian cell with 3 % of its DNA cytosine residues in methylated form, about 10% of hydrolytic deamination would occur at 5-methylcytosine residues and 90% at cytosine residues

(Lindahl, 1993). The hydrolytic reaction occurs without any sequence specificity and varies between species (Lindahl, 1993). In contrast to depurination, the double-helix structure offers protection against hydrolytic cytosine deamination such that this reaction only occurs 0.5-0.7 % of the rate of ssDNA (Lindahl, 1993). Hydrolytic conversion of cytosine to uracil in DNA at neutral pH requires direct deamination by alkali-catalysed hydrolysis and also must be attacked by water on the protonated base in a general acid-catalysed reaction (Shapiro, 1981). Cells may also undergo catalytic conversion of cytosine to 5-methylcytosine by DNA methyltransferase (Lindahl, 1993). A covalent bond is formed by the enzyme at the carbon-6 (C6) position of the cytosine, generating a transient dihydropyrimidine reaction intermediate that is highly susceptible to spontaneous deamination (Lindahl, 1993).

Deamination of DNA purines is a minor reaction when compared to the hydrolytic conversion of cytosine to uracil (Lindahl, 1993). Depurination results in a deoxyribose without a base termed an apurinic site or APsite. Adenine is converted to hypoxanthine in DNA at 2-3 % of the rate of cytosine deamination (Karran *et al.*, 1980). The deamination of adenine is considered a mutagenic lesion since this moiety forms a more stable base pair with C than with T (Lindahl, 1993). The rate of depurination of guanine to xanthine in DNA has not been precisely determined but is suggested to be similar to, or slower than, adenine deamination (Lindahl, 1993). Thought rarely generated, the xanthine-deoxyribose bond is particularly susceptible to spontaneous hydrolysis leading to the rapid conversion of a xanthine residue to an apurinic site in a few days in a non-enzymatic reaction at 37°C (Lindahl, 1993).

Hydrolysis also takes place enzymatically by nucleases, which are either exonucleases or endonucleases. Exonucleases target the terminus of a polynucleotide strand while endonucleases cleave phosphodiester bonds within a polynucleotide strand. There are 5' exonucleases, which

cleave the DNA from the 5'-end of the DNA chain, and 3' exonucleases that cleave the DNA from the 3'-end of the chain (Voet *et al.*, 1998). Exonucleases and endonucleases vary in hydrolysis specificity and thus some hydrolyse ssDNA while others target dsDNA. Endonucleases can either cleave randomly or with high specificity. Endonucleases that cleave dsDNA at random often make interactions with the backbone of DNA. Restriction endonucleases, are able to recognise a specific base sequence of four to eight bases within dsDNA and cleave both strands of the duplex at this precise location (Voet *et al.*, 1998).

Physical forces such as temperature variations and physical shearing also damage DNA. When required, the double stranded conformation may be denatured, through the application of heat or chemical treatment (Hummel, 2003). When carried out using heat, denaturation is fully reversible by decreasing the temperature leading to renaturation or reannealing (Hummel, 2003). Freezing & thawing is known to disrupt cellular structures including mitochondrial membranes, exposing mtDNA and nDNA to degrading enzymes (Ross *et al.*, 1990). Lack of protection by histones and dense packaging leads to preferential degradation of mtDNA compared with nDNA, most likely due to increased accessibility to DNAses (Ross *et al.*, 1990). It is also possible that its unique structure (like the DNA-RNA hybrids in the control region) and potential motifs in the sequence of the mtDNA make it more susceptible to enzymatic degradation than nDNA (Ross *et al.*, 1990). Tissue studies show that mtDNA is degraded much faster than nDNA after a tissue was thawed and kept at room temperature or at 4°C whereas, in fresh tissue, the relative amount of mtDNA remained constant (Berger *et al.*, 2001).

VIII. METHODOLOGICAL BACKGROUND

i. Sample Preparation

a. Extraction

The methodological process utilised to release nucleic acids from within a cell, termed DNA extraction, is suggested to be the most crucial step in DNA analysis (Herrman, 1994). Despite its importance, the DNA extraction method is not one "written in stone". The method used often depends on the tissue type, quantity and quality of the sample at hand. Comparative analysis between extraction methods has been addressed in the literature and in the Paleo-DNA laboratory and is beyond the scope of this project and thus emphasis will be placed only on the methods employed.

The DNA extraction method used is ultimately determined by the type of sample to be analysed and the molecule to be isolated. The isolation of RNA for reverse transcriptase processes is achieved through cell lysis followed by an ethanol precipitation protocol (McPherson *et al.*, 2000). Plasmids, bacteriophage cosmids and artificial chromosome DNA can be prepared using any rapid mini-preps to purification through cesium chloride gradients (McPherson *et al.*, 2000). In the case of genomic DNA, any method can be used to isolate plant, animal and bacterial DNA (McPherson *et al.*, 2000). For larger genomic DNA fragments, greater than 1000 bp, it is preferable to use a commercially available extraction kit in order to save time and money (McPherson *et al.*, 2000). Fragments in the range of 200 to 1000 bp do not require such stringent extraction procedures since they undergo preferential amplification (McPherson *et al.*, 2000).

Specific extraction protocols are required for pathological, forensic and archaeological samples. For pathological and forensic samples, DNA can usually be extracted from fresh tissues or blood using boiling alkaline, a proteinase K digestion, a Chelex® incubation or a guanidinium incubation (McPherson *et al.*, 2000). The extraction of DNA from archaeological samples is a much more scrupulous process since degeneration has most likely occurred (Hoss *et al.*, 1993). Archaeological cases usually involve the study of chloroplast or mitochondrial DNA or genomic ribosomal RNA genes since they are in multiple copies per cell (McPherson *et al.*, 2000).

In general, liberation of the DNA from the cell begins by chemically perforating the cell using extraction buffers containing Tris-HCl, ethylene-diamine- tetra-acetic-acid (EDTA), detergents and/or enzymes (Herrman, 1994). The Tris-HCl, EDTA chelates with calcium ions in the cell wall weakening it and allowing easy extraction (Herrman, 1994). The detergents will also destabilise the cell wall by disrupting the phospholipids in the membrane, while the enzymes digest any of the membrane stabilising or trans-membrane proteins which cause the breakdown of the membrane. Sodium or potassium salt containing buffers as well as non-ionic detergents are used to stabilise the free nucleic acids through the creation of an isotonic environment since they stabilise the DNA and the buffer respectively (Herrman, 1994). Depending on the sample type, the cell's protein fraction may need to be digested using proteinase K or boiling procedures in order to degrade or digest the cellular debris (Herrman, 1994). As mentioned, there are numerous physical, chemical and enzymatic extraction methodologies that successfully obtain DNA, and should be compared in order to attain an optimal extraction methodology for each sample type.

The amount of DNA needing to be extracted for further analysis depends on the application and the source of the template (McPherson *et al.*, 2000). Obtaining DNA from modern samples is far less complicated than obtaining DNA from degraded samples. A general established guide of 3×10^5 template molecules has proven to be successful. This would require approximately 1μg of human genomic DNA, 10 ng yeast genomic DNA, 1-2 pg of plasmid DNA or 20 pg of bacteriophage λ DNA (McPherson *et al.*, 2000). These quantities are in no way constant since some applications require an increased template number while decreasing the PCR cycle number and the viability of the templates may vary due to damage and modification reducing the available DNA to be amplified (McPherson *et al.*, 2000).

Traditionally, protocols used in the extraction of ancient DNA from fragile specimens were no different from those used for extraction of modern materials. Extremely low copy numbers of DNA in these ancient specimens meant increased sensitivity to inhibition decreasing the efficiency of further analyses. The source of these samples may substantially increase inhibitors like buried archaeological or faecal samples. In 1990, Boom *et. al* surmounted this obstacle through post-extraction purification using silica particles. These silica beads, in the presence of guanidinium isothiocyanate, a chaotropic agent, form salt bridges to negatively charged polymers such as DNA, allowing contaminants to be washed away before recovery of the DNA with a suitable elution buffer such as water (Hoss *et al.*, 1993). These methods enable DNA purification directly from crude extracts of many ancient materials while minimising potential losses of ancient DNA likely taking place during standard procedures involving phenol extraction and ethanol precipitation.

b. Purification

There are many techniques employed as a means of DNA purification. Some methods are directly linked to DNA extraction of a sample whereas others are very distinct post-extraction procedures. The first method of DNA extract purification involves the use of silica particles that have a high DNA binding affinity (Boom et al., 1990). This procedure is based on the observation that DNA will bind to silica or glass particles in the presence of high concentrations of the chaotropic agent guanidinium thiocyanate (GuSCN) (Boom et al., 1990). Centrifugation is used to amass the silica particles into a pellet, which is then washed to remove all impurities from the silica particles. Once washed, the silica particles are then dried in order to eliminate all remaining wash buffers. Finally, the purified DNA is eluted from the silica particles into aqueous solution through the application of heat. This purification protocol decreases the likelihood of co-purifying PCR inhibitors however the silica particles themselves may act as PCR inhibitors thus requiring their full elimination prior to PCR (Yang et al., 1998). This purification tool is widely used since it is sensitive, reproducible, rapid, simple, and requires no specialised equipment or specialised knowledge of biochemistry (Boom *et al.*, 1990). Since it was first published in 1990, many researchers have modified this protocol to fit their specific needs. The procedural modification made by Höss, *et. al.* in 1993 may be the most extensively employed (Hoss *et al.*, 1993).

The silica particle method has more recently been adapted to function in a column. This facilitates handling of the samples, reduces purification time and minimises the presence of residual silica particles that may act as PCR inhibitors. The most readily available silica based column is the QIAquick™ column by QIAGEN®. This kit allows rapid purification of pre-PCR DNA extracts as well as post-PCR reaction clean-up with 99.5 % removal precision (QIAGEN,

2002b). The QIAquick purification protocol relies on the adsorption of the DNA to the silica-membrane in the presence of high salt while contaminants pass through the column. This kit includes specific binding buffers to ensure the efficient binding of single- or dsDNA as small as 100bp and the quantitative (99.5 %) removal of fragments smaller than 40 nucleotides long. Impurities such as salts, enzymes, unincorporated nucleotides, agarose, dyes, ethidium bromide, oils, unwanted primer and detergents such as DMSO and Tween® do not bind to the silica membrane but flow through the column (QIAGEN, 2002b). Once the DNA is bound to the silica-membrane, any remaining impurities are washed through the column by addition of an ethanol-containing buffer combined with centrifugation. Further centrifugation steps are required in order to ensure the complete removal of ethanol to prevent PCR inhibition. Elution of the DNA from the silica-membrane requires low salt concentrations under basic conditions. Elution buffer may include water or the provided buffer EB (10 mM TrisCl, pH 8.5). Elution in TE is not recommended since it may lead to inhibition of subsequent enzymatic reactions (QIAGEN, 2002b).

The QIAquick purification kit is invaluable since it allows for simultaneous separation of DNA from potential PCR inhibitory substances while concentrating the DNA and all in less than 5 minutes. However, the volume held by the QIAquick column (750 μL) may be considered a shortcoming since it obliges multiple loading when purifying the entire DNA extract volume (Yang *et al.*, 1998). The DNA yield from this column procedure depends on three factors: the volume of the elution buffer, how the buffer is applied to the column and the incubation time of the buffer on the column. Extended incubation of the column at room temperature during the elution step may increase DNA concentration by 1.7 times (QIAGEN, 2002b).

Post-PCR samples clean up has been drastically simplified since the development of chromatography columns. These columns employ gel-filtration resin that separates molecules based on molecular weight. The resin consists of spheres with uniform pores which retain the impurities or dye terminators while the DNA fragments are excluded and recovered in the flow-through (QIAGEN, 2002a). Columns such as the micro Bio-Spin® P-30 Tris Chromatography Column by BIO-RAD or the DyeEx 2.0 Spin Kit by QIAGEN® allow the swift removal of dye terminators (dRhodamine, DYEnamic ET and BigDye™ teminators), unincorporated nucleotides and desalt nucleic acids, proteins and peptides. The 30 kDa columns have a sample application volume of 20 to 75 μL promising a 99 % removal of ddNTPs and a 95% recovery of applied DNA greater than 22bp (BIORAD, 2002) while the DyeEx columns are limited to 20 μL of sample (QIAGEN, 2002a). Efficient sample purification using the DyeEx columns requires smaller sample volumes since larger volumes increases not only the recovery of DNA fragments but also the level of contamination with dye terminators and nucleotides (QIAGEN, 2002a).

c. Sample Storage

A purified DNA extract may require immediate analysis and/or storage for future study or duplication of analysis. Few storage protocols have been established to eliminate or reduce degradation of the DNA during storage periods. One such protocol provided by the supplier (Qbiogene) of a silica solution used for purification during a phenol-based extraction, proposes storing the samples in pure water or TE-buffered solutions (Hummel, 2003). This is accomplished once the extraction protocol is carried out, by incubating the DNA-silica solution at 56°C for 10-15 minutes, followed by a short centrifugation and transfer of the elute to a new sample tube (Hummel, 2003). A study performed by Hummel, *et. al.* (2003) following this protocol demonstrated that low copy number samples stored in pure water, at normal refrigerator

temperatures started to degrade significantly after only 6 to 14 weeks (Hummel, 2003). The study clarified that samples stored as a DNA-silica solution showed reduced levels of degradation, especially when stored at –20°C (Hummel, 2003).

Sample storage may also be required prior to extraction. Since storage conditions vary according to sample type, it is impossible to target one protocol as being the best. Roon, *et. al.* (2003) have demonstrated freezing at -20°C is the best choice for storage of hair samples over time, provided freeze-thaw cycles are avoided. DNA extraction from hair should take place within six months of sample collection or sooner if larger mitochondrial fragments are to be amplified (Roon, 2003). Blood samples stored at –20°C without any previous treatment or stored at 4°C for longer than 4 days showed a 30 to 40 % decrease in DNA extraction yield (Gustincich *et al.*, 1991). A larger scale study showed that blood samples are best stored at 4°C when extraction is to take place within four weeks (Cushwa *et al.*, 1993). Storage of cells containing extrachromosomal circular DNA is best accomplished at -70°C in the form of a pellet or in liquid nitrogen (Marshall *et al.*, 1993). Studies performed on tissue samples proved storage at -70°C while minimising the number of freeze-thaw cycles to be best (Berger *et al.*, 2001). In all cases of sample storage, samples should be protected from UV radiation and moisture at all times (Lindahl, 1993).

ii. Polymerase Chain Reaction

The invention of the Polymerase Chain Reaction (PCR) by Kari Mullis in 1985 has lead to many major developments in molecular biology, more specifically in DNA research and forensic analysis. Mullis, having designed the PCR during a late night drive, attributes this important discovery partly to chance, naiveté and lucky blunders (Mullis, 1990). Prior to the

invention of the PCR, researchers relied on cloning to amplify trace amounts of DNA however the necessary use of biological entities was very restrictive (SunSITE, 1997). Replication of a duplex DNA region using two DNA primers, designed so that their 3'-ends pointed towards each other, was thought up by Khorana and colleagues in 1971, but repeated cycling wasn't given any consideration (McPherson *et al.*, 2000). The ability of PCR to act as an in vitro "DNA photocopier" allows for its tremendous versatility in the field of DNA analysis (McPherson *et al.*, 2000). Applications include, the human genome project, single sperm analysis, molecular archaeology, molecular ecology and behaviour research, infectious disease diagnosis and molecular epidemiology, drug discovery, as well as multitudes more (White, 1996). Later innovations such as thermostable DNA polymerases (Saiki *et al.*, 1988) and computerised temperature cycling instruments (Wittwer *et al.*, 1990) allowed the process to be completely automated once the reagents were mixed in a tube (McPherson *et al.*, 2000).

PCR relies on enzymatic amplification of specific DNA sequences; the key being two oligonucleotide primers hybridising to opposite strands and flanking the region of interest in the target DNA (Erlich, 1989). DNA polymerase accomplishes exponential amplification of the target region through repeated cycles of template denaturation, primer annealing, and extension of the annealed primers (Erlich *et al.*, 1991). Each PCR cycle gives rise to double the initial number of template DNA, that is to say 20 cycles would yield 1 million fold amplification of the target DNA (Casey, 1992).

A standard PCR can be described as having three phases: exponential, linear and plateau (ABI, 2002). During the exponential phase there is exact doubling of the target DNA at every cycle and the reaction is very specific and precise (ABI, 2002). Exponential amplification occurs because all the reagents are fresh and available, therefore pushing the kinetics of the reaction to

favour doubling of the amplicon (ABI, 2002). For the duration of the linear phase, also referred to as the high variability phase, the reaction components are being consumed causing the reaction to slow and products begin to degrade (ABI, 2002). The final PCR phase is the plateau phase at which point the reaction has stopped, no more products are being made and product degradation transpires (ABI, 2002). A graphical representation of a basic PCR reaction can be seen below.

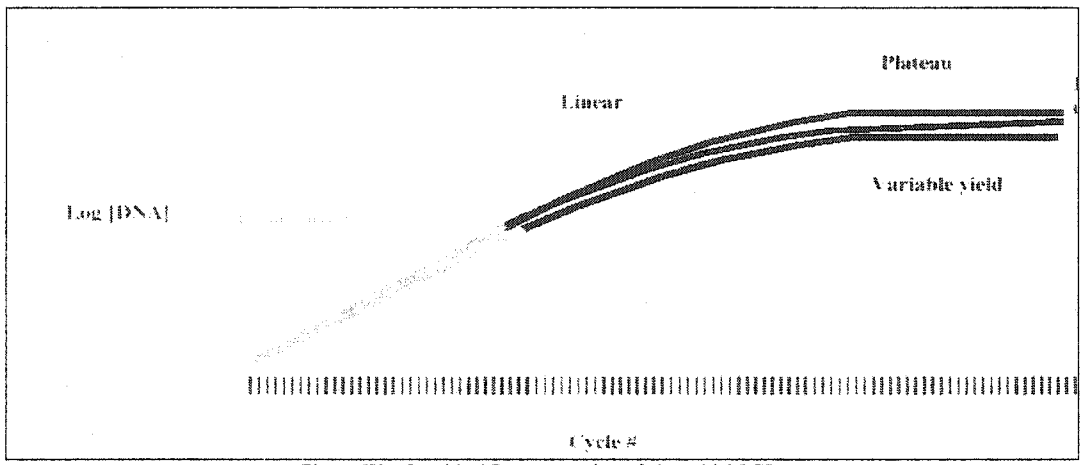


Figure IX - Graphical Representation of sigmoidal PCR.

Despite being referred to as a conceptually simple process by the scientific community, including Mullis, PCR requires specific reagents and precise experimental protocols for specific and optimal amplification (Mullis, 1990). The necessary reagents include a PCR buffer, deoxynucleotide triphosphates (dNTPs), magnesium, oligonucleotide primers, DNA polymerase, template DNA and water. Optimization of the desired product yield and quality is accomplished through adjustment of the concentrations of the above-mentioned reagents and reaction parameters. Due to the various possible optimization conditions, general conditions will be given for a few PCR types; exact optimised conditions employed in this work will be specified in

the procedure section of this paper. All PCRs will be described using thermostable DNA polymerase (*Thermus aquaticus (Taq)*.

a. Standard PCR

These general PCR requirements can be used to amplify a variety of target DNA sequences for numerous applications. PCR reagents can be obtained in a kit prepared by a licensed supplier, however some reagents can be prepared independently. The PCR buffer should contain, but is not limited to: 100 mM Tris-HCl (pH 8.3 at 25°C); 500 mM KCl, 15 mM $MgCl_2$ or $MgSO_4$ (not necessarily in buffer); 1 mg mL^{-1} gelatin; 0.1% Tween-20; and 0.1% NP-40 (McPherson *et al.*, 2000). The prepared buffer can then be stored at -20°C (McPherson *et al.*, 2000). The Tris-HCl reagent is the key component in this PCR buffer in that it is a dipolar ionic buffer with a temperature dependent pH; therefore the pH throughout the PCR will range from 6.8 and 8.3 (McPherson *et al.*, 2000). The KCl component aids in primer/template annealing, although at high concentrations can lead to undesirable nonspecific products (McPherson *et al.*, 2000). One source claims that the elimination of KCl and the gelatin is beneficial (Erlich, 1989).

The concentration of $MgCl_2$ in the PCR mixture has proven to be a major determining factor of specificity and yield of amplification (Erlich, 1989). The Mg concentration present determines the efficiency of the DNA Polymerase therefore a fine range of 1.2 –1.3 mM *free* Mg should be used (McPherson *et al.*, 2000). Excess Mg has been found to cause numerous DNA Polymerase errors while a shortage of Mg causes unspecific amplification (Henegariu *et al.*, 1997). Due to the particular Mg requirements, many PCR buffers are prepared without $MgCl_2$ so that the optimised amount may be added independently (McPherson *et al.*, 2000). The amount of *free* Mg in the PCR mixture is also determined by the concentration of dNTPs since they quantitatively bind Mg^{2+} (Erlich, 1989). The equimolar binding between the dNTPs and Mg^{2+}

makes the joint consideration of the concentration of both these reagents crucial to achieving the desired final concentration of each in solution (McPherson *et al.*, 2000). Since it is an equimolar binding, it can easily be stated that you require twice as much $MgCl_2$ than dNTPs.

The dNTPs, dATP, dCTP, dGTP and dTTP, needed to extend the primer, are added in equimolar concentrations ranging from 50 to 200 μM of each, allowing for the synthesis of 6.5 and 25 μg of DNA respectively (Erlich, 1989). Augmenting the dNTP concentration above 200 μM increases the misincorporation rate of the DNA Polymerase while diminishing the dNTP concentration below 50 μM affects the efficiency of the PCR (McPherson *et al.*, 2000).

The oligonucleotide primers are the starting point of PCR and their selection is therefore crucial to its function. The primers are oligonucleotide sequences complementary to regions of a known sequence which are on opposite strands of template DNA and the 3'(OH)-end points towards the other primer (McPherson *et al.*, 2000). DNA polymerase extends the primers from their 3'-end towards their 5'-end by incorporating the free floating dNTPs (Erlich, 1989). When selecting PCR primers you must consider the following rules:

1. Primer should be between 20 and 30 nucleotides long allowing for good specificity for a unique target sequence (McPherson *et al.*, 2000).
2. Primer should contain approximately equal numbers of each nucleotide (McPherson *et al.*, 2000).
3. Primer should have a 35 % to 65 % G-C content. A string of adenines or thymines can be added at the 5'-end if the primer is overly guanine-cytosine rich. If overly A-T rich, do the same with guanines and cytosines (Alkami Biosystems, 2002).
4. Primer sequences with significant secondary structure due to internal complementarities must be avoided (Erlich, 1989).

5. Primers should begin and end with 1 to 2 purine bases (Alkami Biosystems, 2002).
6. Primer pair annealing temperature is generally calculated as 5°C lower than the estimated melting temperature To calculate T_a : [4(G + C) + 2(A + T)] - 5°C (Suggs *et al.*, 1981).
7. Primer sequence doesn't have to be an exact complement to the template DNA, but the first three bases at the 3'-end should be a perfect match to the template (McPherson *et al.*, 2000).
8. Primer pairs should be checked against each other for complementarity, most importantly, avoid primers with 3' overlaps in order to reduce the frequency of primer-dimer (Erlich, 1989).

Primer-dimer is a result of the primer extending either on itself or on another primer in the PCR (McPherson *et al.*, 2000). This phenomenon occurs principally when very few copies of template DNA are submitted to a high number of amplification cycles (Erlich, 1989). If primer-dimer occurs at an early cycle it can overwhelm a reaction and become the predominant product(McPherson *et al.*, 2000). Primer-dimer can be detected at the bottom of an electrophoresed polyacrylamide gel since it is the sum of the two primers in length, that is anywhere from 40 to 60 nucleotides in length (Erlich, 1989). Primer construction can vary depending on its purpose, it is therefore crucial to experiment with different oligonucleotide sequences either experimentally or through computer simulation (Alkami Biosystems, 2002).

Throughout PCR, specific nucleotides are added to the primer by the DNA polymerase to extend the DNA chain according to the base pairing rules established by Watson and Crick (A:T and G:C) (Watson *et al.*, 1953). Initially, PCR was designed using the Klenow fragment of *Escherichia coli* (E. coli) DNA polymerase I to amplify specific targets from human genomic DNA (Erlich *et al.*, 1991). The Klenow fragment had two enzyme activities, 5'→3' DNA synthesis activity and 3'→5' exonuclease activity (McPherson *et al.*, 2000). Although Klenow

fragments successfully perform PCR, they are inactivated at high temperature such as those necessary in the denaturation step of PCR therefore requiring new aliquots to be added to the PCR mixture after the denaturation step of each PCR cycle (Erlich *et al.*, 1991). These tiresome additions prevented PCR from being an automated concept until a thermostable DNA polymerase; DNA polymerase isolated from the thermophilic bacterium *Thermus aquaticus* was employed (Saiki *et al.*, 1988). DNA Polymerase is a 94 kDa protein that has 5'→3' exonuclease activity as well as 5'→3' DNA Polymerase with a processivity of 50-60 nucleotides at an extension rate of 50-60 nucleotides per second (nt s^{-1}) (McPherson *et al.*, 2000). This enzyme has a half-life of approximately 40 minutes at 95° C allowing it to perform 50 cycles in a typical PCR (McPherson *et al.*, 2000). Use of the Klenow fragment restricted the size of the amplicon to 400 nucleotides, whereas DNA polymerase allows for amplification of an amplicon as large as 10kilobases (Erlich *et al.*, 1991). In a standard PCR, it is recommended that 2.5 units of DNA polymerase be utilised but can be adjusted according to desired results (Erlich, 1989).

The initial source of template DNA used by Mullis was a 25 nucleotide target fragment of a plasmid. However, since then, PCRs have been performed using a variety of sources such as genomic DNA, mRNAs, cDNAs, libraries, phage, cosmid, BAC and YAC clones (McPherson *et al.*, 2000; Mullis, 1990). The application and the source of the template DNA determines the amount required for a PCR but generally 3 x 10^5 template molecules is a good starting point (McPherson *et al.*, 2000). This amounts to 1 μg human genomic DNA, 10 ng yeast genomic DNA, 1ng *E. coli* genomic DNA, 1-2 pg of plasmid or M13 DNA or 20 pg of bacteriophage λ DNA (McPherson *et al.*, 2000). Molecular archaeological and ancient samples may not contain this amount of template, due to degradation over time; therefore PCR conditions such as sample

volume, DNA polymerase volume and cycle number may require adjustments. Water is added to the PCR mixture in order to get the reaction to the desired volume, most often 25 or 50 μL.

Typical samples go through three major phases during PCR cycling; denaturation, annealing and extension (Mullis *et al.*, 1987). The exact temperatures and time of exposure to these conditions requires optimization depending on the desired product. The following guidelines are used most often. Foremost, ssDNA must be obtained through the denaturation of dsDNA requiring the sample to be heated to 94°C for 1 to 2 minutes (Erlich, 1989). Templates having a high guanine and cytosine (GC) content may require increasing the denaturation temperature to 96°C in order to ensure complete denaturation of duplex molecules (McPherson *et al.*, 2000). However, in order to retain the highest DNA polymerase activity in the reaction it is practical to limit the sample's exposure time to elevated temperatures (Erlich, 1989).

The annealing temperature determines the stringency of binding of the primer to the target DNA sequence therefore establishing the presence or absence of non-specific products (McPherson *et al.*, 2000). As a general rule, the higher the annealing temperature, the more stringent is the binding meaning that it will only bind to the exact target sequence (McPherson *et al.*, 2000). It is feasible to start with an annealing temperature of 55°C for a typical 20-base oligonucleotide primer with a guanine and cytosine content of about 50 % (Erlich, 1989). The annealing does not require much time, 30 to 60 seconds, since hybridisation occurs almost instantaneously due to the extreme molar excess of primers in the PCR mixture (Erlich, 1989).

The extension phase is done at the optimal functional temperature for the specific DNA polymerase used and therefore may vary (McPherson *et al.*, 2000). General extension conditions for most polymerases is 72°C for 1 minute each cycle followed by a final PCR setting of 72°C for 2 minutes to ensure complete synthesis of all molecules (McPherson *et al.*, 2000). Annealing

and extension of the primers may sometimes be done at the same temperature depending on the desired product specificity (Erlich, 1989). However, the ranges now available for PCR by changing the enzymes, buffers or constituents can be denaturation between 90°C and 104°C for 15 seconds to 10 minutes, annealing between 30°C and 80°C for 10 seconds to 5 minutes and extension between 50-80°C for 30 seconds to 60 minutes with additional denaturing or extension steps added as required.

Although PCR can amplify a low copy number of template DNA into millions of copies in a matter of hours, the amplification reaction is not infinite (Erlich, 1989). The kinetics of template DNA amplification using PCR resembles a sigmoidal curve, i.e. begins with a lag phase of primer scanning and initial product formation, followed by an exponential accumulation of product, and finally a late phase or plateau where product accumulation halts (see Figure IX) (McPherson *et al.*, 2000). The number of cycles in a PCR should be kept to a minimum required to produce sufficient product for analysis since the late phase is inevitable (Erlich, 1989). The onset of the late phase depends primarily on the initial number of target DNA copies present in the sample, the complexity of the template and by the total amount of DNA synthesized (Erlich, 1989). For example, plasmid templates generally require 25 cycles whereas genomic DNA requires between 30 and 40 cycles (McPherson *et al.*, 2000).

The ability of the PCR to amplify infinitesimal amounts of template DNA into millions of copies offers endless applications however it can also be detrimental if the incorrect sequence is amplified (McPherson *et al.*, 2000). This resulting contamination often originates from product carry over from a previous PCR, exogenous DNA, cloned DNA molecules carrying the target gene, and original template DNA (Erlich *et al.*, 1991). Great care must be taken to avoid all types of contamination since its elimination can be time consuming and costly (Erlich *et al.*,

1991). Aliquoting reagents, using a prepared premix of PCR reagents, dedicating specific equipment, using positive-displacement pipettes or barrier tips and physically separating the reaction preparation area from the product analysis area allow for the abolishment of all contamination or at least leads to its significant decrease (Erlich *et al.*, 1991). More in depth processes such as uracil N-glycosylase, UV irradiation or psoralen and isopsoralen treatment can also be used to inactivate DNA synthesized after each PCR cycle in order to prevent carry-over contamination (McPherson *et al.*, 2000).

b. Hot Start

A PCR, where standard reagents are mixed at room temperature, may result in high background and low specific product yield (Birch, 1996). Despite having an optimal extension temperature of 72°, DNA polymerase was found to retain a considerable enzymatic activity well below this temperature (Erlich *et al.*, 1991). Therefore, if reagents are kept at room temperature during PCR preparation, non-specific annealing occurs between partially denatured template strands and primers, creating non-specific product, or between two primers creating primer-dimer until the mixture reaches a temperature of roughly 65°C (McPherson *et al.*, 2000). These non-specific products are then partially extended and stabilised by the DNA polymerase prior to the reaction mixture attaining 72°C for extension of specifically annealed primers, therefore resulting in the mass amplification of non-specific product (Erlich *et al.*, 1991). The use of Hot Start PCR, which relies on the physical or chemical separation of the DNA polymerase until the desired temperature is attained, is the simplest way to overcome this difficulty (Birch, 1996).

The simplest, and most tedious, approach is the physical separation of the DNA polymerase from the reaction mixture prior to attaining the desired temperature. One option is to prepare the reaction mixture in tubes without the DNA polymerase, then submit them to the

initial denaturing step at 95°C for the required time period. Once this step is completed the required amount of DNA polymerase can then be added to each of the reaction tubes while maintaining a reaction temperature higher than 70°C (McPherson *et al.*, 2000). This method is not preferred for processing a large number of samples due to time involvement, human error, and increased probability of contamination (McPherson *et al.*, 2000).

An alternative method is the use of a physical barrier between the DNA polymerase/template mixture and the other reagents using a wax barrier or commercially available wax beads (McPherson *et al.*, 2000). The melting temperature of the beads determines the temperature at which reagents will be mixed with the DNA polymerase.

A third approach to prevent the creation and amplification of non-specific products is to chemically inactivate the DNA polymerase by means of neutralising antibodies (McPherson *et al.*, 2000). When bound, the antibodies prevent the DNA polymerase from reacting, however once the high temperature of the initial denaturing step is attained, the antibodies denature along with the dsDNA thereby releasing the thermostable, active DNA polymerase and allowing for polymerisation (McPherson *et al.*, 2000).

Although the above-mentioned approaches all serve the same purpose, the method of choice involves the use of a DNA polymerase that is naturally inactive at room temperature, AmpliTaq Gold®. AmpliTaq Gold® is a modified, inactivated form of AmpliTaq polymerase, a thermostable DNA polymerase commonly used in PCR (Birch, 1996). AmpliTaq Gold® regains its enzymatic ability once submitted to high temperatures and can therefore replace the polymerase in an existing PCR to make it a hot start PCR (Birch, 1996). This exchange requires the simple addition of an initial denaturing PCR heat cycle, one example being 95°C for 15

minutes, in order to activate the polymerase, or running the PCR for a extra 10 cycles (Birch, 1996).

c. Multiplex PCR

Multiplex PCR is a variant of the traditional PCR, involving the amplification of a single locus, which allows for simultaneous amplification of two (duplex) or more loci in the same reaction (Henegariu *et al.*, 1997). Multiplex PCR was first used in 1988 in order to screen for deletions in the muscular dystrophy locus (Chamberlain *et al.*, 1988). Since then, the multiplex PCR has extended to many DNA analysis applications such as analysis of mutations (Shuber *et al.*, 1993) and polymorphisms (Mutirangura *et al.*, 1993), quantitative assays (Mansfield *et al.*, 1993), typing of autosomal and Y-chromosomal short tandem repeats (STRs), determination of sex, reverse transcription PCR (Crisan, 1994), and identification of viruses (Heredia *et al.*, 1996; Markoulatos & Georgopoulou *et al.*, 2000; Markoulatos & Mangana-Vougiouka *et al.*, 2000; Markoulatos *et al.*, 1999), bacteria (Hendolin *et al.*, 1997) and parasites (Harris *et al.*, 1998). Multiplex PCR greatly improve data validation, especially when used for analysis of ancient DNA, since they are a step towards individualisation of the data set (Hummel, 2003). The more information obtained simultaneously from a multiplex assay, the less likely it is that this can happen only by chance, as with contamination, in a repeat analysis (Hummel, 2003).

As with the traditional PCR, multiplex conditions must be optimised to yield desired results, however this optimisation can be quite problematic since there is often poor sensitivity and specificity and/or preferential amplification of certain specific targets (Markoulatos *et al.*, 2002). Multiplex PCR relies on the use of multiple primer sets simultaneously and so can easily lead to the production of primer-dimer, if primer-to-template ratio is not optimal (Markoulatos *et al.*, 2002). The most favourable ratio has proven to be a 1×10^7 molar excess of primer with

respect to template (Markoulatos *et al.*, 2002). A successful multiplex analysis must have good primer design that prevents nonspecific product generation, extensive primer-dimer formation and preferential amplification as a result of primer imbalance characteristics (Hummel, 2003). Primer design parameters such as homology of primers with their target nucleic acid sequences, length, guanine-cytosine content and concentration are also key in minimising primer-dimer occurrences in a multiplex reaction (Brownie *et al.*, 1997). The hot start methods previously described may also be applied to a multiplex PCR in order to further reduce primer-dimer formation (Chou *et al.*, 1992).

Preferential product amplification can be reduced by utilising primers with virtually identical optimum annealing temperatures and without significant homology to each other allowing each primer to bind its target sequence with the same efficiency (Markoulatos *et al.*, 2002). Preferential product amplification due to fluctuation in the interactions between the reagents used in early PCR cycles is termed PCR drift and most often occurs in the presence of very low template concentrations (Wagner *et al.*, 1994). The mechanism which favours amplification of certain templates due to the properties of the target sequence, the sequence flanking the target or the entire genome is referred to as PCR selection (Markoulatos *et al.*, 2002). PCR selection stems from properties such as guanine-cytosine content variations within a sequence, creating secondary structures that lead to preferential accessibility of targets within genomes and the gene copy number within a genome (Markoulatos *et al.*, 2002).

The sensitivity and/or specificity of a multiplex PCR can be greatly improved by increasing the concentration of other PCR elements such as PCR buffer, dNTPs, $MgCl_2$ and enzymes that are used in simple PCRs (Markoulatos *et al.*, 2002). Each of these elements should be optimised separately in order to determine the most favourable settings to yield the desired

multiplex PCR products. The primers selected for the multiplex must follow the primer selection rules previously outlined for a standard PCR. The concentration of each primer to be used in the multiplex usually must vary in order to obtain equal product yields (Henegariu *et al.*, 1997). It is best to begin with equimolar concentrations of each primer then adjust the concentrations of each primer independently according to the results as seen using gel electrophoresis or a capillary electrophoresis size detection system (Henegariu *et al.*, 1997). Generally, for low copy number or high complexity DNA, the primer concentration should be 0.3-0.5 μM whereas for high copy number or low-complexity DNA, the primer concentration should be 0.04-0.4 μM (Markoulatos *et al.*, 2002).

Magnesium Chloride ($MgCl_2$) and dNTP concentrations and ratio are crucial to multiplexing. Any $MgCl_2$ in solution has a high binding affinity for dNTPs and DNA, however to work properly, DNA polymerase requires free magnesium. We must thus ensure that the $MgCl_2$ to dNTP ratio is such that enough $MgCl_2$ remains free in solution to activate the DNA polymerase (Henegariu *et al.*, 1997). Through much study, it was found that 200 μM to 400 μM of each dNTP is optimal in 1.5-2 mM of $MgCl_2$ (Henegariu *et al.*, 1997). Much lower product yield was observed when dNTP and Mg^{2+} concentrations were below 200 μM and 1.5 mM respectively. Excessive Mg^{2+} in solution also decreases product yield since it stabilises the DNA, preventing complete denaturation from occurring and also decreases specificity through the stabilisation of erroneous annealing of the primer to non-target regions (Markoulatos *et al.*, 2002).

The amount of template DNA and DNA polymerase must also be adjusted in a multiplex PCR. The optimal total DNA quantity to be used in a multiplex has been found to be between 30 and 500 ng per 25 μL reaction (Henegariu *et al.*, 1997). If the amount of template DNA is below

this range (in the pg range), efficient and specific amplification can be obtained by further lowering the annealing temperature by as much as 10-12°C (Markoulatos *et al.*, 2002). The quantity of DNA polymerase used in a multiplex will determine its efficiency. The most efficient experimental enzyme concentration is approximately 2.5 units per 50 μL reaction (Markoulatos *et al.*, 2002). Increasing enzyme quantities above this range results in preferential amplification of various loci and a slight increase in background noise due to the high glycerol concentration in the stock solution (Henegariu *et al.*, 1997).

The PCR buffer concentration must also be modified in order to obtain amplification of the desired sequence fragments. Increasing the buffer concentration from 1x to 1.4x improves the efficiency and specificity of the multiplex reaction significantly (Markoulatos *et al.*, 2002). Optimal yield of longer amplification products can be achieved by decreasing the salt concentration, i.e. the buffer concentration, whereas short amplification products are best amplified in the presence of high salt concentrations (Henegariu *et al.*, 1997).

Multiplex PCR parameters can differ greatly from those of a standard PCR and thus must be precisely adjusted to obtain the desired amplification product. Denaturation temperature and time most often remain the same (94°C for 4 minutes) as those of the standard PCR but may vary according to fragment sizes. Annealing and extension temperatures must be adjusted according to the product size required. To obtain optimal amplification of short products, it is recommended to decrease the annealing and extension temperatures by 1°C until the desired result is obtained (Henegariu *et al.*, 1997). For favourable amplification of longer products, the annealing and extension temperatures must be increased in the same way along with the time allotted for extension to occur (Henegariu *et al.*, 1997). Gradually increasing the annealing temperature can also lead to a reduction in non-specific product amplification (Henegariu *et al.*,

1997). For more favourable amplification of products of all sizes, the extension temperature should be set in the range of 62-65°C and the annealing temperature should be decreased step-wise by 2°C (Henegariu *et al.*, 1997). Time devoted to the optimization of the above-mentioned parameters is not lost since at long last it leads to time efficient and cost-effective operations.

Multiplex amplification can be greatly improved in the way of efficiency and specificity through the use of PCR additives. As previously mentioned, additives, such as Dimethyl Sulfoxide (DMSO), glycerol, formamide, and betaine were found to facilitate denaturation by relaxing the DNA (Markoulatos *et al.*, 2002). The adjuvant employed depends on the overall multiplex conditions and therefore varies greatly. For example, in some cases 5 % DMSO and glycerol were found to yield conflicting results while Bovine Serum Albumin (BSA) (0.8 μg/μL) increased efficiency significantly (Henegariu *et al.*, 1997).

d. Real Time PCR

Prior to real time PCR, DNA quantification was accomplished by combining PCR amplification with one of many possible methods, these being agarose gel electrophoresis and ethidium bromide staining, fluorescence labelling and analysis using polyacrylamide gels, and radioactive labelling and Southern blotting or detection by photoimaging (Giulietti *et al.*, 2001). Despite requiring intensive and laborious post-PCR manipulation, the above techniques also make use of hazardous chemicals and convey a potential risk for laboratory contamination (Giulietti *et al.*, 2001). Further difficulties include ensuring that the PCR was within the linear range of amplification and finding a suitable method for product detection once the linear amplification was accomplished (Schmittgen, 2001).

Higuchi *et. al.* (1993) performed the first type of real time DNA quantification using ethidium bromide and a video camera as a detection system (Higuchi *et al.*, 1993). Since then,

real time PCR has changed drastically; ethidium bromide has been replaced by various non-toxic chemistries and the video camera replaced by a laser detector system. These new methods provide the ability to monitor the real-time progress of a PCR allowing ultimately for quantification of templates such as nDNA, cDNA, mRNA or any other species of DNA (Schmittgen, 2001). Real time PCR yields data within 2 to 3 hours, depending on the thermocycling program performed, thus reducing the waiting period between experiments (Schmittgen, 2001). There are many additional advantages to using real time PCR such as high throughput, enhanced sensitivity, closed tube system, reduced result variation, elimination of post PCR manipulations and simultaneous multiplex reactions (Schmittgen, 2001).

There are currently only a few different methods used in real time for absolute quantification, all of which are based on the measurement of fluorescence during the progression of a PCR (Klein, 2002). Each method enables us to create a curve of PCR product versus the emitted fluorescence in order to establish the exponential phase of the PCR (Klein, 2002). Since these methods are based on the theoretical fact that there is a quantitative relationship between the amount of starting target sample and the amount of PCR product at any given cycle number the initial copy number can be calculated from the curve (ABI, 2002). A typical real time output is as shown in Figure X.

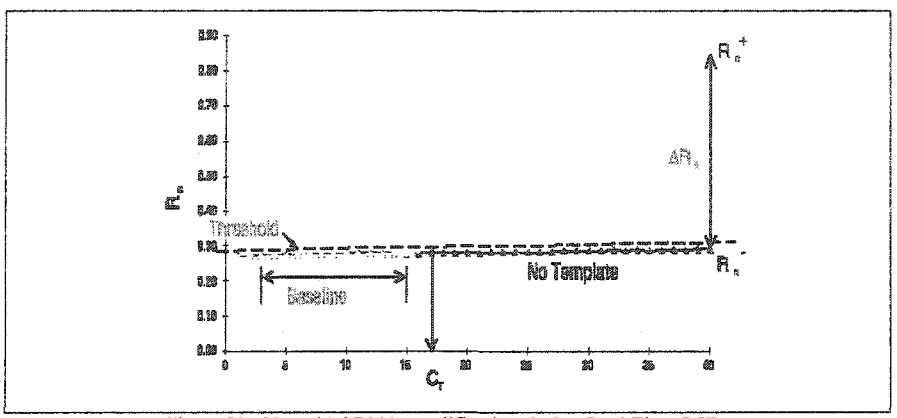


Figure X - Sigmoidal DNA amplification during Real Time PCR.
Figure from Applied Biosystems DNA/RNA Real-Time Quantitative PCR bulletin.

PCR amplification is best detected during the early phase of the reaction, more specifically in the exponential phase (ABI, 2002). The reporter system to be used is chosen by considering its test sensitivity, availability and cost.

The simplest and most cost effective detection system used in real time PCR involves the use of the intercalating dye SYBR Green. This dye binds the Minor Groove of dsDNA, increasing the intensity of the fluorescent emissions as more double stranded amplicons are produced (ABI, 2002). This technology can be applied to already established PCR conditions however since the dye is not sequence specific, PCRs require careful optimisation to minimise binding between the dye and non-specific products (Klein, 2002). Non-specific products may be identified using a melting curve analysis (Klein, 2002). The emitted fluorescence is proportional to the amount dsDNA present at any given time and thus allows for the calculation of the initial template copy number.

The other methods used as real time detection systems are based on the employment of additional fluorescently labelled oligonucleotides. This hybridisation probe chemistry relies on the 5'→ 3'-exonuclease activity of DNA polymerase and the use of dual-labelled oligonucleotide probes which emit a fluorescence signal only on cleavage based on the Fluorescent Resonant Energy Transfer (FRET) principles (Giulietti *et al.*, 2001). The sequence specific probe is fluorescently labelled with 6-FAM at its 5' end and has a fluorescence quencher at its 3' end. During the extension phase of real time PCR, the DNA polymerase enzyme cleaves the non-extendable FRET probe distancing the fluorophore from the quencher thus leading to varying degrees of fluorescence (Giulietti *et al.*, 2001). This fluorescence is then detected and quantified by the real time instrument employed. Advantages of this detector system include superior

specificity due to individual probe design and multi-coloured fluorescent detection however; the sensitivity of this detector has not yet been established.

Another reporter system used for real time PCR relies on oligonucleotide probes, termed molecular beacons, which emit fluorescence when hybridized to a target sequence of DNA. A molecular beacon resembles a hairpin loop having a fluorophore at one extremity bound to a quencher at the other. Once the hairpin loop section of the molecular beacon is hybridized to the target region of the template DNA, it undergoes a conformational change. This new conformation results in fluorescence since the fluorophore and quencher are no longer bound together. The cycle number at which the level of fluorescence rises above a background threshold value is inversely proportional to the log of the initial number of template copies (Burgos *et al.*, 2002).

e. PCR Inhibition

PCR inhibition is a major source of PCR failure. PCR failure due to inhibition is recognised by the lack of any primer dimers, lack of any smears or any other nonspecific amplification products (Hummel, 2003). Common inhibitors include various components of body fluids and reagents encountered in clinical and forensic science (e.g., haemoglobin, urea and heparin), food constituents (e.g., organic and phenolic compounds, glycogen, fats, and Ca^{2+}), and, environmental compounds (e.g., phenolic compounds, humic acids, tannins, maillard products, fulvic acids and heavy metals) depending on the taphonomy of the sample (Hummel, 2003; Wilson, 1997). Other, more widespread inhibitors include constituents of bacterial cells, non-target DNA and contaminants, and laboratory items such as pollen, glove powder, laboratory plasticware and cellulose (Wilson, 1997). Inhibition may be seen in extracted samples as a yellow to dark brown staining of the sample materials before and during extraction

and by specific characteristics, such as in spectrophotometric analysis (Hummel, 2003). The lanes on an agarose gel containing PCR inhibition fluoresce in a bright turquoise colour when illuminated at 254 nm UV light (Hummel, 2003). Another source of inhibition is collagen, which makes up a major portion of the organic fraction of bone material. This inhibitor can be identified as an autofluorescent smear migrating along with DNA of about 500 to 1000 bp on an unstained agarose gel (Hummel, 2003).

PCR inhibition may be total or partial and can manifest itself as complete reaction failure or as reduced sensitivity or detection (Wilson, 1997). Inhibitors generally act at one or more of three essential points in the reaction. First, they interfere with the cell lysis necessary for extraction of DNA thus not allowing for full exposure of nucleic acids as targets or amplification. This inhibition may be prevented by boiling the sample in order to separate the structural and DNA-binding proteins from the DNA (Wilson, 1997). Secondly, inhibitors interfere by nucleic acid degradation usually due to hydrolysis, non-enzymatic methylation oxidative damage and enzymatic degradation or sequestration mainly by Mg^{2+}. Nucleic acid sequestration and degradation may be overcome by physicochemical separation of target DNA from destructive compounds immediately after cell lysis (Wilson, 1997). Last of all, inhibitors may impede polymerase activity for amplification of target DNA (Wilson, 1997). For example, humic compounds interfere with lytic enzymes (Jacobsen *et al.*, 1992), bind to DNA causing template inhibition and proteins (Xia *et al.*, 1995), and interfere with the binding between target DNA and polymerase (Tebbe *et al.*, 1993). Urea may denature the DNA polymerase leading to enzyme inhibition of the PCR (Wilson, 1997). Proteinases and denaturants used for cell lysis were found to inactivate polymerase when DNA purification is inadequate (Wilson, 1997). The

use of additives in a PCR such as BSA or DMSO restrict inhibition by factors, including proteases, by acting as an alternative substrate for catalysis by these enzymes (Wilson, 1997).

When PCR inhibition is suspected or detected, the DNA extract requires an additional pre-PCR clean-up step. Several simple methods have been developed to decrease or eliminate the occurrence of PCR contamination. Some methods are simple including dialysis against large volumes of solvents or centrifugation in CsCl gradients, but involve the sacrifice of substantial amounts of the original sample and other materials (Moreira, 1998). Enhanced methods are highly specific and directed only against concrete contaminants and usually involve expensive materials; these include ion-exchange columns, glass bead extraction, immunomagnetic separation, size-exclusion chromatography, anion-binding resin or spin columns (Wilson, 1997).

f. Additives

There are many additives that aid PCR in diverse and complex ways, the biochemistry of some affects is still uncertain. Betaine (N,N,N-trimethylglycine = [carboxymethyl]trimethylammonium), an isostabilising agent, equalises the contribution of GC- and AT-base pairing strengthening the hybridisation and thus the stability of the DNA duplex and increasing the yield and specificity of PCR products (Don *et al.*, 1991). The addition of betaine may alter the melting temperature of the template DNA in certain cases, thus altering the optimal annealing conditions of a particular reaction (Frackman *et al.*, 2002). Dimethyl Sulfoxide (DMSO) at 2-20 % may be necessary for amplification of some templates, and is proposed to reduce secondary structure and is particularly useful for GC rich templates. However, at 20 % DMSO has been shown to reduce *Taq* polymerase activity by up to 50 % and should be cautiously applied (Gelfand, 1988; Henegariu *et al.*, 1997). Bovine serum albumin (BSA) is a widely used PCR additive due to its versatily and economical value (Kreader, 1996).

BSA has proven particularly successful when attempting to amplify ancient DNA or templates which contain PCR inhibitors, including melanin or ethanol (Barnes *et al.*, 2000; Giambernardi *et al.*, 1998) however, some caution its use due to potential bovine contamination. The use of formamide as a PCR additive has received mixed reviews. Gelfand (1988) has reported that formamide concentrations of 1-5 % and 10 % have not reduced the *Taq* polymerase activity, in contrast Sarkar *et. al.* (1990) claims that 1.25 % formamide is as effective as 2.5 % and 5 % and that in PCR utilising 10 % formamide amplification does not occur (Gelfand, 1988; Sarkar *et al.*, 1990). The use of tetramethylammonium chloride as a PCR additive eliminates the need for individual hybridisations while increasing PCR specificity in templates with differing G-C content (Jung *et al.*, 2002; Ossewaarde *et al.*, 1992). When added in the place of dGTP in the PCR mix, 7-deaza-2'-deoxyguanosine (dC_7Gtp) aids in the amplification of long template DNA with high GC content; particularly when attempting to amplify DNA templates of poor quality (Jung *et al.*, 2002). The T4 gene 32 protein (gp32), a ssDNA binding protein, facilitates PCR by binding with denatured strands of DNA retarding its re-annealment, while stimulating DNA polymerase (Kreader, 1996). Despite increasing reaction costs, gp32 has proven its worth since it enhances long PCR product yield while relieving inhibition at concentrations as low as 20 ng/ µL (Kreader, 1996). PCR specificity and efficiency can be greatly increased through the addition of glycerol at 5 % to 10 % (v/v), although advantages may vary, thus requiring experimental use (Henegariu *et al.*, 1997; Lu *et al.*, 2004; Varadaraj *et al.*, 1994). Polyethylene glycol (PEG) is a useful additive when the DNA template concentration is very low, as it promotes macromolecular association by solvent exclusion facilitating the binding of the DNA polymerase to the template (Pomp *et al.*, 1991). Non-ionic detergents such as Triton X-100, Tween 20 and Nonidet P-40 along with gelatin are routinely used PCR additives due to their

ability to prevent *Taq* polymerase enzyme aggregation and decrease secondary structure optimising amplification (Varadaraj *et al.*, 1994). Well-established proprietary additive mixtures include QIAGEN/Q-Solution; Novagen's Nova*Taq*™ PCR Optimisation Buffer and many more are available from their respective companies, to enhance PCR.

g. DNA Sequencing

DNA sequencing techniques are key tools in many fields including archaeology, anthropology, genetics, biotechnology, molecular biology, and forensic sciences. Four sequencing methods have been developed over the past thirty years: Sanger's method, Maxxam and Gilbert method, pyrosequencing (PPi) and single molecule sequencing with exonuclease. The first sequencing method, conceived by Sanger and Coulson in 1975, was called 'plus and minus' and relied on *Escherichia coli* DNA polymerase I and DNA polymerase from bacteriophage T4 (Englund, 1972, 1971) with different limiting nucleoside triphosphates. The resulting products were resolved by ionophoresis on polyacrylamide electrophoresis (PAGE). The 'plus and minus' method was found to be inadequate and was quickly replaced by Sanger and his co-workers in 1977 with a new breakthrough method for sequencing oligonucleotides via enzymatic polymerisation. This method, initially known as the chain termination method or dideoxynucleotide method consists of a catalysed enzymatic reaction that polymerises the DNA fragments complementary to the template DNA of interest (Sanger *et al.*, 1977). DNA synthesis begins with the annealing of a ^{32}P-labelled primer to the template followed by catalytic polymerisation of dNTPs onto the DNA by DNA polymerase. The growing DNA chain extents by polymerisation until the enzyme incorporates a modified nucleoside, called a dideoxynucleoside triphosphate (ddNTP) (Sanger *et al.*, 1977). This method was performed in four different tubes each containing one of the four ddNTPs. All generated fragments had the

same 5'-end, however the residue at the 3'-end was determined by the ddNTP used in the reaction. Once all four reactions were complete, the mixture of different sized DNA fragments was resolved by electrophoresis on a denaturing polyacrylamide gel in four parallel lanes. Autoradiography allowed the unknown sequence to be visualised. An alternative method is to use radioactively labelled ddNTPs rather than a labelled primer.

The Sanger method has two different approaches that are most utilised: shotgun and primer walking (Griffin *et al.*, 1993). First, the shotgun or random approach is a hit and miss process where there is no control of the region that is going to be sequenced. This process begins with random fragmentation of the DNA into 2 to 3 kb sections. The fragments are then inserted into a vector and replicated in a bacterial culture. Next, numerous positive amplifications are chosen and sequenced. The shotgun sequencing approach generated overlaps in many regions and high levels of redundancy (Adams *et al.*, 1996). The second approach to the Sanger method, primer walking, involves the direct sequencing of unknown DNA within sites in which the sequence is known. A first sequencing reaction is performed on an amplified vector containing the target DNA, using the primers that hybridise to the vector sequence and polymerise the strand complementary to the template (Studier, 1989). Of the newly generated sequence, a second priming sire is then chosen following the same direction (Martin-Gallardo *et al.*, 1992). A genome-sequencing project cannot be realised solely by the shotgun approach and eventually, some part of the sequence has to be generated by primer walking due to its focused nature. This second approach to the Sanger method is valuable since it reduces redundancy (Voss *et al.*, 1993) however; it requires the synthesis of each new primer, which is lengthy and costly.

There have been many DNA labelling strategies designed to simplify ddNTP detection. The first label design used ^{32}P (Biggin *et al.*, 1983) which proved to be hazardous and thus needed to be replaced. The first alternative, designed in 1989, was based on chemiluminescent detection with biotin streptavidin system in which the 5'-end of an oligonucleotide linked to biotin was used as the primer in the sequencing reaction (Beck *et al.*, 1989; Cherry *et al.*, 1994). This method offered many advantages including direct sequencing from the PCR products, elimination of the cloning step (Debuire *et al.*, 1993; Douglas *et al.*, 1993) and the ability to multiplex several reactions on the same gel and detect one at a time with appropriate enzyme-linked primers (Gillevet, 1990). A second alternative allowed all four sequencing reactions to be performed in one lane through the use of four different fluorescent dyes (Smith *et al.*, 1986). The four dyes were each appended to the 5'-end of the primer and each labelled primer was associated with a particular ddNTP. This dye-primer chemistry underwent a considerable advancement with the introduction of energy transfer (ET) dyes (Ju & Kheterpal *et al.*, 1995; Ju & Ruan *et al.*, 1995). The ET dyes are comprised of two dyes per primer, one donor and the other an acceptor dye. The donor can either by a fluorescein (FAM™) or a cyanine (Cy5) derivative (Hung *et al.*, 1996) at the 5'-end. The discriminating dyes, the acceptors, are optimally located about 10 bases along the primer and include FAM™, JOE™, TAMRA™ and ROX™ (Ju & Kheterpal *et al.*, 1995). These dyes are evenly excited by a single wavelength (488nm) and undergo minimal electrophoretic mobility shifts making their use highly advantageous.

Complete automation of the Sanger method was hindered by the complexity of automating gel preparation, sample loading and post-electrophoresis gel treatment. These difficulties were overcome through the replacement of gel electrophoresis with capillary

electrophoresis (CE). CE is a fast technique for separation and analysis of biopolymers which employs narrow-bore β fused silica capillaries and high electric field (Cohen *et al.*, 1987). CE is approximately ten times faster than slab gel electrophoresis since it can contain a higher electric field and thus a faster separation. The analysis time decreased further with the use of gel-filled capillaries with laser-induced fluorescence (Cohen *et al.*, 1990). Once separation temperature (Kleparnik *et al.*, 1996), polyacrylamide polymerisation (Goetzinger *et al.*, 1998), base-calling software (Brady *et al.*, 2000), sample purification (Ruiz-Martinez *et al.*, 1998)and injection (Salas-Solano *et al.*, 1998) were optimised, CE analysis of biopolymers became common practice.

Maxam and Gilbert developed a second sequencing methodology based on chemical degradation in 1977. This method relies on specific chemical agents that randomly cleave end-labelled DNA fragments at adenine, cytosine, guanine or thymine positions (Maxam *et al.*, 1977). The chemical attack occurs in three steps: base modification, removal of the modified base from its sugar, and DNA strand breakage at that sugar position (Maxam *et al.*, 1977). The reaction products are then separated using PAGE and read manually. This method enables sequencing of either dsDNA or ssDNA from chromosomal DNA. This type of sequencing can either begin with digestion with an appropriate restriction enzyme (Maxam *et al.*, 1980) or with an inserted or rearranged DNA region (Maxam, 1980). The DNA template is then end-labelled on one of the strands, originally with ^{32}P (Maxam *et al.*, 1980) but eventually with [^{35}S]dideoxyadenosine 5'-(α-thio)triphosphate ([^{35}S]ddATPαS) and terminal deoxynucleotidyltransferease due to their increased lifetime, low-emission energy, increase in autoradiograph resolution and higher stability after labelling (Ornstein *et al.*, 1985). Nonetheless, this labelling technique was quickly replaced with a less toxic chemiluminescent

label. This label involved the transfer of the chemically cleaved DNA fragment from a sequencing gel onto a nylon membrane followed by selective hybridisation to DNA oligonucleotides with alkaline phosphatase or with biotin, leading directly or indirectly to the deposition of the enzyme. The main advantages of the Maxam-Gilbert and other chemical methods when compared to the Sanger method are that a fragment can be sequenced from the original DNA fragment, instead of from enzymatic copies. Also, no subcloning and no PCR reactions are required, and that this method is less susceptible to mistakes with regard to sequencing of secondary structures or enzymatic mistakes (Boland *et al.*, 1994). Finally, some of the chemical protocols are recognised by different authors as being simple, easy to control, and the chemical distinction between the different bases is clear (Negri *et al.*, 1991). Despite its numerous advantages, the Maxam-Gilbert sequencing method holds specific negative aspects. A minor downfall of this method is that the chemical reactions of most protocols are slow and involve the use of hazardous chemicals, which require special handling care. A major drawback is that the occurrence of incomplete reactions decreases the read-lengths through the induction of electrophoretic mobility polidispersion.

A third DNA sequencing method, termed pyrosequencing, is a real-time technique based on the detection of the pyrophosphate (PPi) released during the DNA polymerisation reaction (Hyman, 1988; Nyren *et al.*, 1985; Ronaghi *et al.*, 1996). Initially used for continuous monitoring of DNA polymerase activity in 1985, This reaction begins with the trial of each dNTP in the nucleic acid polymerisation reaction. When DNA polymerase incorporates one of the dNTPs into the chain extension, PPi is released. Conversion of PPi to ATP via ATP sulphurylase is quick to follow emitting light through the firefly luciferase that catalyses luciferin into oxyluciferin. The number of dNTPs incorporated per chain at that step is proportional to the

average number of emitted photons per chain in a given step. The DNA sequence can then be determined simply by noting if incorporations occur and by counting the number of incorporations in a given attempt. Alternatively, the amount of light emitted can be measured by avalanche photodiode, photomultiplier tube or with a charged-coupled device camera. At present, there are two distinct pyrosequencing tactics: solid-phase sequencing and liquid-phase sequencing. The solid-phase approach, which employs a three-enzyme mixture, requires binding of the template to a solid support, such as a magnetic bead, in order to avoid a decrease in signal as well as a template-washing step between nucleotide additions to remove the non-incorporated dNTPs and ATP resulting from sulfurylase activity (Ronaghi *et al.*, 1996). In liquid-phase sequencing, a four-enzyme approach, a nucleotide-degrading enzyme, apyrase, is added to eliminate the washing steps; the enzyme degrades the unreacted nucleotides and ATP produced (Ronaghi *et al.*, 1998).

Pyrosequencing has displayed many advantages. Primarily, this technique eliminated the need for labelled primers, labelled nucleotides and gel electrophoresis. Additionally, the solid-phase reaction allows for detection in real time with a cycle time of about two minutes. In addition, the pyrosequencing reaction is performed at room temperature and physiological pH, is adaptable for multiplexed sample processing and short chains can be adequately sequenced since the signal-noise ratio remains relatively high even after forty cycles, (Ronaghi *et al.*, 1998) while being very cost effective (Ronaghi, 2001). This method does however have a significant number of disadvantages, some of which include loss of template and thus decreased signal due to multiple wash steps in the solid-phase approach (Ronaghi *et al.*, 1996) and intermediate product accumulation in phase liquid-phase leading to significantly reduced apyrase activity in later cycles of the reaction (Ronaghi *et al.*, 1998). Pyrosequencing is mainly used in the analyses of

secondary structure, such as hairpin structures (Ronaghi *et al.*, 1999), *de novo* DNA sequencing for short- and medium-length DNA fragments (Nordstrom *et al.*, 2000), mutation detection (Garcia *et al.*, 2000), and analysis of single-nucleotide polymorphisms (Ahmadian *et al.*, 2000).

The final sequencing methodology, single-molecule sequencing, was first devised as a laser-based technique that allows for the fast sequencing of DNA fragments of 40 kb or more at a rate of 100-1000 bases per second (Jett *et al.*, 1989). This system relies on the detection of individual fluorescent nucleotides in a flowing sample stream (Harding *et al.*, 1992; Shera *et al.*, 1990). This method occurs in four distinct stages:

1) The bases within a single fragment of DNA are fluorescently labelled.
2) The labelled fragment is attached onto a microsphere and the supported DNA fragment migrated into a flowing buffer stream (Davis *et al.*, 1991).
3) The labelled DNA is digested with exonuclease that sequentially cleaves the 3'-end nucleotides.
4) The individual fluorescently labelled bases are detected and identified of as they cross a focused laser beam (Goodwin *et al.*, 1997).

Though this innovative sequencing technique yields results faster than all other methods, there are still many problems that remain to be unravelled. Such issue involve improved buffer quality, integration of a selection step, biochemical basis to label complementary DNA strands with four distinct nucleotides and improved polymerases and exonucleases for rapid and efficient sequencing (Stephan *et al.*, 2001).

IX. PROJECT

i. Question

How does the nature and structure of DNA change through the process of degradation?

ii. Purpose

The purpose of this research is to assess the quality and quantity of DNA within a sample so that the nature and structure of DNA could be studied and how it changes through the process of degradation. This project focuses on a number of characteristics of degraded DNA including fragmentation, quantity, and single nucleotide damage. There are numerous mechanisms through which each of these features can occur; although each mechanism was not studied the information generated is just as informative. Fragmentation can indicate single or double strand breaks within the DNA, which can be caused by oxidative, hydrolytic, enzymatic or physical damage. The DNA fragmentation will be evaluated through the design of novel multiplex PCRs coupled with capillary electrophoresis detection and analysis. Single nucleotide damage can be caused by the same process but are manifested in DNA as abasic sites, single stranded nicks, modified bases or cross-linked to other molecules. We will use DNA sequencing followed by detailed data analysis to determine the trend of DNA degradation over time. Sample quantification will indicate the relative recovery and preservation of DNA. The relative quantification of our samples using real time PCR will compare the number of DNA copies within a modern to that within samples of unknown age. This work and further research will more accurately provide an understanding of the nature and structure of DNA in degraded samples especially forensic or archaeological samples.

iii. Methodology

Method design includes optimisation of multiplex PCR, real-time PCR and capillary electrophoresis to detect and characterise DNA damage. This characterisation involves the design of a highly specific multiplex PCRs, which target precisely selected fragments within a DNA target region. This project will include the design of three multiplex PCRs including a mtDNA, a nDNA and a Y-chromosome multiplex. Though all three will be designed and applied concurrently, the mtDNA multiplex will be used in exemplary analysis of fragmentation trends. The quantification analysis of fragment distribution using capillary electrophoresis detection, coupled with Genescan® analysis software, will allow us to determine the extent of fragmentation of various DNA samples. Once designed, all three multiplex PCRs act as a DNA fragmentation assessment tool ultimately determining the integrity of the DNA.

The post-multiplex PCR DNA sequencing of the region of interest will identify single nucleotide damage over time. The fragment size of interest will be targeted using the appropriate primer set then sequenced, using an automated Sanger method coupled with Genescan® Analysis software, in order to uncover any alterations to the individual DNA molecules caused by direct or indirect DNA damage to a known reference sample.

The coupling of these fragmentation and sequence assessments with real time PCR quantification will compare the relative number of copies within a modern DNA sample to that of older samples of unknown integrity. This approach will determine the relative rate of DNA degradation over time using a specified DNA fragment as a model.

iv. Samples

A study will be conducted using DNA extracted from modern blood samples stored in various conditions for various lengths of time in order to simulate the degradation of DNA over

time. A first set of DNA samples will be extracted using the DNA Stat Blood Kit produced by Stratagene®, assessed using the mtDNA fragmentation multiplex PCR and stored in water at -20°C. The fragment distribution of these samples will be evaluated at specific time intervals in order to assess the fragmentation of mtDNA stored over time at specific temperatures. The resulting PCR products will also be employed to evaluate sequence fidelity over time. These products will be sequenced at specific time intervals and compared to the reference sequence. The reference sequence will be a freshly extracted DNA sample from the same individual as the set of twelve.

We will also assess the effect of DNA in assorted buffer solutions. One set (3 extracts) of samples from the same individual will be stored in each of three separate buffers (water, Tris and TE) thus requiring three sorts of sample purification (silica bead, QIAquick columns and P-30 chromatography columns respectively). The use of three different buffers allows us to determine the efficiency of each one at preserving the DNA while stored in solution. Each sample, in each buffer is stored at 4°C and at -20°C for a period of 12 months, allowing for the identification of the most beneficial storage temperature. It is imperative that the samples be well labelled and tracked using forensic analysis procedures and documentation throughout the process to avoid any confusion or false conclusions.

X. METHODS AND PROCEDURES

i. Samples

The fragmentation analysis was carried out on ten DNA extract of whole blood, from the same individual, extracted using the DNA Stat® Blood Kit supplied by Stratagene. These DNA extracts were then purified using the Micro Bio-Spin® P-30 Tris Chromatography Columns with a buffer exchange protocol in order to have purified DNA in three different buffer solutions, Tris, TE and ddH_2O. Eight of the DNA extracts were purified using ddH_2O as a buffer whereas one extract was purified using each TE and Tris. Each extract underwent an intial detection PCR using the Hot Start protocol in order to confirm successful extraction of the DNA. Once the DNA extraction procedure was verified, all extracts were analysed using the mitochondrial fragmentation multiplex. Six of the eight 50µL DNA extracts in ddH_2O were then aliquoted into 15µL volumes for storage at -20°C. The other two bulk extracts in ddH_2O were stored at -20°C immediately after purification and will be used for the freeze/thaw analysis. Both the TE and Tris bulk extracts were stored at -20°C without aliquoting and were used to compare the degradation within certain storage buffers after 14 days of storage.

The effects of multiple freeze/thaw cycles were assessed using the two bulk extracts in ddH_2O stored without aliquoting. The extracts were removed from -20°C storage and allowed to thaw at room temperature until a visual assessment confirmed complete thawing. The thawed extracts were then immediately analysed using the mtDNA fragmentation multiplex protocol and returned to -20°C for further storage. This freeze/thaw procedure was repeated until the DNA extract was fragmented below 133 base pairs in length.

The DNA samples used for DNA quantification through real time PCR were extracted from bone using the guanidinium thiocyanate protocol followed by purification using the silica bead protocol. All four samples, one modern/reference sample and three unknown samples, were extracted immediately prior to quantification in order to minimize possible degradation caused by freeze/thaw cycles and extended storage.

ii. Extraction Protocols

DNA extraction is accomplished through many methods. The sample tissue type and taphonomy often determine the extraction method employed. This research utilises fresh modern blood samples and thus does not require extensive extraction procedures. In this case, the blood is taken within 24 hrs of extraction thus is in contact with minimal amounts of PCR inhibitors. We will employ the DNA Stat Blood kit protocol by Stratagene® and a Guanidinium Thiocyanate extraction protocol.

a. DNA Stat® Blood Kit (Stratagene)

The following protocol allows us to purify genomic DNA from a 25-200 μL sample of whole blood to be amplified in a PCR. The red and white blood cells are removed from the initial sample using the RBC Lysis Solution and the WBC Lysis Solution respectively. Further details on this protocol can be found in the Stratagene DNA Stat™ Blood Kit Instruction Manual.

Required Reagents:

RBC Lysis Solution (1x) [2 vol. sterile H_2O + 1 container of 3X RBC Lysis Solution]

WBC Lysis Solution

Wash Solution (1x) [4 vol. of 100% ethanol + 1 container of 5X Wash Solution]

Elution Buffer

100% Ethanol

1. Prepare 1x RBC Lysis Solution and 1x Wash Solution.
2. Place a provided microspin cup into a 2 mL microcentrifuge tube and add to it 600 µL of 1x RBC Lysis Solution.
3. Add 40 µL of whole blood to the microspin cup.
4. Microcentrifuge at 17,900 x g for 5 minutes.
5. Discard the resulting supernatant and retain microspin cup in same 2mL tube.
6. Add 600µL of WBC Lysis Solution to the microspin cup.
7. Incubate samples at room temperature for 5 minutes.
8. Microcentrifuge the samples at 17,900 x g for 2 minutes.
9. Discard the resulting supernatant and retain microspin cup in same 2 mL tube.
10. Add 600 µL of 1x Wash Solution to the microspin cup.
11. Microcentrifuge the samples at 17,900 x g for 2 minutes.
12. Discard the resulting supernatant and retain microspin cup in same 2 mL tube.
13. Repeat wash steps 10 through 12.
14. Spin the samples at 17,900 x g for 2 minutes in order to dry the fiber filtrate.

15. Discard the 2 mL tube containing only the supernatant and place the microspin cup into a clean 2 mL tube.
16. Add 50 µL of Elution Buffer directly onto the fiber matrix of the microspin cup and incubate samples at room temperature for 5 minutes.
17. Centrifuge the samples at 17,900 x g for 2 minutes.
18. Discard microspin cup and store the extracted DNA samples at -20°C.

b. Guanidinium Thiocyanate Extraction

The protocol outlined below allows us to isolate genomic DNA, from a sample of whole blood preserved on an FTA® card, to be amplified in a PCR. This extraction protocol was performed on the unknown samples quantified by real time PCR.

Required Reagent:

Guanidinium Thiocyanate (GuSCN) solution

1. Select 3 FTA cardpunches and place into 1.5 mL tube.
2. Add 500 µL of GuSCN solution
3. Incubate at 56°C overnight under gentle agitation of an Eppendorf thermomixer™. (product referred to as GuSCN extract)
4. Proceed with silica bead purification protocol.

iii. Purification Protocols

a. Silica Bead Purification

The protocol outlined below allows us to purify DNA fragments from primers, nucleotides, enzymes, mineral oil, salts, agarose, ethidium bromide and other impurities in preparation for amplification. The silica bead purification protocol ends with storage of the DNA in water, one of three buffers being investigated.

Required reagents:
Guanidinium Thiocyanate (GuSCN) solution
Silica oxide Beads (SiO_2) - acidified
Working wash buffer
Ethanol (100%)
ddH_2O

1. Boil GuSCN extract at 94°C for 10 minutes, with no agitation.
2. Centrifuge at 17,900 x g for 1 minute.
3. Remove the supernatant and place it in a sterile 1.5 mL tube.
4. Add 900 µL GuSCN solution and 10 µL of silica to the sample.
5. Vortex briefly.
6. Place tubes on ice for 60 minutes and invert to resuspend, acidified silica every 15 minutes.
7. Wipe tubes and Zip spin the samples.
8. Remove and discard the supernatant carefully.
9. Add 500 µL of working wash buffer.
10. Vortex for 1minute to resuspend the silica beads.

11. Zip spin the samples.
12. Remove and discard the supernatant.
13. Repeat wash if required (Steps 9 through 12).
14. Add 200 µL of 100% ethanol and vortex for 1 minute.
15. Zip spin the samples.
16. Remove and discard the supernatant.
17. Speed-vac the silica pellet for 5 minutes (or air dry for 30 min.)
18. Add 100 µL of ddH_2O and resuspend (vortex).
19. Incubate at 56°C for 1 hour with no agitation.
20. Store at 4°C for short term or -20°C for long term.
21. Reheat at 56°C for 1 hour and centrifuge tubes at 13 000 rpm for 1 minute prior to each PCR.

b. Micro Bio-Spin® P-30 Tris Chromatography Columns with Buffer Exchange

These chromatography columns are used in order to obtain our mtDNA in a TE buffer. These Bio-Rad columns are composed of polyacrylamide gel suspended in a Tris buffer with 0.02% sodium azide and thus a buffer exchange is required prior to sample purification. These columns are available in a variety of sizes depending on the sample to be purified. The columns used here, P-30 columns, are able to purify DNA strands from 40 to 50 nucleotides in length.

Required Reagents:
Micro Bio-Spin® Columns
Exchange Buffer

1. Snap off tip of column and place column in a provided 2.0 mL microcentrifuge tube.

2. Centrifuge column for 2 minutes in a microcentrifuge at 1 000 x (g) to remove the Tris packing buffer.

⇒ *Buffer Exchange:*

3. Apply the exchange buffer (TE or ddH_2O) in 500 µL aliquot and centrifuge the column for 1minute at 1 000 x (g) to remove the buffer. Discard the buffer.
4. Repeat exchange buffer application to the column and centrifugation a total of 4 times in order to achieve 99.9 % buffer exchange.
5. Place column in clean 1.5mL tube.

6. Carefully apply the sample (40 µL) directly to the centre of the column.
7. Centrifuge the column for 4 minutes at 1 000 x (g).
8. Following centrifugation, the purified sample is now in the exchanged buffer (TE or ddH_2O).

c. Dyex Purification Columns

Purification of a PCR product is required prior to sequencing PCR in order to eliminate dye terminators, dNTPs, DNA primers, buffers and other low molecular weight materials from a sequencing reaction. Once the purification is complete, DNA sequencing may continue using the ABI Prism™ 3100 Genetic Analyser.

1. Gently vortex the spin column to resuspend the resin.
2. Loosen the cap of the column a quarter turn. This is necessary to avoid a vacuum inside the spin column.
3. Snap off the bottom closure of the spin column, and place the spin column in a 2 ml collection tube (provided).
4. Centrifuge for 3 min an approximately speed of 36,000 x g.

5. Carefully transfer the spin column to a clean centrifuge tube. Slowly apply the sequencing reaction (10–20 µl) to the gel bed.

Notes: Pipette the sequencing reaction directly onto the center of the slanted gel- bed surface of the column. Do not allow the reaction mixture or the pipette tip to touch the sides of the column. The sample should be pipetted slowly so that the drops are absorbed into the gel and do not flow down the sides of the gel bed. Avoid touching the gel-bed surface with the pipette tip. This protocol is suitable for sequencing reactions with volumes of 10–20 µl. For easier handling, more reproducible pipetting, and reduced error with sample volumes <10 µl, we recommend adjusting the volume to 20 µl using distilled water, before application to the gel-bed. It is not necessary to remove mineral oil or kerosene prior to cleanup of dye-terminator sequencing reactions. It is not necessary to replace the lid on the column.

6. Centrifuge for 3 minutes at the calculated speed.

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

8. Desiccate the sample in a vacuum centrifuge and proceed according to the instructions provided with the DNA sequencer.

iv. ***Amplification Protocols***

a. Primers

Primer selection is of great importance in the study of DNA. In this research, a target area within the mtDNA sequence was chosen arbitrarily to represent the mtDNA as a whole. The target area was chosen to be within the HVR I region of the mtDNA since it is well established and reagents and resources are abundant and easily accessible. All selected primers were chosen following the above specifications. The HVR I region was evaluated for possible primer locations and primer binding and interactions were simulated using Amplify® (Engels, 1993), see Figure XI. The chosen primers display complete binding to their target and minimal primer-dimer associations. See selected primer information in Table 3.

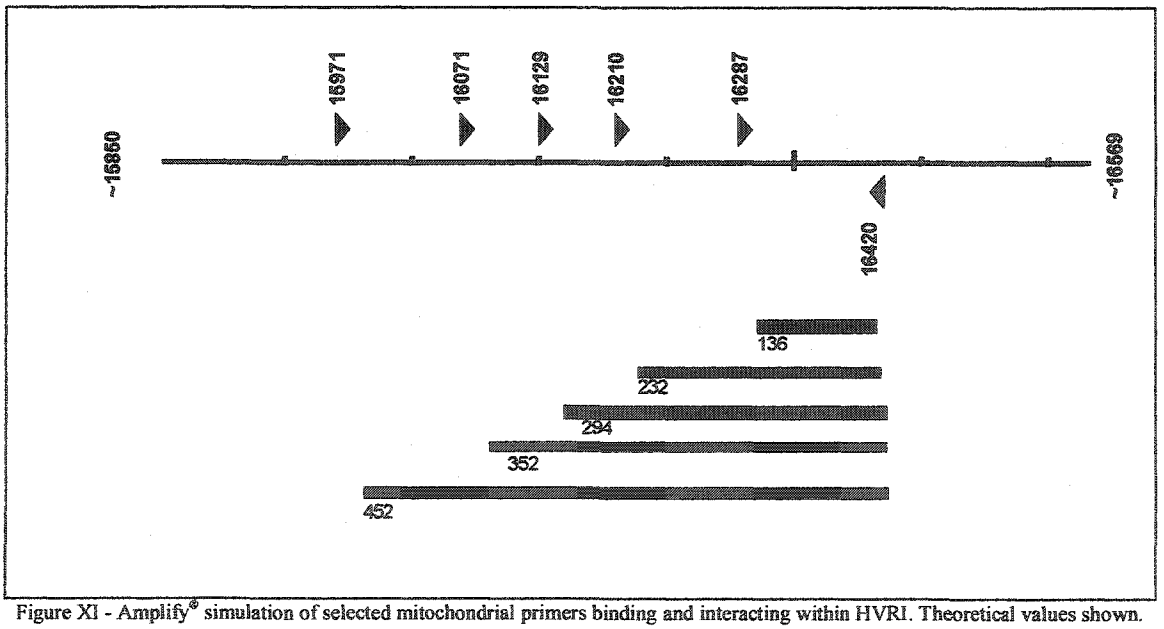


Figure XI - Amplify® simulation of selected mitochondrial primers binding and interacting within HVRI. Theoretical values shown.

Table 3 - Selected mtDNA multiplex primers

Primer name and sequence	Length (bp)	Tm (°C)	% GC Content
mt15971F TTA ACT CCA CCA TTA GCA CC	20	58.35	45
mt16071F CCC ATC AAC AAC CGC TAT G	19	57	53
mt16129F GGT ACC ATA AAT ACT TGA CCA C	22	58	41
mt16210F CCC ATG CTT ACA AGC AAG TA	20	56	45
mt16287F CTA CCC ACC CTT AAC AGT AC	20	58	50
mt16420R TGA TTT CAC GGA GGA TGG TG	20	58	50

The nuclear DNA multiplex PCR was designed for the human beta globin gene. This gene is responsible for the secondary structure of polypeptide chains in adult heamoglobin (Saiki *et al.*, 1985). The primers were designed following the above mentioned primer selection rules with the exception of the HBB1F primer which was taken from published work (Saiki *et al.*, 1985). Various samples were analysed using this mulitplex (data not shown) however this assay was not used in the time study and thus is not the focus of this study.

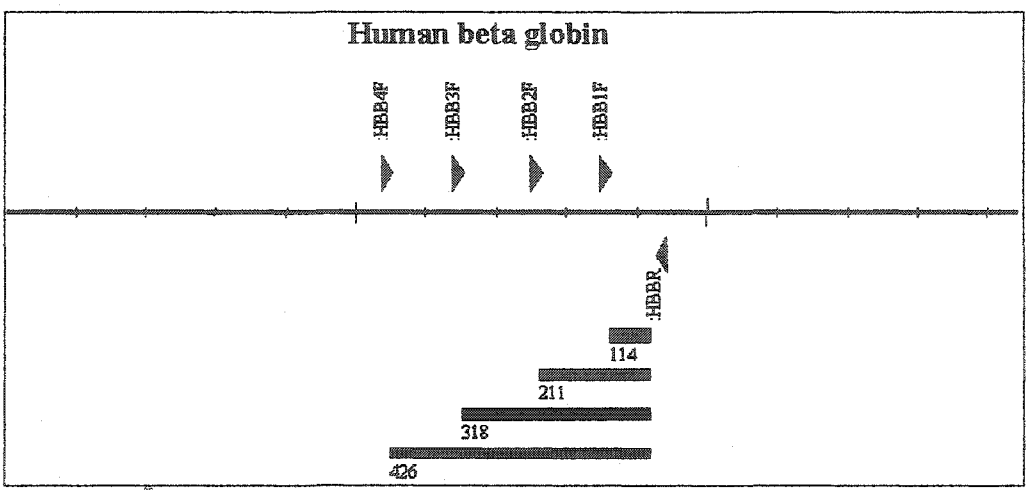


Figure XII - Amplify® simulation of chosen human beta globin primers binding within the HBB gene. Theoretical values shown.

The Y-chromosomal fragmentation multiplex detection system was designed using the amelogenin gene located on both the X- and Y-chromosomes. This assay was not used in our time study and thus is not the main focus of this research.

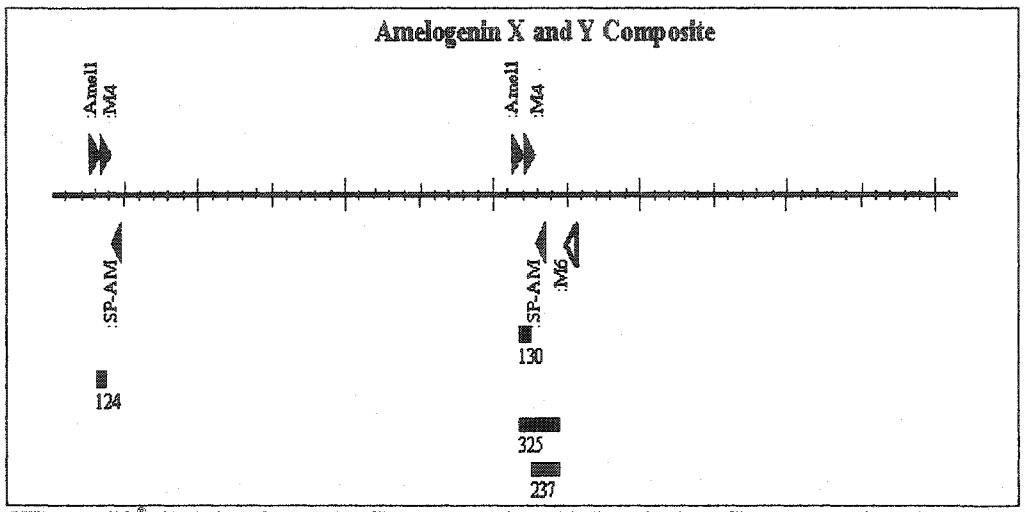


Figure XIII - Amplify® simulation of selected Y-Chromosome primers binding with the Y-Chromosome. Theoretical values shown.

b. Hot Start

The initial detection PCR of all modern and aged samples was performed using a PCR mixture with the final concentrations seen below.

Components	Final Concentration
10X PCR Buffer, Minus Mg	1x
10mM dNTP mixture	200μM
50mM MgCl2	2mM
mt16210F (10μM)	0.2μM
mt16401R (10μM)	0.2μM
Template DNA	n/a
Platinum Taq DNA Polymerase	1.25 U
ddH_2O	n/a

All sample amplification was carried out in an Eppendorf Mastercycler®. The samples were amplified using the following thermal profile:

Temperature (degrees C)	Time	Repeat
94.00	10:00	1
94.00	1:00	35
60.00*	1:00	
72.00	2:00	
72.00	2:00	1
4.00	Hold	

* This annealing temperature has been optimised for the primers used in this specific PCR and do not directly correlate to the target template.

c. *Fragmentation Multiplex Protocol*

1. Combine the following components in a sterile 0.5mL tube:

Components	Volume (mL)	Final Concentration
10X PCR Buffer, Minus Mg	2.00	1x
10mM dNTP mixture	0.40	200 μM
50mM MgCl2	0.80	2 mM
Primers (10 μM): mt15971F	0.28	0.14μM
mt16071F	0.28	0.14 μM
mt16129F	0.20	0.1 μM
mt16210F	0.06	0.03 μM
mt16287F	0.05	0.025 μM
mt16420R*	0.80	0.4 μM
Template DNA	1.00	n/a
Platinum Taq DNA Polymerase	0.20	1 U
ddH_2O	13.93	n/a

The above primer concentrations have been experimentally optimised for this multiplex PCR. Preparation of a master mix is highly recommended for multiple reactions, to minimise reagent loss and to enable accurate pipetting.

2. The PCR should be performed using the following thermal profile:

Temperature (degrees C)	Time	Repeat
94.00	2:00	1
94.00	1:00	45
60.0**	1:00	
72.00	2:00	
4.00	Hold	

* Primer is fluorescently labeled using 6-FAM for detection using Genescan®.

** This annealing temperature has been optimised for this specific multiplex PCR and thus do not directly reflect the melting temperatures of each individual primer.

d. DNA Sequencing Protocol

1. Combine the following components in a sterile 0.2 mL tube for each purified PCR sample to be sequenced:

Component	Volume (mL)
Big Dye Terminator v.3.1	3.0
Primer: mt16401R	0.3
PCR product	7.0

2. The PCR should be performed using the following thermal profile:

Temperature (degrees C)	Time	Repeat
96.00	0:30	40
50.00	0:15	
60.00	4:00	
4.00	Hold	

3. Purify the PCR product using DTR columns.

4. Desiccate the purified product.

5. Perform sequence detection using the ABI Prism™ 3100 Genetic Analyser.

v. Quantification Protocols

a. Real Time Quantification – SYBR Green Protocol

1. Prepare the ABI 7000™ software prior to preparing the PCR reaction using the SYBR® Green template file.

2. Add the following components to a sterile 0.5mL tube:

Components	Volume (μL)	Final Concentration
10X PCR Buffer, Minus Mg	12.50	2.5x
10mM dNTP mixture	1.00	200μM
50mM MgCl2	1.50	1.5mM
Primer (10μM): mt16210F	1.00	0.2μM
mt16420R	1.00	0.2μM
Dimethyl Sulfoxide (DMSO)	1.00	2%
Template DNA	1.00	n/a
Platinum Taq DNA Polymerase	0.40	2 U
SYBR® Green(10X)	1.00	0.5x
ddH_2O	29.6	n/a

Note: Preparation of a master mix is highly recommended for multiple reactions, to minimise reagent loss and to enable accurate pipetting.

3. The PCR should be performed using the following SYBR® Green thermal profile:

Temperature (degrees C)	Time	Repeat
50.00	2:00	1
94.00	2:00	1
94.00	0:30	40
60.00 *	0:30	
72.00	1:00	
72.00	2:00	1

Post extension storage at 4°C is not an option with the ABI7000.

* This annealing temperature was experimentally optimised for this specific target region.

b. Relative Quantification

The relative quantification is used to compare the comparative amounts of DNA between two samples through the assessment of their threshold cycles (C_T) as determined through real time PCR. This type of quantification requires the use of a reference sample to which all unknowns will be compared. This study uses a modern control as the reference sample and thus allows us to determine the relative amount of degradation of unknown samples over time. The threshold cycle of each sample is determined by performing the above protocol in combination with the ABI Prism® Sequence Detection System. The relative quantification of the unknown samples are determined using equation (Equation 3) where $\Delta Ct = Ct_{observed} - Ct_{reference}$ (Applied Biosystems, 2001). The resulting value is the fraction of DNA relative to that of the reference sample thus unknown samples are a fraction of the reference, set to 1.

Equation 3

$$RelativeQuantification = 1 / (2^{-\Delta Ct})$$

vi. Detection Protocols

a. Polyacrylamide Gel electrophoresis

PAGE gels were employed in order to visualise our PCR product and determine its amplification size. PAGE was used rather than agarose gels since it easily resolves fragments of DNA differing in length by a single base pair. The following protocol includes pouring, loading and running of the gels.

Pouring the gels:

Required reagents to make 12 - 5% Polyacrylamide gels:

5X TBE	20 mL
Acrylamide	12.5 mL
ddH_2O	66.5 mL
TEMED	90.0 µL
10% APS	900.0 µL

1. Prepare 10% APS solution by mixing 0.1 g APS to 1 mL of ddH_2O.
2. Place a 125 mL Pyrex flask in an icebox and allow cooling.
3. Set up gel cassettes and combs ahead of time.
4. Add reagents in same order as they are listed above and mix well.
5. Fill gel cassettes from one corner using a transfer pipette ensuring that the bottom groove is completely filled and does not have any air bubbles.
6. Push comb into the gel making sure there are no bubbles.
7. Allow gel to set for 1 hour at room temperature.

Gel apparatus preparation:

1. Obtain a gel cartridge and remove the strip of tape and the gel comb.
2. Place the gel cartridge into the gel apparatus.
3. Fill the apparatus with 1X TBE running buffer. Make sure the running buffer covers the wells inside the apparatus, and is one third full in the outside cartridge compartments.
4. Flush the wells with a Pasteur pipette to remove any air bubbles.

Loading and Running the PAGE:

1. Sample preparation: 7 µL of PCR product mixed with 3 µL of 6X loading dye.
2. Load a size standard and sample into gel wells.
3. Set voltage to 110V and the time to 55 minutes on the electrophoretic power supply.
4. Once the gel has run, stain it with ethidium bromide ($EtBr_2$) for 15 minutes.
5. Rinse gel with ddH_2O then view it on the transilluminator (wavelength UV B) and photograph.

b. Capillary electrophoresis size detection system

An ABI Prism Genetic Analyzer™ 310 equipped with the GeneScan ™ software was employed in order to enhance size detection of the various bands during the optimisation of a multiplex PCR. High precision DNA fragment sizing using this method relies on the presence of an internal fluorescence-based lane standard named GENESCAN-500 LIZ® developed by Perkin-Elmer's Applied Biosystems Division. This size standard is designed for sizing DNA fragments in the 35-500bp range and provides 16 single-stranded, uniformly spaced fragments. Further details on this protocol can be found on the ABI website (Applied Biosystems, 2000).

Required Reagents:

Deionized formamide

Labelled size Standard (Liz 500)

PCR product of interest

1. For each sample, combine 12 µL of deionized formamide with 0.5 µL of size standard (Liz 500).
2. Distribute 12 µL of the above mixture into 0.5mL tubes.
3. Add 2 to 3 µL of PCR product to each tube.
4. Vortex and centrifuge each tube.
5. Denature the samples by heating them at 95°C for 5 minutes.
6. Cold shock them by placing them in freezer for 5 minutes.
7. Load 14 µL of each prepared sample into a 96 well plate.
8. Load plate into clean, calibrated ABI 310 instrument.

XI. RESULTS

i. Fragmentation Analysis

Initial design of the multiplex PCR includes five forward primers with a single reverse primer common to all, thus allowing for the study of five amplicons. Despite the use of the five forward primers on a freshly extracted DNA sample, first attempts showed only four amplicons on the electropherograph (see Figure XIV). The 254bp fragment seen in the simulation PCR (Figure XI) is not seen in the results despite the inclusion of the corresponding primer in the multiplex PCR analysis. The unsuccessful primer was consequently removed in order to minimise primer-primer interactions. The detection of this four forward primer multiplex revealed only two very weak amplicons, a failure of the multiplex PCR optimisation (see Figure XV). All further research was conducted using all five primers.

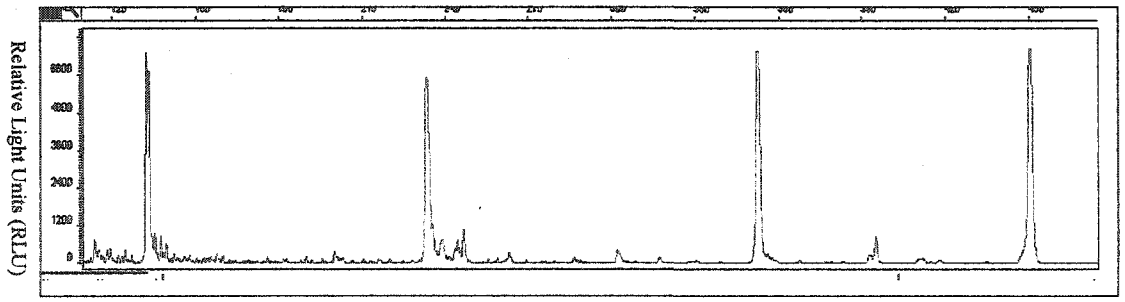


Figure XIV - Electropherograph of mtDNA fragmentation analysis multiplex using five forward primers.

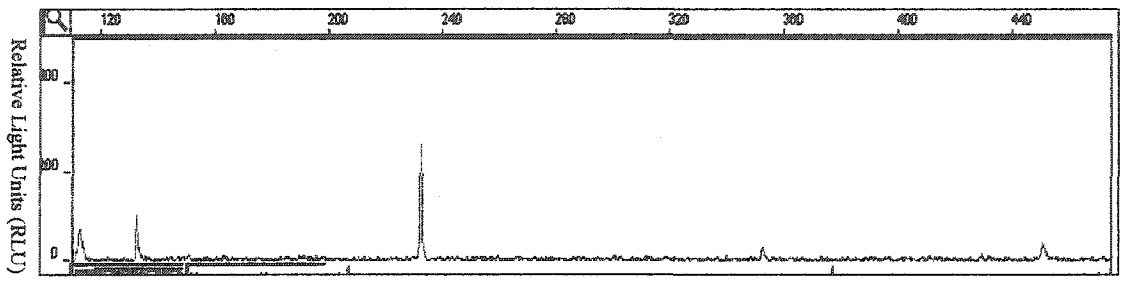


Figure XV - Electropherograph of mtDNA fragmentation analysis multiplex using four forward primers.

The samples analysed for fragmentation were tested each month over a 12-month period. An obvious increase in fragmentation was seen through the study of the four chosen fragment sizes: 133 bp, 232 bp, 352 bp & 450 bp. The differences between the observed fragment sizes compared to those predicted using the simulation software are attributed to the inability of the software to predict exact reaction conditions. The quantities of each fragment are expressed in terms of peak height, a measure of relative fluorescent units (RFU), thus denoting the relative quantity of each fragment. Initially, when the DNA was freshly extracted, all four fragments were present in the same quantities, as reflected by the RFU, since the mtDNA is completely intact. As time progressed, all fragments were not present in equal quantities and a pattern of degradation emerged. The largest fragment (450 bp) was the first to decrease significantly in concentration after 3 months, followed by the second largest (352 bp) after 3.5 months, then the third largest (232 bp) after 6 months and then the smallest (133 bp) after 9 months, as seen in Figure XVI. After 9 months only the smallest fragment length was present in detectable quantities to finally become undetectable after 12 months.

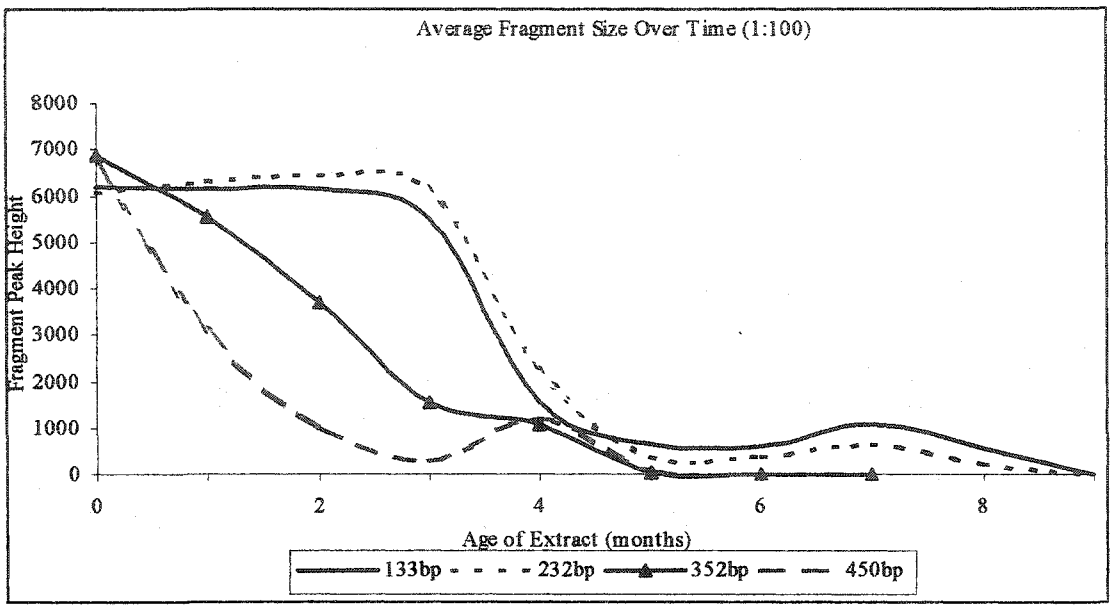


Figure XVI - Fragmentation of aged DNA extracts over time

A monthly survey was performed of the stages of DNA fragmentation over 12 months. As seen in Figure XVII, the freshly extracted DNA samples had approximately equal concentrations (100 %) of each fragment size of interest. The decrease in large fragments becomes very apparent after 3 months, as seen in Figure XVIII. At this point, the 450 bp fragment has dropped off by 95.8 %, the 352 bp dropped off by 77.63 % while the second smallest fragment (232 bp) has increased slightly to 100.9 % of its original concentration and the smallest fragment (133 bp) dropped by 10.9 %. Figure XIX demonstrates the fragmentation of the DNA extract after four months of storage.

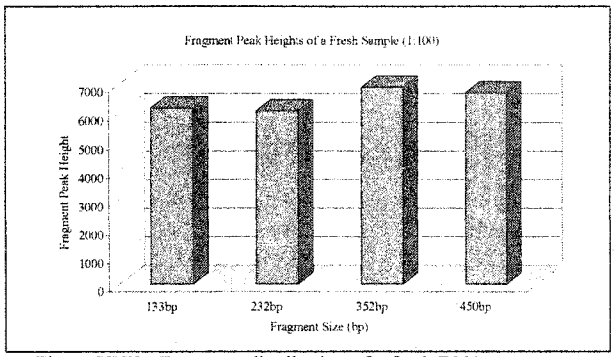


Figure XVII - Fragment distribution of a fresh DNA extract.

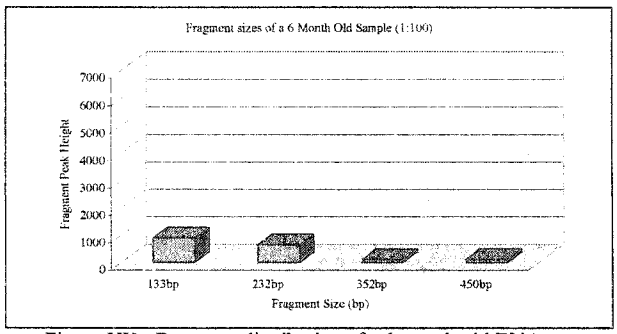


Figure XX - Fragment distribution of a 6-month-old DNA extract

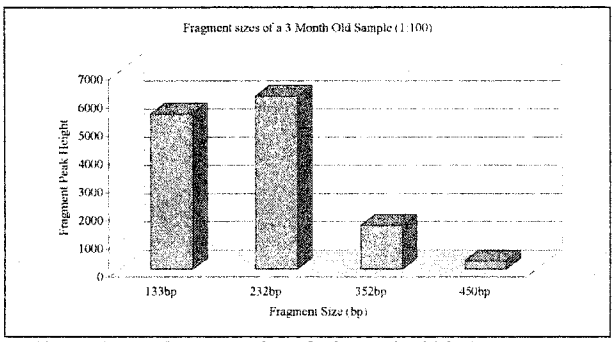


Figure XVIII - Fragmentation of a 3-month-old DNA extract

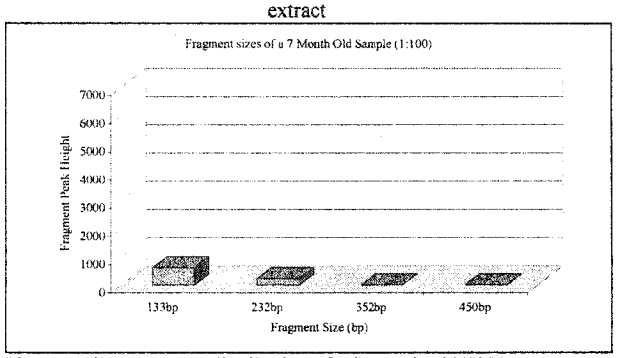


Figure XXI - Fragment distribution of a 7-month-old DNA extract.

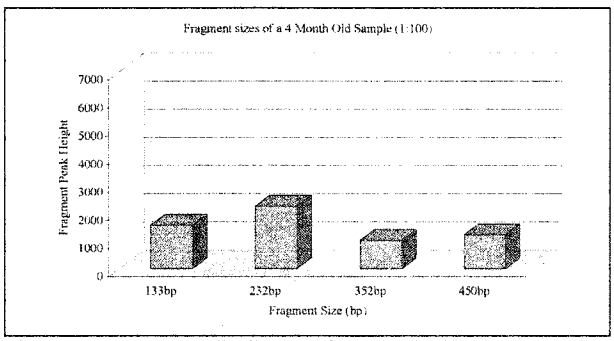


Figure XIX - Fragment distribution of a 4-month-old DNA extract.

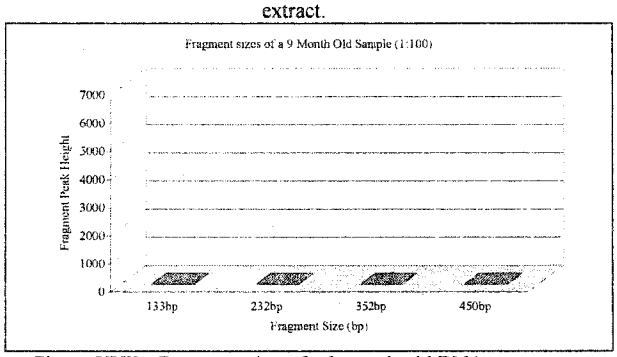


Figure XXII - Fragmentation of a 9-month-old DNA extract.

This graph shows a significant decrease (450 bp dropped by 82 %, 352 bp dropped by 85.3 %, and 232 bp by 63.4 %) in the amount of all fragments from their initial intensity but more importantly a large decrease (74.9 %) in the amount of 133 bp fragment. After six months, see Figure XX, fragmentation of the mtDNA region of interest continues to increase leading to low levels of the 352 bp (98.5 % decrease) and 450 bp (98.4 % decrease) fragments and eventual absence of their detection after seven months (Figure XXI). The smaller fragment sizes, 133 bp

and 232 bp, are also decreasing in intensity to 10.9 % and 14.7 % of their initial intensity after six months and 3.7 % and 9.9 % after seven months respectively. The analysis at nine months, results seen in Figure XXII, show a complete fragmentation (all fragments 0 % of initial intensity) of the mtDNA into small (<133 bp), undetectable fragments.

The overall distribution of fragments during DNA degradation was assessed through the analysis of the fragmentation distribution over time. These assessments will alow us to test for viable DNA template and/or the presence of single and double stranded nicks. The combined peak height of the four fragment sizes within our multiplex analysis yielded a peak height equal to that of the overall amount of 133 bp fragments. The fragment distribution observed at specific time intervals can be seen in Figure XXIII. The fragment distribution of the reference sample was analysed prior to any type of storage. This yielded an equal distribution of the 133 bp, 232 bp, 352 bp & 450 bp fragments at 24 %, 24 %, 27 % and 26 % respectively yielding a combined peak height of 25,841units. When the same sample was analysed after three months, the four fragment sizes are no longer present in same quantities. The total peak height of 13 455 units for this sample is made up of 40.89 % of 133 bp fragment, 45.57 % of 232 bp fragment, 11.46 % of 352 bp fragment and 2.08 % of 450 bp fragment. After four months of storage, the total peak height of the sample is 6002 and is composed of 25.84 % of 133 bp fragment, 37.07 % of 232 bp fragment, 16.93 % of 352 bp fragment and 20.16 % of 450 bp fragment. After six months of storage, the overall peak height of 1768 units is broken down as follows: 50.90 % of 133 bp fragment, 37.46 % of 232 bp fragment, 5.67 % of 352 bp fragment and 5.98 % of 40 bp fragment. At the seven-month time interval, only the two smaller fragments 133 bp and 232 bp amplified and contribute 73.15 % and 26.85 % respectively, to the overall 840 unit peak height. The analysis at the final time interval of nine months does not yield peak heights and thus no

fragments, of any length, remain in the solution. The overall trend of DNA degradation in solution indicates an exponential degradation of all fragments sizes over a storage period of nine months, see Figure XXIV. The combined peak heights of all four fragment sizes for samples at specific time intervals displays a quick rate of degradation with an exponential drop at the 3.5 month time interval. After nine months of sample storage, the DNA in solution has fully degraded into fragments smaller than 133 bp.

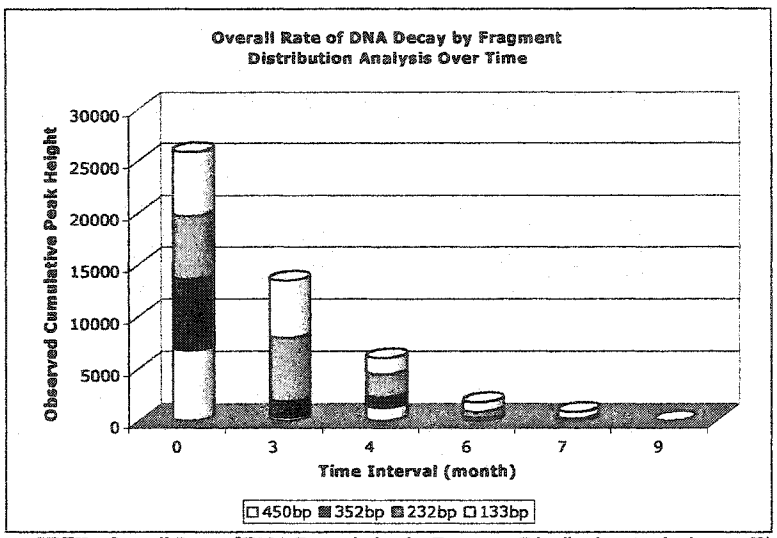


Figure XXIII - Overall Rate of DNA Degradation by Fragment Distribution Analysis over Time.

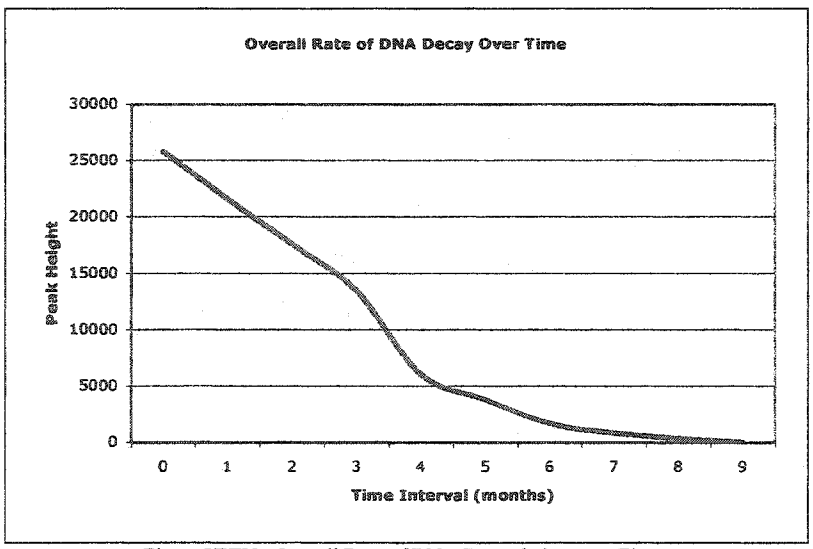


Figure XXIV - Overall Rate of DNA Degradation over Time.

The analysis of DNA storage in three separate buffers: water, Tris and TE yields very significant results, seen in Figure XXV. First, using Tris as a storage buffer yields similar intensities of each DNA fragment size in a freshly eluted sample. The DNA eluted into water also yielded similar intensities of each fragment size. In comparing the Tris and water samples, we see that they are within the same range of peak heights, as well fragment size distribution. The sample in TE however displays very low and unbalanced levels of each fragment size. After 14 days of storage at 4°C, the distribution is altered drastically, see Figure XXVIII. The fragment distribution of the sample stored in ddH_2O remains stable throughout the storage period, in contrast to the samples stored in Tris and TE. In Figure XXVII, we see a substantial change in fragment distribution after 14 days of storage at -20°C. The observed fragmentation is much higher in the mtDNA samples stored in ddH_2O and TE compared to the Tris. The analysis of samples stored in Tris display a more consistent intensity of each fragment size when compared to both the ddH_2O and TE samples.

The study of storage conditions also includes the comparison of samples eluted into a specific buffer, either water, Tris or TE, and stored at different temperatures, 4°C or –20°C. Storage of DNA in water at 4°C or -20°C results in similar peak heights with a slight drop of the 349 bp fragment peak height, see Figure XXVI, when stored at -20°C. Storage of DNA samples in a Tris buffer at 4°C or –20°C yields very similar electropherograph peak height, as seen in Figure XXIX. When DNA is stored at 4°C and –20°C in a TE solution, the fragment distribution of the two samples is nearly identical, see Figure XXX.

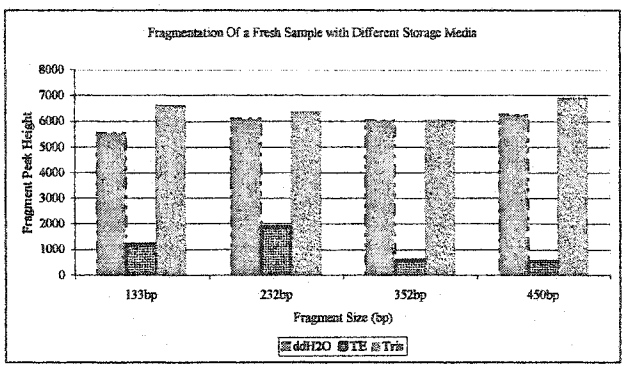


Figure XXV – Fragmentation of fresh extracts in various solutions.

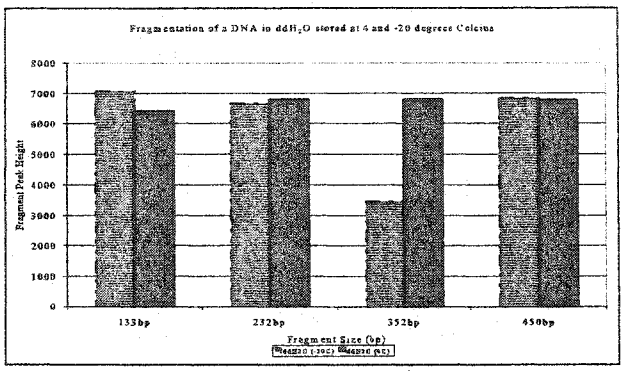


Figure XXVI – Fragmentation of 14 day-old extracts stored in ddH_2O at 4°C and -20°C.

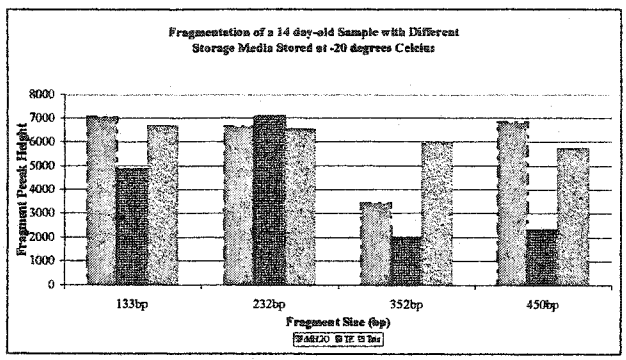


Figure XXVII – Fragmentation of 14 day-old extracts in various solutions sored at -20°C.

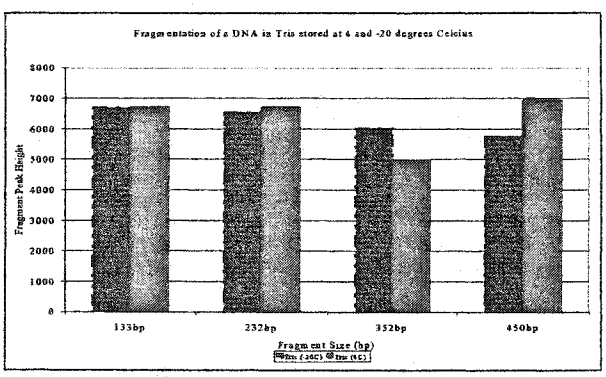


Figure XXIX – Fragmentation of 14 day-old extracts stored in Tris at 4°C and -20°C.

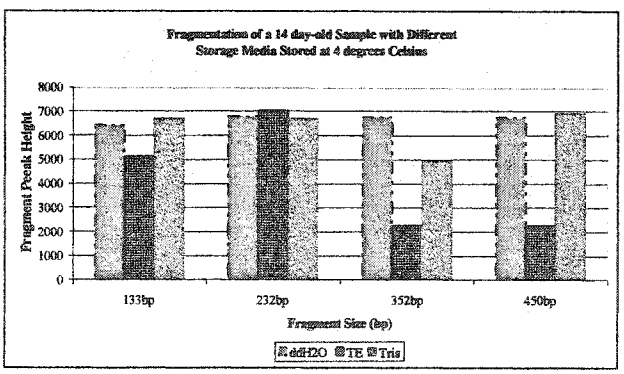


Figure XXVIII – Fragmentation of 14 day-old extracts in various solutions stored at 4°C.

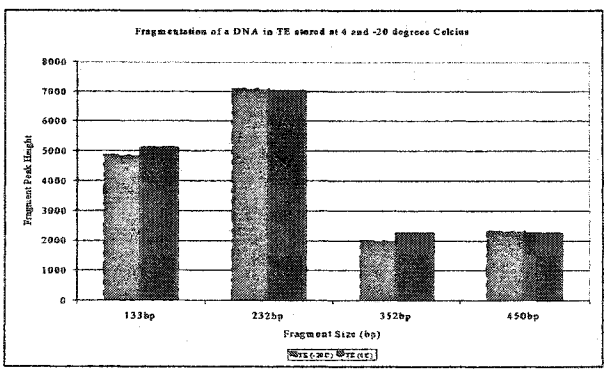


Figure XXX - Fragmentation of a 14-day old extract stored in TE at 4°C and – 20° Celsius.

The effects of freeze/thaw cycles were examined using specific samples stored at -20°C and thawed at specific intervals. This study reveals a large decrease in the number of 133 bp and 232 bp fragments remaining after a number of freeze/thaw cycles. The larger fragment, 232 bp long, degrades much more quickly than the smaller, 133 bp long, fragment, as seen in Figure XXXI. After six cycles of freeze/thawing, very few copies of each fragment size remains undetectable.

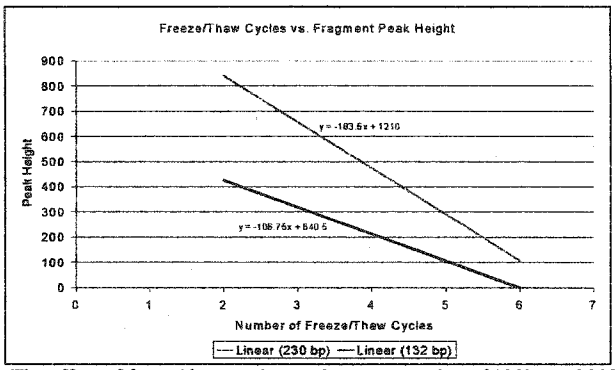


Figure XXXI - The effect of freeze/thaw cycles on the concentration of 133bp and 232bp fragments.

ii. Sequence Analysis

The sequences of 12 distinct DNA samples from the same individual were obtained using the Sanger Method through the Big Dye Terminator (v.3.1) kit, and the ABI 3100™ using Genescan™ Software. The sequencing reaction had the target region starting with the mitochondrial DNA position 16420 and going towards the start of the HVRI sequence. This target sequence was found to have a break down of 37.4 % cytosine, 32.2 % adenine, 19.4 % thymine and 10.9 % guanine as seen in Table 4.

Table 4 - Base content of sequenced target region (of HVR I nucleotide postions mt16210 to mt16420)

Base	Target Sequence Percentage (%)
Adenine (A)	32.2
Cytosine (C)	37.4
Guanine (G)	10.9
Thymine (T)	19.4

The resulting sequences were closely examined for any misread error by the software. The evident read errors were corrected, however, the questionable errors were not altered. Samples with excessive uncertain error were discarded and not used in this study. The 12 DNA samples were stored at -20°C for 1 month at which time the DNA was sequenced a second time. This second sequence was then compared manually and with a biological sequence alignment editor, BioEdit®, to the reference sequence, a fresh extract of this same individual. A significant number of modifications were observed when comparing these 12 sequences to the reference sequence, however only those with an incidence of 20 % or higher, as seen in Table 5, were considered to be authentic. All observed base substitutions are found in Appendix A.

Table 5 - Observed Base Modifications after 1 month of storage at -20°C.

mtDNA position	change	Type	Occurrence (%)
16210	A - G	transition	**20.0**
16221	C - G	transversion	**46.7**
16228	C - T	transition	**26.7**
16250	C - G	transversion	**20.0**
16262	C - G	transversion	**20.0**
16270	C - G	transversion	**20.0**
16286	C - T	transition	**66.7**
16290	G	insertion	**20.0**
16292	C - G	transversion	**20.0**
16293	C - G	transversion	**50.0**
16295	C - G	transversion	**41.7**
16301	A - C	transversion	**35.7**
16303	pyrimidine	insertion	**73.3**
16331	G - C	transversion	**26.7**
16335	C	insertion	**25.0**
16340	C - G	transversion	**33.3**

As seen in Table 6, the base mutations are predominantly transversion type (78.6 %) alterations while transition mutations are more infrequent (21.4 %). The most common transversion is C - G with an incidence of 64.3 %, followed by the A - C and G - C transversions both taking place 7.1 % of the time. The most frequent base modification observed is the modification of cytosine seen 78.6 % of the time, followed by adenine at 14.3 % and finally guanine at 7.1 % occurrence, see Table 7. There were no modifications of thymine observed, thus it is not included in Table 7.

Table 6 - Percentage of Observed Types of Base Substitutions

Type of Base Substitution	Percentage Observed (%)
Transition	21.4
Transversion	78.6

Table 7 - Observed modifications of the individual bases.

Base Modified	Overall Observed Percentage (%)
Adenine (A)	14.3
Cytosine (C)	78.6
Guanine (G)	7.1

iii. Quantification Analysis

Real time PCR using SYBR® Green allows for the quantification of DNA through the detection of emitted fluorescence, since it is proportional to the amount dsDNA present at any given time during amplification and thus allowing for the calculation of the initial template copy number. The real time analysis of two recently extracted modern DNA samples using the SYBR® green detection system reveals the threshold cyle number (Ct) values of 24.40 and 25.87 (see Figure XXXII and Table 8). The two PCR controls analysed in this same PCR reveal no amplification thus yield undetectable Ct values. The real time analysis of three newly extracted, unknown DNA samples yields Ct values of 36.41, 37.68 & 39.12 (see Figure XXXIII and Table 8). The modern control included in this real time analysis, results in a Ct of 24.40 while the incorporated PCR control displays no amplification consequently resulting in an undetectable Ct value.

The relative quantity of DNA contained in the the three unknown samples was determined using Equation 3 with the modern sample as the reference sample. Unknown samples 1, 2 and 3 contain 4.12×10^3, 2.70×10^4 and 9.95×10^3 times less DNA than the reference sample respectively (as seen in Table 8, calculated using Equation 3).

Table 8 - The relative quantity of DNA found unknown samples in respect to a reference sample.

Sample	Ct	Relative Quantity
Modern	24.40	1.00E+00
Unknown 1	36.41	-4.12E+03
Unknown 2	39.12	-2.70E+04
Unknown 3	37.68	-9.95E+03

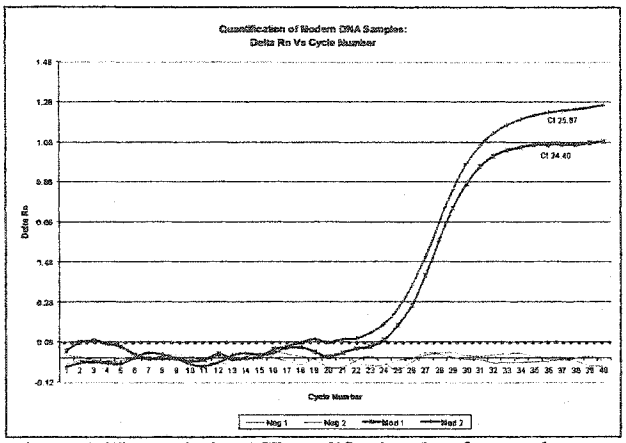


Figure XXXII - Real Time PCR amplification plot of two modern DNA samples with two PCR controls.

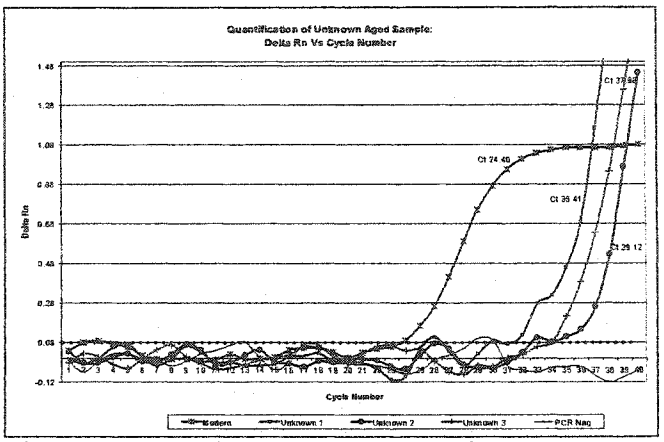


Figure XXXIII - Real Time PCR amplification plot of 3 unknown DNA samples, one modern sample and one PCR control.

XII. DISCUSSION

In this project, methods were developed to assess the quality and quantity of DNA within a sample so that the nature and structure of DNA could be studied and changes through the process of degradation could be identified. This project focused on a number of features inherent in degraded DNA including fragmentation, quantity, and single nucleotide damage. There are numerous mechanisms for each of these features; although each mechanism was not studied the information generated is just as informative. Fragmentation can indicate single or double strand breaks within the DNA, which can be caused by oxidative, hydrolytic, enzymatic or physical damage. Single nucleotide damage can be caused by the same process but are manifested in DNA as abasic sites, single stranded nicks, modified bases or cross-linked to other molecules. The quantification analysis will indicate the relative recovery and preservation of DNA. This work and further research will more accurately provide an understanding of the nature and structure of DNA in forensic or archaeological samples.

i. Fragmentation Analysis

The assessment of DNA fragmentation over time was performed to identify possible sources and mechanisms of DNA damage. The evaluation of DNA fragmentation over time is not a widely studied topic and thus required the design of a novel assessment tool. The method of choice was a multiplex PCR targeting a number of small converging amplicons, forming a larger, uniform DNA fragment. The use of these smaller amplicons contained within the larger fragment allows us to track the degradation of the large DNA segment into sections at specific time intervals. The design of such a multiplex would allow us to evaluate the size of the DNA fragments over time for any type of DNA, including mitochondrial and nuclear DNA as well as

for the Y-chromosome. The approach to multiplex optimization of each multiplex is the same since all the same problems are encountered. The design of this novel multiplex reaction involving these target sections allowed us to evaluate the fragmentation of each section at one time, minimising analysis time and costs. This technique may be applied as a screening process for forensic, or archaeological samples of unknown quality. The multiplex fragmentation analysis would quickly and easily reveal the degree of fragmentation, thus allowing for further analysis using appropriate amplicon sizes decreasing case result turnover times. This advantageous method was designed for three specific target DNA, mitochondrial, nuclear and specifically for the Y-chromosome however only the mitochondrial multiplex was used in the time study of fragmentation. The mitochondrial multiplex PCR will serve as our in-depth analysis example to reduce repetition, but all concepts and techniques may also be applied to the nuclear DNA and the Y-chromosome.

The assessment of fragmentation over time required that all fragment sizes be present in equal quantities initially in order for each fragment to be representative. The equal distribution of all fragment sizes posed the biggest difficulty since the smaller amplicons preferentially amplify thus prevailing over the larger ones. To overcome this obstacle, the multiplex PCR was optimised by employing distinct primers and primer concentrations for the various amplicon sizes, by altering the concentration of magnesium chloride ($MgCl_2$) and of each dNTP, by using specific annealing and extension temperatures, by using positive control DNA samples as well as by incorporating PCR additives including BSA, DMSO and the extra oligonucleotide primer.

First and foremost, target amplicons and thus primer sets must be chosen very carefully during the design of a multiplex PCR to ensure equal amplification of each fragment length. Adherence to the primer selection rules including primer length, nucleotide content, GC content,

secondary structure, target complementation and primer annealing temperature is compulsory. The launch of the multiplex optimisation process involved many potential overlapping forward primers capable of constructing the convergent fragment sizes. A large number of primer sequences and primer combinations were evaluated and used in the multiplex detection amplification system. Within the evaluated primers, most of them met some of the primer selection rules, however, only a few met all selection rules and were thus chosen to be used in the multiplex reaction design. The concentration optimisation of the chosen primers began by using equimolar concentrations of each forward primer and twice this concentration of the reverse primer. The reaction required a higher concentration of the reverse primer to prevent substrate exhaustion since it was common to each target amplicon. Primer concentrations were altered in the following trials through the analysis of the previous resulting electropherograph. A lower peak height result signalled for an increase in concentration of that primer whereas a decrease in primer concentration was required with the observation of extremely high peaks. These chosen primers required many concentration, temperature and reagent trials in order to identify the best combination of PCR conditions.

During the design of this multiplex the reagent concentrations were optimized. Standard single-plex PCRs have been designed and perfected using specific reagent concentrations, however a multiplex PCR requires reagent concentrations specific to the amplicons of interest. This multiplex PCR required an increase in the concentration of each dNTP from the traditionally used 100 μM to 200 μM for optimal equal amplification of all four-fragment lengths. More specifically, this increase was necessary to prevent substrate limitations thus ensuring continuous amplification of the four target amplicons within this multiplex reaction. Standard PCRs do not require such elevated levels since there is only one target amplicon. This

increase in dNTP concentration requires us to consider of the magnesium levels included in the optimising a multiplex PCR. The magnesium concentration used in a PCR must be proportional to the concentration of dNTPs added to the reaction since they undergo quantitative, equimolar binding to each other. The concentration of free magnesium is crucial to a multiplex reaction as well as a traditional PCR since it acts as a stabilising agent, increasing the specificity and yield of amplification. In the optimisation process of this fragmentation multiplex, the magnesium concentration, in the form of $MgCl_2$, was increased from the standard 1mM to 2mM in order to ensure remaining free magnesium. PCR buffer concentrations were also considered throughout the optimisation of the multiplex however deviations from the norm were of no benefit.

The temperatures, for annealing and extension, employed in a PCR play a key role in the specificity and yield of amplicons of interest, especially in terms of a multiplex PCR. The use of several primer sets in a multiplex PCR requires extensive temperature optimisation trials. These PCR temperatures are involved in determining the binding specificity of the primers as well as the overall product yield. Increasing the annealing temperature can increase the efficiency of a weakly binding primer pair that produces a large amplicon product. Decreasing the annealing temperature can increase the efficiency of a weakly binding primer pair that produces a smaller amplicon product. The extension temperatures can be changed in a way to aid the production of PCR products in a multiplex reaction. So that decreasing the extension temperature will increase the production of the larger amplicons consequently, increasing the temperature would improve the amplification of the smaller amplicons. Our temperature optimisation trials, using gradient PCR, identified the best annealing and extension temperatures for the final primer sets used for fragmentation analysis.

The use of a control sample was necessary during the optimisation process since it theoretically eliminates the presence of degraded fragments thus validating the results. The control sample with which the multiplex was designed is modern human DNA in an aqueous, pH neutral solution having been extracted immediately prior to a trial run. The analysis of the control prior to any storage conditions allows us to evaluate the effects of such storage without a preceding period of exposure.

Finally, standard and multiplex PCRs occasionally require the use of additives to increase their yield and specificity. Our first attempts at developing this multiplex reaction seemed to malfunction since the use of five forward DNA primers yielded an electropherograph (Figure XIV) with only four peaks. Upon removal of the primer thought to be unproductive, the reaction failed even further producing small, insignificant peaks (Figure XV). The primer was thus re-introduced into the reaction acting as a synthetic oligonucleotide additive, enhancing the overall multiplex reaction yield. The unexpected amplification enhancement provided by the oligonucleotide seems unlikely. It would normally be thought that the addition of another primer would increase the interaction with the other multiplex primers affecting the efficiency of the multiplex reaction. This primer may aid the specificity of DNA binding in the other primers of the multiplex reaction. PCR additives such as BSA, DMSO, betain, salmon sperm DNA, and glycerol are often tested when expected amplification are not achieved. These additives were included in a few optimisation trials but did not provide any further enhancement of the reaction. We suspect the added synthetic oligonucleotide improves the hybridisation of the primers and increases the efficiency of the multiplex as does the addition of denatured salmon sperm (Pang *et al.*, 1996; Sambrook *et al.*, 1989).

Through the fragmentation analysis of the chosen fragment sizes, we discovered the trend by which mtDNA degrades over time. When the DNA samples were newly extracted, all fragments were present in roughly equal quantities. This confirms that the sample DNA was intact at the beginning of this investigation and that very little fragmentation had occurred previously. The monthly analysis of these samples revealed a considerable trend in DNA degradation. The larger fragments (352 and 450 bp) begin to break down into smaller segments, 232, 133 bp or smaller. Figure XVI suggests that degradation of all fragment sizes occurs at approximately the same rate despite the appearance of more small fragments. This phenomenon is brought about by the fragmentation of the larger fragments into pieces less than 350bp long. This pattern of degradation holds true until all of the large and small sections are fragmented into short (<133 bp) undetectable oligonucleotides.

Both the larger DNA fragments, 352 bp and 450 bp, display an elevated rate of degradation. The degradation of the 450 bp fragment occurs mainly over the first three months of storage with only 4.18 % of its original peak height remaining after this time (see Figure XVIII). Further analysis during smaller time intervals are required to pin point the precise time of exponential degradation, however, this data is consistent with that of Hummel, (2003) who identified a significant degradation of samples after only 6 to 14 weeks of storage. This large fragment shows significant degradation at each of the time intervals leading to its eventual disappearance (0 % of initial peak height) after seven months (see Figure XXI). There are many possible explanations for this rapid degradation including various types of DNA damage and an increased surface area undergoing attack. The 352 bp fragment undergoes exponential degradation between the third and fourth months of storage, being reduced to only 22.37 % of its initial peak height at the three-month mark and further reduced to 14.74 % after the fourth

month. This fragment continues to break into smaller pieces at each time interval analysis until it dies out (0 % of initial peak height) after seven months. As expected, the 450 bp fragment shows a higher rate of degradation than the 350bp fragment.

The smaller fragments, 232 bp and 133 bp, display a slower rate of degradation than both the 450 bp and 352 bp fragments, with exponential drops at approximately 4 and 5 months respectively. After three months of storage, the 232 bp fragment average peak height is slightly higher (100.93 %) than that of the initial control result (see Figure XVIII). This result is not surprising and indicates the degradation of the larger fragments into segments smaller than 350 bp, which are amplified as a 232 bp fragment. The average peak height for this fragment size drops suddenly to 36.63 % of its original peak height at the four-month time interval (see Figure XIX). This rapid drop in average peak height suggests the acceleration of the break down of the 232 bp DNA fragments. In other words, prior to the four-month storage period larger fragments were breaking down into smaller segments leading to an accumulation of 232 bp products. At the four-month mark, the breakdown of large fragments slows due to a decrease in their concentration thus leading to an increase in the degradation of smaller 232 bp segments. The 133 bp fragment peak intensity drops considerable between the third and fourth month like all other segment sizes. At the three-month mark, the fragment is 89.10 % of its original peak concentration (peak height) and after four months of storage it has a peak height of 25.12 % its original concentration. This degradation is as expected since at this time large fragments continue to break apart into 200 to 400 bp fragments as well as 100 bp fragments. As expected, the 133 bp fragment displays the slowest rate of degradation and persists over nine months whereas the large 352 bp and 450 bp fragments have completely degraded after 6 months. The

trends observed for all fragment lengths indicate that degradation of DNA is occurring throughout the storage period however results may be unique to under these storage conditions.

The overall rate of degradation studied through the distribution of fragment sizes over time, seen in Figure XXIII, indicates an exponential degradation of DNA over a short period of time. This graph allows us to visualise the distribution of fragment sizes in terms of the overall observed peak heights. The cumulative peak height of each fragment size within a specific sample at a specific time interval is equal to the amount of 133 bp fragments present in the solution. In other words, through addition of all peak heights for one sample we can determine the overall peak height of the smallest fragment of interest, 133 bp in this case. This holds true since the larger fragments are made up of the smaller fragment; thus each large fragment contains the 133 bp fragment. As expected, an increase in storage time leads to a decrease in overall peak height or concentration however, the fragment distribution within this overall peak height also varies. As seen in Figure XXIII, the initial sample (0 months) shows an even distribution of each fragment size whereas at the three month mark the two smaller fragments make up approximately 86 % of the overall peak height. This trend continues until only the two smaller fragments are present and contributing to the overall peak height. After four months of storage, results seem to indicate an increase in the amount of the 450 bp fragment relative to that present after the three-month period. This is most likely an artefact of the methodology, most likely a fragment amplification competition anomaly. After seven months of storage, the 133 bp fragment accounts for the majority (73.15 %) of the fragments within a given sample with the only other contributor being the 232 bp fragment (26.85 %). These results clearly display the degradation of the DNA region of interest into only small fragments after seven months and their further degradation to undetectable lengths after nine months. The results after nine months of

storage time indicate a zero peak height meaning that all present DNA fragments are smaller than 133 bp.

The degradation of DNA may come about through many DNA damage mechanisms and pathways. These specific observed fragmentation trends suggest the occurrence of single- and/or double-stranded nicks most likely caused by oxidative or hydrolytic DNA damage. These DNA nicks cause large DNA fragments to degrade into smaller and smaller segments depending on the number of nicks within a given region. The identification of the exact source and cause of this DNA damage would require the differentiation between single and dsDNA nicks. The denaturation step included in PCR denatures the dsDNA prior to amplification making it very difficult to differentiate between ssDNA and dsDNA nicks and thus all fragments appear to be single stranded. The DNA structure identification would require the elimination of the denaturation step within the multiplex PCR.

The study of DNA, in various storage solutions, over time identified a degradation trend. DNA extracts in three buffer solutions, ddH_2O, Tris and TE were stored at 4°C and -20°C in order to determine the differences between storage conditions. A control sample, newly extracted DNA, for each solution was analysed prior to storage in order to determine the maximum attainable peak height. This study revealed that DNA eluted in ddH_2O gave peak heights averaging around 6000 with a roughly equal fragment size distribution. The DNA in Tris solution yields peak heights averaging 6500 with each fragment size within a close range of peak height. The extracts stored in ddH_2O and Tris yielded similar when analysis takes place immediate following the extraction procedure. This is as expected since the multiplex PCR was optimised using water based DNA samples. The analysis of the DNA in TE generated average peak height values in the range of 500 to 2000, only 18 % of the total average peak height in

ddH_2O. This shows a high variation in the peak height of each fragment size. The use of TE as an elution or storage buffer is cautioned throughout this field of research since high concentrations of TE have been shown to inhibit a PCR. TE contains EDTA, which chelates the magnesium in the PCR mix making it unavailable during the reaction (QIAGEN, 2002b). Our experiment contained low enough concentrations of TE to prevent complete inhibition of the multiplex PCR however; the reduced peak heights are as expected since some magnesium may be chelated thus reducing the PCR product yield. TE proves to be much less efficient of an storage buffer than ddH_2O and Tris when the multiplex fragmentation analysis is conducted immediately following the extraction procedure.

The examination of storage temperature effects on DNA in the three different buffer solutions, ddH_2O, Tris and TE, demonstrates a valuable association between the degradation process and the storage temperature. The 14 day storage at 4°C of the DNA stored in water resulted in the highest average peak height values (Figure XXVIII). This sample yielded equally elevated peaks for each fragment size. The water sample stored at -20°C shows a similar pattern of peak heights with the exception of the unexpected dip at of the 352bp fragment. This sudden dip is more likely a cause of competitive bind of the primer or other experimental conditions which would warrant further replications. We would expect that the DNA in water samples stored at -20°C would display higher peak heights than that stored at 4°C. Rather, both samples display similar average peak heights after 14 days of storage.

The DNA samples in Tris stored at 4°C and -20°C yielded similar average peak heights and fragment distribution to that of the DNA in water samples. Both samples, one stored at 4°C and the other at -20°C, have similar peak heights for the 133 bp and 232 bp fragments, however they differ significantly on both the 352 bp and 450 bp fragments. The samples stored at 4°C

show a slow decline in peak height starting with the 352 bp fragment and ending with a peak height about 1000 units lower than the 133 bp fragment. As expected, this shows fragmentation of the larger products during the storage period at 4°C. These findings are consistent with the undocumentated common practice procedures used in the field of degraded DNA studies where researchers discourage the storage of DNA at 4°C over long period of time. The DNA in Tris sample stored at -20°C show maintenance of the high peak heights, again with the exception of the 352 bp fragment. The dip of the 352 bp fragment may be an artefact of the multiplex methodology since this method was optimised using samples in water. The methodology may require some modification if it were to be continually used to assess DNA fragmentation of samples stored in Tris. It is doubtful that we would observe such a steep decline in the 352 bp peak height while the 450 bp remained at a higher level. The overall fragment distribution of the DNA sample stored in Tris at -20°C, while disregarding the 352 bp experimental error, displays elevated average peak heights with an even distribution of fragment sizes. This indicates very little degradation over the storage period of 14 days at -20°C thus suggesting a reliable solution for the storage of DNA.

The samples stored in TE buffer at two different temperatures, 4°C and -20°C, identifies a very important trend in DNA degradation during storage. Figure XXX shows the fragment distribution and yields for the sample stored in TE for 14 days at both temperatures. The results indicate identical trends for both the 4°C and the -20° samples. We see a relatively high peak for the smallest fragment of 133 bp followed by a higher 232 bp-fragment peak height, next the 352 bp-fragment peak height drops and finally the 450 bp fragment has a relatively low peak height. The 133 bp and 232 bp fragment peak heights are unexpectedly high displaying a higher average peak height at this time interval than the fresh extract sample. This does not indicate an increase

in the overall DNA (see Figure XXIII) rather it simply indicates the larger proportion of the smaller fragments over the larger fragments within the samples. This change in proportion is more likely a product of the larger pieces of DNA fragmenting into smaller ones. This may also be an artefact of the methodology as observed for the 352 bp peak height drop of the DNA stored in Tris solution. These observations are not an indicator of contamination because all PCR controls were negative. The low amplitute fragmentation pattern observed for the fresh TE samples may suggest an elevated concentration of TE, compared to that of the stored ones, causing PCR inhibition. Perhaps the storage at both 4° and -20°C diminishes the inhibiting features of TE allowing for larger product yields. The lower 325 bp- and 450 bp fragment peak heights are as expected since we anticipate some damage to have occurred over the storage period.

The final degradation assessment employs the fragmentation multiplex tool in the evaluation of the effects of frequent sample freeze/thaw cycles. This analysis assesses the importance and effects of repeated handling of bulk DNA extracts. The peak heights of the two smallest fragments, 133 bp and 232 bp, were measured within specified DNA samples eluted in water after a specific number of freeze/thaw cycles in order to determine the freeze/thaw effects on fragmentation (Figure XXXI). These small fragment sizes were chosen for this study in order to minimise possible methodological restraints involving competitive binding of the smaller fragments over the larger ones. The 232 bp fragment shows a noteworthy drop (87.1 %) from a peak height of 843 units after 2 cycles of freezing and thawing to 109 units after 6 cycles. This decline corresponds to a linear slope of –183.5 units and thus an inverse relationship exist between the peak height and the number of freeze/thaw cycles. In other words, the average sample peak height decreases with each increase in the number of freeze/thaw cycles. These

results also suggest a negative relationship between peak height and the number of freeze/thaw cycles thus having a negative slope of −106.75 units. The larger of the two DNA fragments (232 bp) displays a more negative slope than the smaller 133 bp fragment. As expected, this increased slope indicates the break down of the larger 232 bp fragment into smaller ones. These results confirm that numerous freeze/thaw cycles induce the degradation of DNA into smaller and smaller pieces. As in the fragmentation analyses previously described, it is extremely difficult to pin point the exact type of DNA damage leading to this type of degradation and fragmentation. The observed fragmentation does however require the occurrence of either single or double stranded breaks, which can transpire as a result of oxidative, hydrolytic or physical damage. The results of this study suggest the occurrence of physical strand damage caused during the expansion/condensation of the water molecules surrounding the DNA. The repeated conversion of water from one physical state to the next causes the spatial displacement of the DNA, thus leading to double or single strand breaks depending on the extent of structural dislocation. The degree of single versus double stranded breaks would require further investigation.

This study provides the freeze/thaw cycle effects on the integrity of DNA, which has been suspected by many researchers without direct assessement. The observed freeze/thawing affects on stored DNA has caused many procedural differences to the handling of DNA in solution. Some fields of research who recognise the effects of DNA storage in solution will discard their DNA extracts after a certain period of time (six weeks), while other areas of research will aliquot their DNA containing solutions. The freeze/thaw effects assessed here indicate that the storage of bulk DNA extract is not favourable since the bulk solution must be thawed at the time of each analysis. Throughout analysis the samples stored in bulk have undergone numerous freeze/thaw cycles, but without taking in to account the fragmentation of

DNA stored in solution would render the data incomparable. Results replication may be difficult to accomplish after an unknown number of freeze/thaw cycles due to extensive DNA damage and degradation. Most DNA extraction protocols require the elution of the DNA into large volumes of buffer. This would require aliquoting the bulk DNA extract into smaller, one analysis volumes thus minimising the number of freeze/thaw cycles and reducing DNA degradation. This procedure would allow the independent storage of smaller volume aliquots permitting some to be stored for longer periods than others, for the purpose of further analysis or replication.

This analysis of DNA fragmentation over time is not limited to mtDNA rather it also includes nDNA and Y-chromosome DNA. The method for determining the fragmentation in nDNA has been established through the design of a multiplex PCR using the human β–globin and Y-chromosome sequences. The human β–globin gene was chosen because the target region is well studied and provides information on disease. The Y-chromosome targets were chosen to assess the fragmentation of the Y-chromosome for further STR typing methodologies. The design of these two additional multiplex PCRs was successful, however, time constraints did not permit the time interval assessments required to identify trends in degradation.

ii. Sequence Analysis

Single base pair damage and modification was assessed in this research by sequence analysis. This involves the study of the effects of damage on individual bases within a fragment of interest. This single base pair damage and modification could lead to DNA fragmentation through weakening of the DNA backbone, as evaluated through multiplex PCR. This study incorporates DNA sequencing with visual and computational base alignment enabling the identification of single base modifications resulting from DNA sample storage. This study may

also reveal a number of "hot spots" or repeated patterns of base modifications at the same sequence location either confirming or expanding those found by Parsons, *et. al.* (1997). Twelve DNA extracts from one individual were sequenced at specific time intervals to identify the precise location, frequency and type of base damage occurring over the storage period. The DNA samples studied were taken from the same individual simplifying data analysis by allowing simultaneous comparison of sequences to the same freshly extracted reference sequence. These samples were first analysed using the fragmentation assessment multiplex PCR followed by the sequencing of the multiplex PCR product using the reverse primer. The reverse sequencing of the multiplex PCR product is made possible since all amplicons within the multiplex share a common reverse primer, otherwise the sequence analysis of these samples would require a separate, fragment specific PCR. The 133bp fragment was chosen for this in-depth base analysis since each of the sequencing reactions dropped out after approximately 140 bp. This short target region (mt16210 to mt16420) will be evaluated which may generate models for base alterations within the other hypervariable regions of the mtDNA genome.

The target region sequences were aligned and compared to the reference sequence from the same individual in order to identify any base substitutions arising during sample storage. A compilation of the most common (>20 % occurrence) base substitutions is seen in Table 5 whereas a complete list of observed substitutions is found in Appendix A. The more frequent base substitutions observed within the target fragment occur between mt16210 and mt16340. A base substitution may be a transition, where the base is substituted by another of the same type, for example purine substituted by another purine, or a transversion where the base is substituted by a different type of base, as in the substitution of a purine by a pyrimidine. As seen in Table 6, transversions made up 78.6 % of all observed substitutions whereas transitions had a minimal

incidence of 21.4 %. These rates of substitution are opposite what would be expected for rates of substitution *in vivo*. The observation of a higher transversion rate is one characteristic that researchers have used to indicate DNA damage rather than mutation generated through evolution. Base substitutions come about through a multitude of mechanisms and pathways including enzymatic, oxidative, hydrolytic, and physical pathways. Oxidative damage is caused either by exposure to UV light or through the Fenton reaction. In general, base transversions occur through an oxidation pathway involving oxidative attack of the base whereas base transitions require hydrolytic attack. Our samples were not exposed to UV light throughout the experiment thus our results imply the occurrence of oxidative base damage due to the presence of an oxidant within the sample. Additional base analysis is required to evaluate and assess this further.

The sequencing reaction allowed for the identification of base modifications throughout the 133 bp fragment of the multiplex PCR. The most frequently substituted base is cytosine (78.6 %), followed by adenine (14.3 %) and finally guanine (7.1 %). No repeated thymine base substitutions were observed in any of the sequences. The elevated, dominant rate of cytosine substitution within this target fragment is quite unexpected. All previous literature involving base modification in solution identify guanine as the most susceptible to DNA damage and thus substitution (Lindahl, 1993; Rodriguez *et al.*, 2000; Schweigert *et al.*, 2000). This deviation may be attributed to the skewed base distribution within the target region: cytosine (37.4 %), adenine (32.2 %), thymine (19.4 %) and finally guanine (10.9 %), see Table 4. Earlier literature does however claim a dependence of cytosine deamination on the pH and temperature of the elution solution (Lindahl *et al.*, 1973b). These reports also convey a very low level of guanine and adenine deamination in samples contained in weakly acidic buffers and exposed to elevated

temperatures. This pH and temperature dependence may also contribute to the elevated base substitutions observed in this study. The denaturation step during the multiplex PCR may lead to elevated rates of cytosine deamination (Shapiro *et al.*, 1966). Our samples are stored in a water buffer and thus deamination would need to occur through an addition-elimination mechanism with water as the principal base catalysing the reaction as suggested by Lindahl and Nyberg (1974). The increased rate of cytosine substitution is possibly due to the combination of the shear number of cytosine residues compared to that of guanine throughout the fragment sequence studied and exposure to elevated temperatures and slightly acidic solutions.

The majority of cytosine base modifications are C – G transversion (81.8 %) and thus the above factors may not account for all cytosine residual substitutions. The observed transversion may be the product of oxidative attack of the cytosine residue leading to the formation of 5-hydroxy cytosine or 5-hydroxyuracil intermediates, as per observed pairing rules for modified bases found in Table 1. The formation of these two modified bases accounts for the transversion of a cytosine residue into a guanine residue within the sequence since they bind to cytosine rather than guanine, thus distorting the base reading during sequence analysis. The 5-hydroxycytosine and 5-hydroxyuracil residues both bind to cytosine residues rather than following the standard binding pattern of their antecedent cytosine residue. The sequence analysis performed by the Genescan software detects the bound cytosine residue thus allocating a guanine base at that precise location.

Base modification generated through oxidation requires an oxidant within the DNA extract solution. The oxidation of the cytosine bases may also be a consequence of exposure of the sample to UV light. The sample storage facility (-20°C) is equipped with a sliding glass door thus allowing light, both natural and artificial, to enter at all times throughout the day. As

established by previous studies, exposure of water to ionising radiation leads to the formation of hydroxyl radicals, solvated electrons (e_{aq}^-) and H atoms (Dizdaroglu, 1992). The UV exposure of the water based buffer, acting as a storage medium for the DNA extracts, may lead to the formation of damaging radicals thus providing an oxidant within the DNA extract. Oxidative damage through UV irradiation of water leads to the formation of many modified DNA bases, most to that of cytosine glycol (Dizdaroglu, 1992). Cytosine glycol, an oxidation intermediate, has a relatively long half-life allowing it to be further oxidised into either 5-hydroxycytosine or 5-hydroxyuracil. This succession of oxidative mechanisms corroborates the suggested indirect formation of either 5-hydroxycytosine or 5-hydroxyuracil from abundant cytosine residues within the sequence of interest. The conflict between the distribution of base modifications seen in this study and that of previous works is attributed to the abundance of cytosine residues within the target sequence. Previous studies claim excessive formation of 8-OHGua to be the indication of oxidative DNA damage however our research was conducted on DNA samples in solution containing an overpowering number of cytosine residues. Oxidative base damage is observed throughout our target sequence affecting all bases as indicated by the diversity of base modifications: A – G, A – C, C – G, G – C, C – T. The overall base oxidation results in this study differ from other research which finds a 95% higher modification rate within guanine and adenine (Lindahl, 1993). Our study identifies a higher rate of oxidative damage or base modification in cytosine which may simply reflect the different percentage of bases within the sequence.

The sequence analysis performed on the multiplex PCR product with special focus on the 133bp fragment may lead to the discovery of hot spots within this segment of the mtDNA genome. The resulting DNA sequence revealed a significant number of base substitutions within

the 16210 – 16340 region of the mitochondrial genome. The frequently observed base exchanges identify possible hot spots at the following mitochondrial base positions 16210, 1622, 16228, 16250, 16262, 16270, 16292, 16295, 16286, 16290, 16293, 16295, 16301, 16303, 16331, 16335, and 16340, frequencies are as seen in Table 5. The most frequent base modification is a pyrimidine insertion at position mt16303 occurring in 73.3 % of all observed sequences. This unforeseen base insertion has not been previously documented in any previous publications and thus is most likely an artefact of the multiplex or sequencing methodology. The most frequently observed base modification overall is a C – T transition at position mt16286, occurring in 66.7 % of sequences. This base substitution is indicative of hydrolytic DNA damage, which is defined as the breaking of the N-glycosylic bond through the addition of water, forming an abasic site followed by a modified base. The most numerous base substitutions are C – G transversions occurring at positions 16221, 16250, 16262, 16270, 16292, 16293, 16295, and 16340. These specific locations within the mitochondrial genome are possible 'hot spots' for the occurrence of oxidative DNA damage under these particular experimental conditions. These possible 'hot spot' locations do not correlate with any established by Parsons *et. al.* (1997) due to methodological differences. Our 'hot spot' assessment studies DNA in solution (ddH_2O) whereas other research does not (Gilbert *et al.*, 2003; Parsons *et al.*, 1997). The comparison of these two data sets would be statistically incorrect since they were performed under different experimental circumstances and evaluate two separate forms of DNA.

iii. Quantification Analysis

The quantification of DNA recovered within a sample is considered proportional to the amount of damage that DNA may have undergone. The preceding analytical methods have assessed the integrity and damage levels within DNA, the quantification of the DNA is therefore

also part of this research. The analytical method used to assess the quantity of DNA was real time PCR. DNA quantification using real time PCR requires the use of a photo-detection system since the quantity of DNA is derived from the level of light emitted by the sample in a spectrophotometer. There are three types of established detection systems employed in real time PCR: SYBR green, molecular beacons and a probe-based FRET system. We initially chose a probe-based FRET system for our analysis since it would allow for highly specific amplification and quantification of our target region, however this system did not have the sensitivity required in our analysis of low copy number DNA. Modifications have since been improved this system, but not optimally or in time for this research. The next trial was run using the SYBR green detection system and proved to have the necessary sensitivity and so our quantification protocol was developed using this detection system in conjunction with the ABI Prism® 7000 Sequence Detection System. The SYBR green detection system was chosen since it is a well-established, highly sensitive method requiring very little target region specific design; it is highly cost effective and readily available. These characteristics allowed us to utilise the previously developed multiplex primers in the quantification protocol therefore facilitating the correlation of quantification assessment results with the fragmentation analysis findings.

The quantitative assessment was conducted using the smallest fragment size (133bp) within the multiplex as a model, however, the established pattern is applicable to all fragment lengths. The use of this short 133bp-target fragment (mt16210 to mt16420) is advantageous since it can be amplified with increased specificity while employing a short annealing and extension time thus increasing the sample turnover time. A freshly extracted modern DNA sample was used as the reference to which the unknown samples were compared. The real time PCR of this duplicated reference sample displayed a conventional sigmoidal amplification as

seen with low quantity threshold values as expected, see Figure XXXII. The observed Ct values of 24.40 and 25.87 are indicative of modern DNA samples containing a multitude of copies of DNA. This real time amplification plot shows the eventual slowing of the reaction after approximately thirty cycles leading to an amplification plateau after thirty-five cycles. The cycle number at which the reaction plateau is observed is dependent on the type of DNA polymerase used in the PCR. Supporting research is completed but not included since it is beyond the scope of this research.

The real time amplification of the reference sample included the simultaneous amplification of duplicate negative control samples. Extreme caution was required to prevent contamination of the negative PCR controls. The first few real time PCR trials included only triplicate PCR controls in order to establish a suitable sample and reagent handling procedure to avert contamination. Tremendous difficulty with negative control contamination was overcome through added decontamination steps above and beyond the customary cleaning procedure. Consumables such as pipette tips, mixing and reaction tubes are autoclaved, cross-linked and placed in sterile sample bags upon receipt followed by an additional cross-linking step immediately prior to use. Once effective decontamination techniques were established and the reference sample Ct value was repeatedly duplicated, we were prepared for the analysis of samples of unknown age and integrity.

The comparison of modern DNA real time amplification results with those of samples of unknown integrity allows us to determine a relative rate of degradation of DNA samples stored in solution. The study of the three unknown samples resulted in Ct values of 36.41, 39.12 and 37.68. At first glance these Ct values seem low compared to those of the modern DNA samples and such is the case after calculating the relative quantity of the unknown sample to that of the

reference sample. The calculation of relative quantification of unknown samples 1, 2 and 3 using Equation 3 resulted in levels $2.42x10^{-4}$, $3.71x10^{-5}$ and $1.01x10^{-4}$ times lower than that of the reference sample respectively. These results suggest the sample to be within the same range of integrity and fragmentation. The quantification of these samples relative to the modern reference sample compares the level of damage within the unknown sample. The level of DNA damage is inversely related to the relative quantity, for example a lower relative quantity represents an increase in DNA damage. The relative quantity is not an indication of the age of an unknown sample since sample taphonomy differs.

XIII. CONCLUSION

The methods developed here for the fragmentation analysis identified the increased fragmentation over time in a variety of storage solutions. The fragmentation is increased through the process of freeze/thaw cycles and changes under different storage temperatures. These results suggest methodological changes in order to decrease fragmentation level when storing samples for long periods of time. Sequence analysis identified potential hot spots where an elevated number of nucleotide substitutions and base modifications occur which requires further investigation. The observed nucleotide substitutions are thought to occur primarily through an oxidative pathway with extensive damage occurring to the cytosine residues due to their abundance within the target sequence. Oxidative damage was found in all the nucleotides identifying it as the biggest problem in DNA stored in solution. Quantification using real time PCR proved the decrease in relative quantity of DNA over time. The relative quantity of DNA is inversely proportional to the amount of damage and degradation found within a certain sample.

XIV. FUTURE DIRECTION

This project leads the way for many further analyses of patterns explaining the degradation of DNA. The mtDNA fragmentation patterns were assessed in this study however, future analysis of nDNA and Y-chromosome DNA is required to establish an overall DNA degradation pattern. Once complete, this model for overall DNA degradation in various solutions may be used to establish the correlation between sample taphonomy and specific size fragment distributions. It may also be used to assess damage induced through the process of extraction, analysis using x-rays and other molecular procedures.

This project focused on the distribution pattern of various DNA fragment lengths within DNA samples exposed to various storage times and conditions. Further research is required to characterise the types of damage leading to such fragmentation patterns. A distinction must be made between fragmentation occurring due to single stranded or double stranded breaks. In this research, the denaturation step within the multiplex PCR fragmentation assessment tool prevented such break identification. Future research could employ single nucleotide extension without a denaturation step and with the addition of labelled nucleotides to identify the single stranded fragments within the sample resulting from single stranded nicks. The incorporation of the labelled nucleotides at the site of single strand breaks permits their spectrometric detection leading to base identification. This could be extended with the use of other enzymes to label double stranded breaks as blunt or sticky ends.

This study identified possible base substitution or modification hot spots within the mitochondrial genome using a sequence analysis approach. This analytic method allowed us to visualise any changes occurring to the mtDNA sequence over time however, we were unable to identify the exact mechanism by which these modifications are occurring. Further sequence

analysis and modified base identification could be accomplished through the combination of gas chromatography and mass spectrometry (GC/MS) and again using a variety of enzymes and labelled nucleic acids.

The real time PCR analysis conducted in this project allowed us to make a correlation between the relative amount of DNA found in modern samples and that found in unknown samples. Further real time PCR optimisation and the construction of a standard curve would allow a direct assessment of the absolute concentration of DNA within a certain sample. This absolute quantification tool could then be used to establish a trend for specific sample taphonomy and levels of DNA degradation.

As a whole, our research provides much insight into the rate of degradation of DNA in solution when exposed to various conditions. Each section of this study requires repeated replication in order to confirm the identified trends. The assessment of sample storage in different solutions and temperatures merits profuse replication since optimisation of storage conditions would facilitate the analysis in all fields of DNA research, especially those dealing with samples having a low DNA copy number.

XV. APPPENDIX A

Raw Data: Fragmentation Analysis

Sample	Dilution	Eluent	Date of Extract	PCR Date	Age	Fragment Peak Height			
						133bp	232bp	352bp	450bp
CB	1:100		13/01/2003	15/01/2003	0	5652	5494	6808	5700
						7175	6068	7063	7000
						6170	5974	6787	6860
						5704	6762	6917	7231
AVG						6175	6075	6894	6698
STDV						706.0	522.7	126.4	682.5
CB	-	ddH_2O	8/21/2003	8/21/2003	0	1882	2698	1612	2164
						7093	6227	6396	7331
						7234	7151	6545	6523
						7125	5763	6716	6507
						5625	6942	6840	6803
						4775	6072	4315	7387
						6133	7041	7129	6659
						5732	5763	6594	7032
AVG						5700	5957	5768	6301
STDV						1770.8	1432.0	1888.5	1705.5
CB	-	TE	8/21/2003	8/21/2003	0	1974	2903	718	206
						1623	2375	640	219
						608	787	254	203
						653	950	344	235
						2267	3935	1734	834
						886	2042	781	502
						830	966	152	2153
						989	1263	244	84
AVG						1229	1903	608	555
STDV						636.9	1122.0	513.6	688.4
CB	-	Tris	8/21/2003	8/21/2003	0	6041	6499	4905	7321
						5809	5741	4304	6602
						6087	6014	6792	7002
						6565	5941	6739	7133
						7231	7205	7174	7318
						7058	6234	7143	6885
						7028	6453	4698	6668
						7198	7060	6810	6427
AVG						6627	6393	6071	6920

CB	-	ddH_2O	9/23/2003	9/23/2003	0	4705	7205	7376	6502
						5469	6160	6992	5701
AVG						5087	6683	7184	6102
CB	-	ddH_2O (-20°)	8/21/2003	9/8/2003	0.5 mths	7257	6560	3083	6462
		ddH_2O (-20°)				6845	6758	3818	7211
		ddH_2O (4°C)				6517	7260	6378	7000
		ddH_2O (4°C)				6330	6329	7192	6528
AVG						6737	6727	5118	6800
CB	-	TE (-20°)	8/21/2003	9/8/2003	0.5 mths	5188	6896	2025	1508
		TE (-20°)				4519	7253	1960	3095
		TE (4°C)				5304	6728	3027	3694
		TE (4°C)				4923	7327	1471	821
AVG						4984	7051	2121	2280
CB	-	Tris (-20°)	8/21/2003	9/8/2003	0.5 mths	6196	6713	6771	7028
		Tris (-20°)				7181	6381	5272	4475
		Tris (4°C)				6327	7240	4756	6500
		Tris (4°C)				7104	6180	5214	7382
AVG						6702	6629	5503	6346
AJ	-		6/6/2003	7/17/2003	1 Month	3199	2184	4027	9397
						1726	1855	3600	7341
AVG						2463	2020	3814	8369
RB	-		5/6/2003	7/17/2003	2 Months	3640	1674	3127	9403
						4104	1990	3349	9460
AVG						3872	1832	3238	9432
CB	1:10000		9/12/2002	11/21/2002	2 Months	3309	5964	853	280
CB	1:100		9/12/2002	12/9/2002	3 Months	5502	6131	1542	280
CH	-		4/9/2003	10/27/2003	3 months	1262	0	0	0
						2154	5818	1788	0
AVG						1708	2909	894	0
CB	1:100		9/12/2002	1/8/2003	4 Months	1551	2225	1016	1210
KV	-		6/6/2002	10/27/2003	4 Months	0	7382	5943	7465

					0	7505	6512	518
AVG					0	7444	6228	3992
LL	-	2/20/2003	7/17/2003	5 Months	4157	1795	2062	7113
					4242	1903	2060	6754
AVG					4200	1849	2061	6934
KV	-	2/6/2003	10/27/2003	5 months	0	1147	0	0
					0	468	0	0
AVG					0	808	0	0
CB	1:100	9/2/2002	3/13/2003	6 months	613	410	59	68
					1545	1180	176	122
					542	397	66	127
AVG					900	662	100	106
CB	1:100	12/10/2002	7/17/2003	7 months	1078	367	0	0
					151	84	0	0
AVG					615	226	0	0
CB		12/9/2002	7/17/2003	7 months	3728	1022	4403	9415
					3199	880	4101	9375
AVG					3464	951	4252	9395
TJ	1:100	10/17/2002	7/17/2003	9 months	0	0	0	0
					0	0	0	0
AVG					0	0	0	0
CB	-	1/13/2003	10/27/2003	9 months	375	767	0	0
					108	324	0	0
AVG					242	546	0	0
CB	-	9/4/2002	7/17/2003	10 months	5169	935	3271	9641
					3395	2113	3903	9682
AVG					4282	1524	3587	9662
TM	-	9/16/2002	7/17/2003	10 months	1714	2486	7489	8025
					5866	0	3221	9873
AVG					3790	1243	5355	8949
TJ	1:10	9/9/2002	7/17/2003	10 Months	2576	3059	6686	4573

					2661	3143	7647	8337
AVG					2618.5	3101	7166.5	6455
CB	-	12/12/2002	10/27/2003	10 months	0	0	0	0
					0	0	0	0
AVG					0	0	0	0
TJ	-	9/9/2002	10/27/2003	12 Months	2176	1874	281	0
					0	2056	137	0
AVG					1088	1965	209	0
TM	-	10/11/2002	10/27/2003	12 months	5591	5890	6536	7515
					3766	7029	7313	7239
AVG					4679	6460	6925	7377
TJ	-	9/25/2002	10/27/2003	13 months	4618	7334	7373	6617
					2843	4982	6893	6000
AVG					3731	6158	7133	6309
AL	-	3/18/2002	7/17/2003	16 months	4249	1544	841	4917
					4105	3889	2772	4955
AVG					4177	2717	1807	4936
AL	-	3/18/2002	10/27/2003	19 months	1077	3948	2759	1558
					922	2951	1754	1501
AVG					1000	3450	2257	1530

Raw Data: Fragmentation analysis of diluted (1:100) including percent total peak height and % total peak height

Sample name	Dilution	Date of Extract	PCR Date	Age (mth)	Fragment Peak Height				Total height
					133bp	232bp	352bp	450bp	
CB	1:100	13-Jan-03	15-Jan-03	0	5652	5494	6808	5700	
					7175	6068	7063	7000	
					6170	5974	6787	6860	
					5704	6762	6917	7231	
AVG (initial)					6175	6075	6894	6698	25841
% of total peak height					24	24	27	26	100
CB	1:100	12-Sep-02	9-Dec-02	3	5502	6131	1542	280	13455
% of initial peak height					89.10%	100.93%	22.37%	4.18%	
% of total peak height					40.89	45.57	11.46	2.08	100.0
CB	1:100	12-Sep-02	8-Jan-03	4	1551	2225	1016	1210	6002
% of initial peak height					25.12%	36.63%	14.74%	18.07%	
% of total peak height					25.84	37.07	16.93	20.16	100.00
CB	1:100	2-Sep-02	13-Mar-03	6	613	410	59	68	
					1545	1180	176	122	
					542	397	66	127	
AVG					900	662	100	106	1768
% of initial peak height					14.57%	10.90%	1.46%	1.58%	
% of total peak height					50.90	37.46	5.67	5.98	100.00
CB	1:100	10-Dec-02	17-Jul-03	7	1078	367	0	0	
					151	84	0	0	
AVG					615	226	0	0	840
% of initial peak height					9.95%	3.71%	0.00%	0.00%	
% of total peak height					73.15	26.85	0.00	0.00	100.00
TJ	1:100	17-Oct-02	17-Jul-03	9	0	0	0	0	
					0	0	0	0	
AVG					0	0	0	0	0
% of initial peak height					0.00%	0.00%	0.00%	0.00%	

Raw Data: Sequence Analysis – Observed base substitutions

Age	Sample	mtDNA position	Modification	Type of Substitution
1 month	743	16303	^C	insertion
	16205 - 16360	16331	G - C	transversion
		16333	A - T	transversion
		16335	^ C	insertion
		16340	C - G	transversion
	744	16221	C - G	transversion
	16205 - 16330	16228	C - T	transition
		16286	C - T	transition
		16290	^G	insertion
		16292	C - G	transversion
		16293	C - G	transversion
		16295	C - G	transversion
		16329	C - G	transversion
	745	16295	C - G	transversion
	16205 - 16370	16301	A - C	transversion
		16334	C - A	transition
		16335	^ C	insertion
		16367	C - G	transversion
	746	16228	C - T	transition
	16205 - 16370	16286	C - T	transition
		16293	C - G	transversion
		16303	^ C	insertion
		16367	C - G	transversion
	747	16228	C - T	transition
	16205 -16310	16248	C - T	transition
		16286	C - T	transition
		16290	^ C	insertion
		16293	C - G	transversion
		16295	C - G	transversion
		16301	A - C	tranversion
		16303	^T	insertion
	750	16211	C - G	transversion
	16205 - 16370	16221	C - G	transversion
		16262	C - G	transversion
		16286	C - T	transition
		16295	C - G	transversion

	16301	A - C	transversion
	16303	^C	insertion
	16340	C - G	transversion
	16342	C - G	transversion
751	16250	C - G	transversion
16205 - 16370	16286	C - T	transition
	16293	C - G	transversion
	16295	C - G	transversion
	16301	A - C	tranversion
	16303	^T	insertion
	16327	T - G	transversion
	16339	G - A	transition
	16340	C - G	transversion
	16345	T - A	transversion
752	16286	C - T	transition
16205 - 16370	16290	^C	insertion
	16292	C - G	transversion
	16293	C - A	transversion
	16294	A - G	transition
	16296	C - A	transversion
	16300	A - C	transversion
	16301	A - C	transversion
	16303	^C	insertion
	16331	G - C	transversion
	16340	G - A	transition
	16341	A - C	transversion
	16342	C - A	transversion
	16345	T - G	transversion
753	16210	A - G	transition
16205 - 16310	16214	C - T	transition
	16221	C - G	transversion
	16236	C - G	transversion
	16250	C - G	transversion
	16262	C - G	transversion
	16286	C - T	transition
	16287	C - G	transversion
754	16210	A - G	transition
16205 - 16370	16214	C - T	transition
	16221	C - G	transversion

		16228	C - G	transversion
		16236	C - G	transversion
		16242	C - T	transition
		16248	C - T	transition
		16251	C - G	transversion
		16260	C - G	transversion
		16261	C - T	transition
		16267	C - G	transversion
		16270	C - G	transversion
		16279	C - T	transition
		16282	C - T	transition
		16286	C - T	transition
		16287	C - G	transversion
	755	16221	C - T	transition
	16205 - 16370	16270	C - G	transversion
		16286	C - T	transition
		16293	C - G	transversion
		16295	C - G	transversion
		16303	^C	insertion
		16322	C - T	transition
		16361	C - G	transversion
		16363	C - G	transversion
		16368	C - G	transversion
	756	16211	C - G	transversion
	16205 - 16370	16221	C - G	transversion
		16228	C - T	transition
		16270	C - G	transversion
		16286	C - T	transition
		16293	C - G	transversion
		16296	C - G	transversion
		16303	^T	insertion
		16325	T - C	transition
		16327	T - G	transversion
5 months	39	16303	^C	insertion
	16205 - 16370	16321	G - C	transversion
		16322	C - T	transition
		16323	C - T	transition
		16331	G - C	transversion
		16335	^A	insertion

	16336	A - T	transversion
	16337	T - A	transversion
	16338	A - G	transition
42	16292	C -G	transversion
16205 - 16370	16293	C - G	transversion
	16295	C - G	transversion
	16303	^ C	insertion
	16331	G -C	transversion
	16339	G - A	transition
	16340	C - G	transversion
	16341	A - C	transversion
	16367	C - G	transversion
43	16210	C - G	transversion
16205 - 16360	16250	C - G	transversion
	16251	C - G	transversion
	16262	C - G	transversion
	16295	C - G	transversion
	16301	A - C	transversion
	16303	^T	insertion

Raw Data: Quantification – Real Time PCR

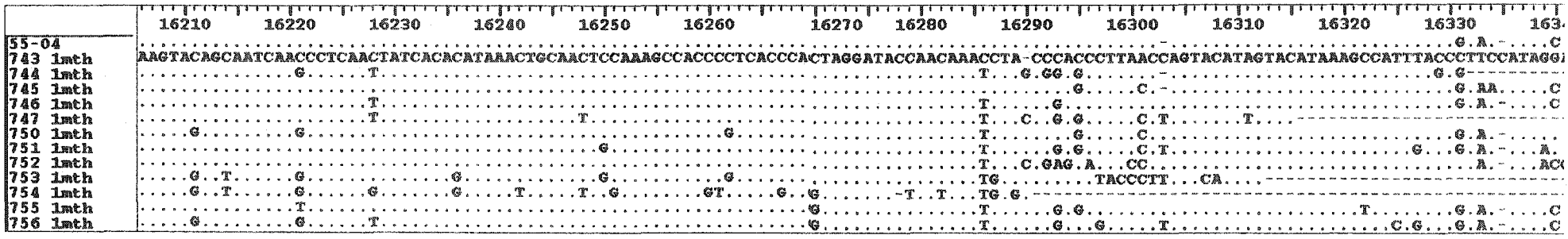
Cycle #	1	2	3	4	5	6	7	8	9	10	11	12
Modern	0.037499	0.07931	0.087431	0.072383	0.057606	0.014307	-0.002613	-0.003759	-0.002356	-0.017305	-0.00805	0.020462
Unknown 1	-0.003935	-0.026357	-0.000754	0.054734	0.074201	0.002311	-0.035366	-0.000533	0.062749	0.028441	-0.021074	-0.018934
Unknown 2	0.034428	-0.022727	-0.023043	0.006517	0.022481	-0.015849	-0.017208	0.020119	0.071801	0.041155	-0.035502	-0.020303
Unknown 3	-0.023253	0.021911	0.007541	-0.030507	-0.050491	-0.002516	0.040409	0.067411	0.003003	-0.024979	-0.056714	-0.048538
PCR Neg	-0.018061	-0.069127	-0.013839	0.022179	0.023808	-0.005984	-0.012413	-0.037209	-0.014632	0.023325	0.03865	0.066502

Cycle #	13	14	15	16	17	18	19	20	21	22	23	24
Modern	-0.003812	-0.002446	0.005572	0.04274	0.052103	0.053838	0.030899	0.003106	0.028512	0.04706	0.057814	0.09103
Unknown 1	-0.037436	-0.036951	-0.028841	0.007617	0.011435	0.025889	-0.020317	-0.023097	-0.024492	-0.053465	-0.107281	-0.093308
Unknown 2	0.015109	0.041875	-0.019058	-0.025911	-0.041929	-0.015305	-0.021459	-0.006911	-0.003	-0.011194	-0.040704	-0.054063
Unknown 3	-0.032932	-0.009466	0.003565	0.017081	0.058727	0.049407	0.020926	-0.026386	0.021013	0.055914	0.061423	0.038087
PCR Neg	0.08261	0.002752	-0.059468	-0.018345	0.070372	0.0669	0.004822	-0.026119	-0.032933	-0.0379	-0.055892	-0.079458

Cycle #	25	26	27	28	29	30	31	32	33	34	35	36
Modern	0.163752	0.263457	0.412461	0.592784	0.751018	0.871894	0.957281	1.009515	1.039532	1.054924	1.066431	1.066532
Unknown 1	0.048053	0.10688	0.038104	-0.039337	0.023044	0.088023	0.072664	0.117814	0.277056	0.318608	0.461878	0.691406
Unknown 2	0.015476	0.072863	0.048668	-0.034098	-0.044732	-0.050149	-0.01962	0.028056	0.104216	0.08197	0.109908	0.14532
Unknown 3	0.04379	-0.015854	-0.067402	-0.082662	-0.038508	-0.043876	0.002511	0.018549	0.057449	0.078255	0.212017	0.388318
PCR Neg	-0.06255	-0.008952	0.016789	0.050591	0.100572	0.091269	-0.038818	-0.060506	-0.069982	-0.046978	-0.042467	-0.041936

Cycle #	37	38	39	40
Modern	1.067555	1.067609	1.075535	1.082885
Unknown 1	1.164489	1.752638	2.494724	3.194799
Unknown 2	0.262626	0.526923	0.970407	1.445271
Unknown 3	0.635527	0.946485	1.360317	1.804681
PCR Neg	-0.088466	-0.119757	-0.091988	-0.058117

Raw Data: Quantification – Sequence analysis



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