Assessing *in vitro* DNA Repair Methods

Written by: Ryan Lehto

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Department of Biology

Lakehead University, Thunder Bay, Ontario, Canada

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Declaration:

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person, except where due acknowledgment has been made in the text.

Ryan Lehto

I. Acknowledgements

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V. List of Abbreviations

A	Adenine
Adna	Ancient DNA
AGE	Agarose Gel Electrophoresis
AP	Apurinic/Apyrimidinic Sites
AMU	Atomic Mass Units
Bp	Base Pair
BER	Base Excision Repair
BSTFA	*N,O* -Bis(trimethylsilyl)trifluoroacetamide
C	Cytosine
CpDNA	Chloroplast DNA
DNA	Deoxyribonucleic acid
DSB	Double Strand Breaks
DsDNA	Double Stranded DNA
Endo IV	Endonuclease IV
ETBr	Ethidium Bromide
Fapy A	2,6-diamino-5-formamidopyrimidine
Fapy G	2,6-diamino-4-hydroxy-5-formamidopyrimidine
G	Guanine
GCMS	Gas chromatography/Mass spectrometry
H2O2	Hydrogen Peroxide
HPLC	High Performance Liquid Chromatography
ICLs	Interstrand Cross Links
MtDNA	Mitochondrial DNA
NER	Nucleotide Excision Repair
PCR	Polymerase Chain Reaction
PAGE	Polyacrylamide Gel Electrophoresis
PK	Protinase K
PTB	N-Phenacylthiazolium Bromide
SIM	Selected Ion Monitoring
SSB	Single Strand Breaks
SsDNA	Single Stranded DNA
T	Thymine
Taq	*Thermus aquaticus*
UDG	*E. coli* Uracil-DNA Glycosylase

VI. Abstract

The DNA molecule is constantly under attack from endogenous and exogenous sources leading to degradation by a variety of forms of damage. In this study current methods of *in vitro* DNA repair methods were evaluated for efficiency and for their relative ability to repair three different experimentally induced forms of damage strand breaks, abasic sites, modified bases and cross-links. GCMS was used to identify and evaluate effectiveness of repair methods for 16 different damaged products or modified bases. The methods were then used on ancient samples from three sites 1) Daklah Oasis, Egypt; 2) Copan, Honduras; and 3) Çayönü Tepesi, Turkey. Success was achieved repairing these ancient samples depending on which method was used and what type of damage was present. These results were consistent with the DNA damaged expected in each of these sets of samples predicted hypothetically from the environment in which these samples were recovered. A new helicase dependant DNA polymerase, Phi 29 had an unexpected DNA repair capability notably on hydrolytic damage while the PreCR™ enzyme repair mix was very effective at repairing the affects of oxidative damage. The DNA repair capabilities of 8 DNA repair systems were characterised and the demonstrated successful retrieval of DNA from 6 ancient DNA samples previously shown to be non-viable for genetic analysis were successfully analyzed and amplified.

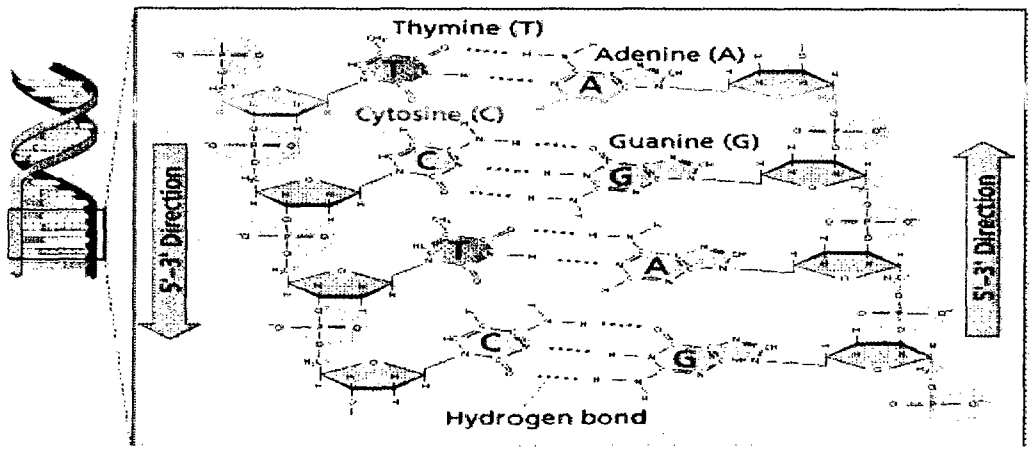
1.0 Introduction

The process of DNA repair is a very complex process that varies widely between organisms and has many interrelated pathways and components. Intact genomes are integral to the preservation of life. Genetic material undergoes rapid degradation after the organism dies. This damage accumulates over time and depends heavily on the manner of death, environmental conditions and age of specimen. While it is possible that some *in vivo* genetic maintenance and repair processes, can be used to repair some of the damage *in vitro*. This can help to increase the quantity and quality of template DNA retrieved from biological material even from samples of considerable age and increases the range of damaged and degraded biological sample available for genetic analysis.

1.1 DNA structure

Deoxyribonucleic acid (DNA) is the primary mechanism for the storage of genetic information. This molecule is the blueprint for life. It controls development, metabolism and drives evolution. It's structure was elucidated by work done by Franklin, Wilkins, Watson and Crick in the 1950s which is considered one of the great breakthroughs in molecular biology (Watson and Crick 1953a). DNA is a polymer consisting of many individual monomers called nucleotides. The nucleotides are composed of three separate parts a heterocyclic nitrogenous base which is attached to a sugar and a phosphate group. The nitrogenous bases are divided into two groups a single ring structure called a pyrimidine and a double ring structure called a purine. The purines include the bases adenine (A) and guanine (G) while the pyrimidines include the bases cytosine (C) and thymine (T) (Watson and Crick 1953a) These nucleotides are arranged in an double helix, winding around an axis in a right-handed spiral like the railing of a spiral staircase with the nitrogenous base towards the inside of the helix (Figure 1). The DNA

backbone is an alternating sugar-phosphate sequence. The helical structure is arranged with two anti-parallel chains running in the 5' to 3' directions which was found to be the most stable configuration (Watson and Crick 1953b). The deoxyribose sugars are joined at both the 3'-hydroxyl and 5'-hydroxyl groups to phosphate groups in ester links, also known as "phosphodiester" bonds (Alberts 1998). The bases preferentially pair together in this structure with G binding to C creating three hydrogen bonds within the helix centre and T binding to A which creates two hydrogen bonds. Although weak in themselves the force of the hydrogen bonds in an entire strand of DNA which can be many thousands of base pairs long allows the DNA to bind tightly together and also allow areas to be unzipped to allow for transcription of various genes by only having to overcome localized hydrogen bonding while leaving the rest of the strand intact. The twisting of the double helix create gaps of different sizes one called the minor groove and the other called the major groove in which many of the regulatory proteins attach due to the more exposed nitrogenous bases.



http://library.thinkquest.org/C0123260/basic%20knowledge/images/basic%20knowledge/DNA/DNA%20model%202.jpg

Figure 1. Model of DNA structure.
DNA double stranded molecule with bases arranged in the antisense arrangement of 3' to 5'. Bases are on the inside of the structure bonding the adjacent strand together through hydrogen bonding between complimentary bases while the sugar phosphate backbone located on the outside of the strand uses phosphodiester bonds to attach the bases in sequence inside the strand.

The DNA within the cell provides the template for DNA transcription and DNA replication. DNA transcription is where ribonucleic acids (RNA) are synthesized from the DNA template while DNA replication is where the whole genome is copied to allow for cell division and the formation of gametes. The DNA replication process can be copied *in vitro* by the polymerase chain reaction (PCR) which allows the amplification of specific DNA sequences. This technique developed in the 1980's is also considered a ground breaking moment in molecular biology (Mullis and Faloona 1987). It can create billions of copies of DNA theoretically from a single copy within a few hours depending on how many cycles are used with a doubling of the DNA in every cycle. The process uses single nucleotides (dNTPs) and thermostable DNA polymerases to copy template DNA and for sequencing uses dideoxynucleotides (ddNTPs) which terminates replication in combination with regular nucleotides (Sanger et al. 1977). One major problem with using PCR to amplify highly degraded or damaged DNA is that upon the denaturing step strand breaks that have accumulated cause fragmentation of the molecule which greatly hinders amplification and analysis. Damaged, modified or missing bases are also a major obstacle for the PCR reaction. When the DNA polymerase encounters a modified or missing base they can generate the misincorporation of an erroneous nucleotide or are unable to copy the DNA template causing them to fall off stopping transcription at that point. Changes to base pairing by misincorporations due to the presence of modified bases can also affect the quality of information retrieved and establish mutations in subsequent amplification products (Sikorsky et al. 2007).

1.2 Ancient and degraded DNA

DNA is a relatively unstable biological molecule that needs constant maintenance, from repair enzymes, and the stable environment that is found in the living cell to maintain its integrity and fidelity. Under normal conditions it is extremely rare to find preserved DNA post-mortem. These post-mortem DNA molecules are usually affected by fragmentation, cross-linking and modification. Sometimes if the DNA molecules are maintained in a relatively constant temperature, usually low temperature, and protected from other factors such as pH, water exposure, heat and pressure it is possible for these molecules to survive. The ability to extract and amplify these degraded or ancient DNA (aDNA) molecules allows researchers to reconstruct the past. All the information available to modern forensic science, population genetics, and evolutionary studies can theoretically be applied to extinct or past populations. The upper limit for DNA preservation has yet to be determined and continues to be extended as techniques are improved and new methods are discovered. Fully fossilized material no longer contains organic molecules meaning the organic backbone has been mineralized over the years and is not available for amplification or analysis with any current methodology. There were early spectacular claims of DNA surviving for millions of years (Myr) in plants (Golenberg et al. 1990) and from fossilized dinosaur bones (Woodward et al. 1994) along with the famous amber studies which was thought to have preserved ancient insects and possibly dinosaur blood (DeSalle et al. 1992). These claims were eventually shown to be the result of microbial or human DNA contamination from modern sources which is ubiquitous in the environment and is a constant problem with authenticating aDNA findings (Zischler et al. 1995). Due to the highly fragmented and highly degraded nature of aDNA a variety of methods were employed like high

cycle number PCR, the higher number of cycles in the PCR are needed for amplification by PCR, however this method can often lead to false positives (Yang et al. 2003).

The DNA will be rapidly degraded post-mortem, initially from the enzymes released as the cell dies losing its structural integrity and then later from environmental conditions. Kinetic calculations of hydrolytic damage rates predict that small fragments in temperate regions may survive a maximum of only about ten thousand years (Poinar et al. 1996). If protected in cold dry climates such as the polar ice caps with constant temperatures as low as -50 degrees Celsius, DNA may last considerably longer with reports of DNA being recovered from microbes and viruses trapped in ice core samples over 100 thousand years old (Willerslev et al. 1999). However with the DNA analysis of all of these extremely old samples there is still some controversy about their authenticity. The most successful geographical area in which aDNA has been successfully recovered and authenticated has been in the permafrost areas of the northern hemisphere which have yielded 65 thousand year old bison mitochondrial DNA (mtDNA) (Gilbert et al. 2004) and possibly 300 to 400 thousand year old plant chloroplast DNA (cpDNA) (Willerslev et al. 2003). Other geographical areas have yielded aDNA with varying degrees of success. DNA survival long term depends on the amount and type of damage that accumulates which depends a great deal on the conditions the biological material is found. Generally speaking aDNA fragments are considered to be in the 100 to 500 base pair (bp) range and is one of the factors considered to authenticate the results (Hoss et al. 1996). Different tissue types also have varying rates of decay and preservation characteristics depending on their physical structure, biochemistry, taphonomy and any pretreatments which can preserve the tissue but may also create inhibition which is the second biggest problem in aDNA studies (Burger et al. 1999).

1.3 DNA damage

DNA damage is an extremely common event in the life cycle of DNA but many mechanisms exist to keep it intact within a living cell. If the DNA is contained in a metabolically active tissue then enzymatic damage occurs rapidly after the cell dies and loses its membrane integrity allowing the digestive enzymes to release from their segregated organelles. For preservation of the nucleic acids there has to be a rapid halt to this process such as rapid desiccation, freezing or treatment with an inhibitory substance (Pusch et al. 2003). Even with minimal initial damage and under ideal preservation conditions nucleic acids gradually degrade over time through spontaneous processes such as hydrolysis and oxidation (Hofreiter et al. 2001). Post-mortem DNA damage is characterized by strand breaks, abasic sites, miscoding lesions, modified bases and cross-links which block transcription (Figure 2) (Lindahl 1993).

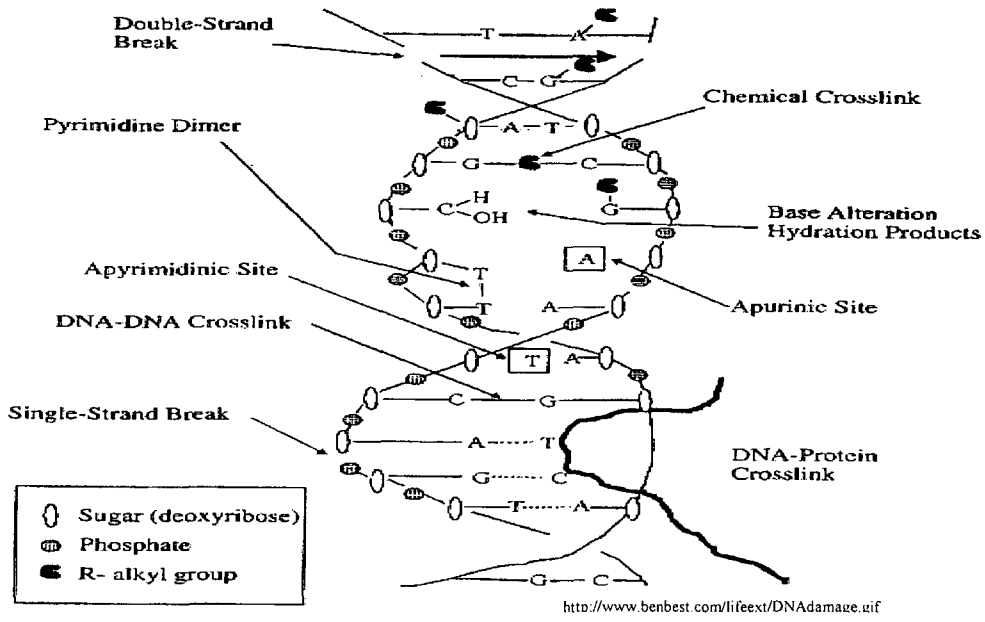


Figure 2. Forms of DNA damage
The most common type of DNA damage *in vivo*, these forms of damage can be caused by multiple sources both endogenous and exogenous and each source of damage may induce more than one type of damage.

1.3.1 Strand breaks

The term strand break can be applied to a wide range of diverse chemical structures. Strand breaks are usually characterized by the loss or modification of the phosphodiester bond in the sugar-phosphate backbone of DNA which results in a loss of integrity (Karimi-Busheri et al. 1998). The phosphodiester bond is vulnerable to hydrolysis, both occur at a fairly slow but steady rate and are constantly being repaired in metabolically active tissue but will accumulate in tissues post-mortem (Lindahl and Wood 1999). Oxidative damage and enzymatic attack can also break the DNA backbone. Single strand breaks (SSB) are lesions on one side of the DNA helix while the far more damaging double strand breaks (DSB) have lesions adjacent to each other on both strands or in the very near vicinity causing a blunt or sticky end shearing of the helix into two fragments. There are many chemically distinct 3' and 5' modifications but to be repaired the 3'-termini have to be returned to hydroxyl groups and 5'-termini to phosphate groups in order for DNA polymerases and ligases to initiate and complete the DNA repair process. DSB repair involves either homologous recombination which requires an additional copy of the DNA sequence on a sister chromatid or by non-homologous end joining which rejoins the broken ends directly but also may incorporate errors and deletions (Dobbs et al. 2008). DSB will inhibit both nucleotide excision repair (NER) and base excision repair (BER) *in vitro* even before the denaturation step and are one of the major obstacles in repairing post-mortem DNA *in vitro* (Calsou et al. 1996).

1.3.2 Abasic sites

The chemical bond between a DNA base and its respective deoxyribose sugar, the glycosidic bond, is subject to chance cleavage by a water molecule in a process known as spontaneous hydrolysis. The result of hydrolysis of the glycosidic bond is the creation of an

abasic site as the base is cleaved. The formation of an abasic site can occur to any of the four bases (A, T, G and C), both purine and pyrimidines, but have been reported at different rates where depurination has a higher rate than depyrimidination (Lindahl and Andersson 1972). Abasic sites can generate misincorporation lesions depending on the DNA polymerase but in most cases will prevent DNA replication if allowed to accumulate and become blocking lesions.

1.3.3 Modified bases

Nucleic acid bases can be modified from their chemical structure in a multitude of ways and each base has many sites which are susceptible to attack or modification (Figure 3). The nitrogen-carbon bond in the heterocyclic ring structure is less stable than a carbon-carbon bond. The presence of heteroatoms of which nitrogen is one, results in significant changes in the cyclic molecular structure due to the availability of unshared electrons and the difference in electronegativity between the heteroatom and carbon. The purines because of their double ring structure contain more heteroatom sites that are chemically reactive, so is often the target for the majority of modified bases in DNA (Table 1). Guanine is the most reactive base with an extra oxygen in the O6′ position of the ring which is also reactive and exposed to certain attack (Garcia-Valverde and Torroba 2005).

The susceptibility of DNA bases to modification depends on its environment. The most common damage types that happen within the cell are alkylation/methylation, oxidation, deamination (hydrolysis) and hydrogenation. Other damage mechanisms exist especially in highly degraded DNA but the most common forms of damage have repair mechanisms *in vivo* which theoretically could be performed *in vitro* (Tuteja et al. 2001). Methylation is the most common form of alkylation in DNA which is simply the addition of a methyl group to a DNA base, usually C in a cell. It is most harmful to living organisms because of its gene

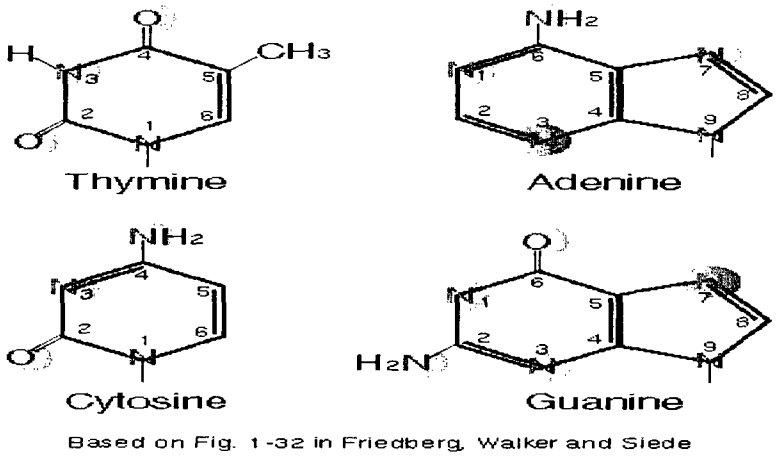


Figure 3. Heteratoms in DNA bases most susceptible to chemical modification
The most active sites on the four main DNA bases that are subject to modifications especially to oxidative damage and also for silation in derivitization for GCMS analysis.

silencing consequence and transcriptional mutations (Razin and Riggs 1980) but still can cause problems *in vitro* by inhibiting PCR, preventing amplification as a blocking lesion or by inducing sequence changes to copies in the PCR reaction. Early on radiation biologists learned that the attack of hydroxyl (·OH) radicals generated by the radiolysis of water had significant alterations to all four bases and the deoxyribose sugar (Teoule 1987). Hydroxyl radicals are also produced through oxidation and enzymatic processes. It has been estimated that as much as 2% of the oxygen consumed through respiration is converted to free radicals such as the ·OH radical. This is part of normal metabolism and is handled by the cell, while healthy and alive, but rapidly accumulates when the cell dies (Aust and Eveleigh 1999). Reactions of the ·OH radical can be classified into three main types: hydrogen extraction, addition and electron transfer. Reactions of the ·OH with the deoxyribose sugar proceeds by hydrogen abstraction forming carbon centered radicals. All five carbons in the deoxyribose are vulnerable to this attack. Under anaerobic conditions the C4′ carbon can undergo β cleavage which leads to stand breakage, generating an abasic site and modifies the sugar (Dizdaroglu et al. 1975). Under aerobic conditions peroxyl radicals are formed by the addition of molecular oxygen. This results in the cleavage of a carbon-

carbon bond and the creation of an alkali-labile site. The C5′ can again undergo β cleavage which generates a strand break then the release of an intact base and an altered sugar occurs. An aldehyde formation at the C5′ can also occur while generating a strand break (Goldberg 1987). The heterocyclic bases in the DNA can be modified through an addition reaction. In pyrimidines the ·OH radical adds to the C5′-C6′ double bond creating base radicals that rapidly undergo additional chemical reactions which results in multitudes of modified bases (O'Neill 1983). In purines the ·OH radical adds to the C4′, C5′ and C8′ positions which can create both oxidizing and reducing types of radicals expanding the additional modification products that may be created (Cadet et al. 1999). The addition to the C8′ can also undergo unimolecular opening of the imidazole ring again offering the opportunity for many possible modifications from additional reactions depending on the substances available and environmental conditions (Dizdaroglu et al. 2008). The oxidized purine bases 2,6-diamino-5-formamidopyrimidine (Fapy-A) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-G) are lethal lesions to cells *in vivo* effectively stopping replications one base prior to the fapy residue. *In vitro* analysis have shown both fapy modifications to be blocking lesions to both *E.coli* DNA Polymerase Klenow Fragment as well as phage T4 DNA Polymerase effectively inhibiting PCR analysis (O'Connor et al. 1988). Deamination is the hydrolysis of an amine group from A, C or G which results in a modified base (Table 1). An acidic, moist environment and elevated temperatures will speed up the rate of hydrolysis (Wolfenden et al. 1998). The deamination of DNA bases occurs more frequently in pyrimidines than in purines but both are equally mutagenic (Mol et al. 1999). In this reaction an oxygen atom is donated from a water molecule. The spontaneous deamination products of A and G are recognizable as unnatural when they occur in DNA and thus are readily recognized and repaired (Table 1). Deamination does

Table 1. Oxidative DNA modified products and the mutations induced

DNA modification	Mutation (base change)	Reference
5-formyluracil	C→T	1, 2
	G→T	1, 2
	T→C	1-4
	T→A	1-4
	T→G	2, 5
5-hydroxyuracil	C→T	2, 6-8
5,6-dihydrouracil	G→A	2, 9
5,6-dihydroxyuracil	C→T	2, 7
	G→A	
5-hydroxy-6-hydrouracil	C→T	2
5-hydroxymethyluracil	C→T	2, 7, 10, 11
Uracil glycol	C→T	2, 6, 8
5-hydroxymethylcytosine	C→T	11, 12
5-hydroxycytosine	C→T	2, 6-8
5,6-dihydroxycytosine	C→T	2
5-hydroxy-6-hydrocytosine	C→T	2
5-formylcytosine	C→T	8, 13
	C→A	8, 13
cytosine glycol	C→T	2
8-hydroxyguanine	G→T	2, 4, 7, 8, 14, 15
	G→C	2, 4, 14, 15
	G→A	14, 15
	A→C	8, 16
8-hydroxyadenine	A→G	2, 14, 17
	A→C	14, 17
2-hydroxyadenine	A→G	2, 8, 15
	A→T	8, 15
	A→C	8, 15
5-hydroxy-6-hydrothymine	T→C	2
thymine glycol	Blocking	2, 8, 18
5,6-dihydrothymine	T→C	2
5-hydroxy-5-methylhydantoin	Blocking	2, 7
trans-1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine	Blocking	2
5-hydroxyhydantoin	Blocking	2, 7
Alloxan	Blocking	2
4,6-diamino-5-formamidopyrimidine (FapyA)	Blocking	2,7
2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyG)	Blocking	2,7
Oxazolone	G→T	2,8

1 (Anensen et al. 2001). 2 (Cooke et al. 2003). 3 (Miyabe et al. 2001). 4 (Zhang 2001). 5 (Zhang et al. 1997). 6 (Kreutzer and Essigmann 1998). 7 (Kasprzak et al. 1997). 8 (Evans et al. 2004). 9 (Liu, Zhou et al. 1995). 10 (Cannon-Carlson et al. 1989). 11 (Hori et al. 2003). 12 (Baltz et al. 1976). 13 (Karino et al. 2001). 14 (Tan, Grollman et al.1999). 15 (Kamiya 2004). 16 (Cheng et al. 1992). 17 (Tuo et al. 2003). 18 (Basu et al. 1989).

not occur with T as there is no amine group but about 3% of the C nucleotides in vertebrate DNA and as much as 25% of plant DNA are methylated to help in controlling gene expression. In mammalian cells C is often methylated on the 5' position in the gene regulation and silencing function making the most common mutation which is the deamination of 5-methlycytosine and formation of the base T (Waters and Swann 2000). These 5-methylcytosine nucleotides can be deaminated, to form the natural nucleotide T. This T would be adjacent to a G on the opposite strand, forming a mismatched base pair which will persist as a mutation in the sequence (Horst and Fritz 1996). Oxidative damaged bases can also generate replication errors, and transcription errors (Table 1) (Anensen et al. 2001; Cooke et al. 2003; Kamiya 2004; Tan et al. 1999).

Hydrogenation is simply the addition of a hydrogen atom to the compound usually at the site of a carbon=carbon double bond. This addition reaction reduces the double bond to a single bond. On some occasions it is catalytic where it breaks a single bond. The T and C bases are the most susceptible to these modifications at the C4′=C5′ double bond. The resonance stability of the C4′=C5′ double bond gives the N-glycosidic bond it resistance to acid hydrolysis. This modified base is now susceptible to depyrimidization and/or stand breakage (Dabkowska et al. 2005). Many divalent metals increase the rate of hydrogenation especially if the DNA is exposed such as the case in buried or treated remains (Cano 1996).

1.3.4 Cross links

Cross-linking of DNA to DNA is a covalent bonding between two bases within one strand (intrastrand) or to a base of an adjacent strand (interstrand). Cross-linking can also occur between DNA and protein and DNA and sugars. All three types have deleterious effects in living organisms and can occur through a variety of exogenous and endogenous agents

Intrastrand DNA cross-linking can cause kinking in the DNA strand which can prevent regulatory proteins from attaching or create a blocking structure for a DNA polymerase or repair protein which will prevent replication and amplification both *in vivo* and *in vitro.* Interstrand cross-links (ICLs) are an extremely toxic class of DNA damage incurred during normal metabolism and spontaneously post-mortem. The ICLs covalently tether both strands of duplex DNA, preventing strand unwinding which is essential for DNA polymerase access for transcription of essential metabolic proteins and for replication *in vivo* and prevents artificial amplification like PCR *in vitro* (Rothfuss and Grompe 2004). In kinetic studies done by Hansen *et al* (2006) on nucleic acids from Siberian frozen sediment core samples from the permafrost, layers ranging from 10,000 to 600,000 years old, they found that ICLs accumulated approximately 100 times faster than SSB. Although this will prevent amplification and retrieval through PCR it may well preserve the integrity of the biological molecule over long periods of time (Hansen et al. 2006). The mechanisms for the covalent bond formations were thought to involve a free radical transfer in the presence of molecular oxygen which is most likely the main mechanism *in vivo* but *in vitro* by exogenous agents by a free radical mechanism that requires no molecular oxygen to be present (Greenberg 2005).

Proteins can become cross-linked to DNA by a variety of agents including ultra-violet (UV) light, metals, various aldehydes and environmental chemicals. The mechanism most often is an oxidative free radical but can also occur through various chemical agents in combination with a metal such as chromium or nickel (Barker et al. 2005).

1.4 Damage detection

Detecting, identifying and quantifying types and severity of damage in DNA has been an active area of research in many fields. Multitudes of methods exist all with advantages and with

major disadvantages; the challenge in this study was to find methods that could work for very small quantities of damaged DNA. The methods had to be sensitive, low cost, quick and without too much specialized equipment allowing multiple and rapid screening.

1.4.1 Gel electrophoresis

Agarose gel electrophoresis is the easiest and most common way of separating and analyzing DNA (Johansson 1972). The DNA is placed in a well within the gel matrix and an electric current is applied. The DNA will then separate out from each other based on size and charge with smaller DNA fragments migrating faster through the gel than the larger ones allowing separation. The DNA is visualized in the gel by the addition of ethidium bromide (EtBr) which intercalates in the groove of the DNA and fluoresces under UV light. Molecular markers of known sizes are run on each gel for comparison. Band intensities can be measured to give quantitative measurements of DNA amount or to assess inhibition and optimization of a PCR reaction. The presence of smearing or bands of unexpected size may also indicate template damage or partial PCR inhibition.

Polyacrylamide gel electrophoresis (PAGE) uses the same principles as agarose gel electrophoresis but uses polyacrylamide for the matrix which because of its synthetic nature allows for a more uniform distribution. It is particularly useful when a higher degree of resolution is necessary even single base pair differences in DNA bands can be distinguished.

1.4.2 Gas chromatography mass spectroscopy

Structural identification of modified bases in DNA has been a much sought after and elusive goal for investigators. High performance liquid chromatography (HPLC) with electro-chemical (EC) detection has been used successfully for the analysis of modified bases in cellular

DNA but the sensitivity is limited and many modified bases could not be observed (Dizdaroglu et al. 1993b). Gas chromatography coupled to mass spectrometry (GCMS) is more universal and provides high sensitivity and selectivity for the characterization and quantification of modified nucleic bases. GCMS with electron ionization is a very reliable and highly sensitive method for the detection and quantification of all four nitrogenous bases in DNA and a large group of their modified products from one sample (Jaruga et al. 2008). DNA being a large molecule needs to be hydrolyzed into individual nucleotides or nucleosides for separation and for discrimination by the GCMS. DNA can be hydrolyzed by a heat/acid method or enzymatically in either case residual hydrolyzing agents become possible contaminants in the process if not removed. After hydrolysis GCMS requires that the polar nucleotides and bases must be converted to thermally stable derivatives that posses characteristic mass spectra (Jenner et al. 1998). One of the most popular methods is trimethlysilylation with *N, O* -bis(trimethylsilyl)trifluoroacetamide (BSTFA). BSTFA is an effective trimethylsilyl donor that reacts with a wide range of polar compounds to replace labile hydrogens on a wide range of polar compounds with a -Si $(CH_3)_3$ group. Therefore, it is widely used to prepare volatile and thermally stable derivatives for gas chromatography and mass spectrometry and is particularly suited to the derivitization of nucleic acids (Dizdaroglu 1990). The presence or absence of oxygen during the derivitization process can also effect the types and amounts of modified products detected so most reactions are done under an inert gas such as nitrogen (Dizdaroglu 1994). Ion profiles were generated for the damaged nitrogenous bases in this study. To locate and identify specific damage products in the derivitized nucleic acid samples with GCMS, ion profiles from previously published work that are known to be representative of that individual product with a very high degree of accuracy and certainty within a fairly complex mixture were used.

1.5 DNA Repair *in vivo*

In mammalian cells there are at least four major pathways for the repair of damaged DNA. First, a simple reversal of the damage called direct reversal (DR); second, base excision repair (BER); third, nucleotide excision repair (NER), including mismatch and transcription-coupled repair; and last, recombination repair including non-homologous end joining. Simple DR repair can be applied to repair modification to the DNA strand that involve the formation of adducts or cross-links whereby the DR repair is simply a cleavage of these structures. Some of these types of mechanisms include alkylation where the added methyl or alkyl group can be chemically or enzymatically removed.

1.5.1 Direct Reversal

The DR repair is the most energy efficient method of DNA repair and does not involve breaking the DNA backbone potentially exposing the DNA to greater damage but there are only a few types of DNA damage that can be repaired in this way. The DR repair system can reverse the UV induced pyridimine dimer formation and remove methyl groups by methyltransferases. The most frequent damage type is the spontaneous addition of a methyl group (CH_3-) to C (Wyatt and Pittman 2006) this additional methyl group can be cleaved using methyltransferases. The formation of pyrimidine dimers, the major type of damage caused by UV light, distorts the double helix and blocks transcription or replication past the damaged site. The DR process called photoreactivation causes direct reversal of the dimerized reaction, thus the original pyrimidine bases are restored (Jagger 1958).

1.5.2 Bypass Damage Repair

Pyrimidine dimers, modified bases and protein cross-links can act as blocking lesions to DNA replication. Many cells have specialized low fidelity and often error prone DNA polymerases that can replicate the damaged DNA section without disassociating from the DNA strand (Johnson et al. 1999). The majority of these DNA polymerases belong to the Y family of DNA polymerases and are present as homologues in many different kinds of organisms. These DNA polymerases all lack 3' to 5' exonuclease activity and are capable of all twelve mismatch base pairings (Kokoska et al. 2002). The presence of error prone DNA polymerases in organisms that need to maintain specific genome integrity does not seem to make evolutionary sense but because their activity is severely limited. As an example the *Sulfolobus solfataricus* DNA Polymerase IV (Dpo4), a thermostable Y family DNA polymerase is able to replicate up to approximately 50 nucleotides per binding event with most binding events only resulting in a few nucleotide inclusions (Boudsocq et al. 2001). This suggests that these enzymes are used to patch DNA in combination with other proofreading enzymes (Godoy et al. 2006).

1.5.3 Enzymatic BER and NER

Base excision repair involves removing the incorrect or modified base from the DNA strand by an appropriate DNA N-glycosylase to create an apurinic/apyrimidinic (AP) site. These hydrolyze the N-glycosidic bond between the base and the deoxyribose sugar of the DNA backbone. The crystal structures of many of the DNA glycosylases have been determined. They are similar to each other, and they suggest a common mode of action (with variations, depending on the specific structure recognized by the glycosylase). It appears that the DNA glycosylases gently pinch the DNA while scanning it, so that the DNA kinks (bends sharply) at positions of instability caused by the mismatching bases. The glycosylases all possess specific binding sites

for the modified bases that they recognize. The DNA kinking, combined with additional pushing by the enzyme, encourages mismatched bases to flip out of the DNA double helix and enter the binding site. If the modified base is recognized by the fit within the binding site of the glycosylase the bond to the deoxyribose in the DNA backbone is cleaved (Roberts and Cheng 1998). Then an AP Endonuclease creates a nick in the backbone of the damaged DNA strand upstream of the AP site, thus creating a 3'-OH terminus adjacent to the AP site. A DNA polymerase then can attach to the DNA upstream and synthesize a new DNA strand by replacing the AP site and finally the end is ligated by a DNA ligase enzyme.

Nucleotide excision repair is a more complicated procedure for dealing with DNA damage (Maddukuri et al. 2007). The NER is one of the most versatile repair pathways which operate in both eukaryotes and prokaryotes. Unlike the other repair pathways NER is capable of removing various classes of damage including cross-links (Balajee and Bohr 2000). The major difference between NER and BER is the way the damage is removed. The NER cuts out the damaged DNA in sections, while in the BER pathway only the base of one nucleotide is excised. In eukaryotes up to 32 nucleotides can be removed at a time with NER (Moggs et al. 1996). The NER pathway in mammals involve at least 30 gene products while bacteria use mainly four enzymes (DeLaat and Meadows 1999). Due to the complexity of the mammalian system and problems that would be involved trying to adapt to an *in vitro* system this project will focus on the bacterial NER. The NER systems recognize the damaged DNA strand and cleave it 3′, then 5′ to the lesion. After the oligonucleotides containing the lesion are removed, repair synthesis fills the resulting gap. The UvrABC is the endonuclease enzyme complex that is responsible for repairing a variety of structurally dissimilar DNA damage products *in vitro* and *in* vivo. The UvrA enzyme within the enzyme complex is involved in recognizing certain damage products

and helping the UvrB enzyme attach. The UvrB is the central component of bacterial NER. It is directly involved in distinguishing damaged from undamaged DNA and guides the DNA from recognition to repair synthesis (Theis et al. 2000). The UrvC combines with UrvB and together excise a 12 to 13 oligonucleotide fragment at either side of the lesion which is released from the DNA by another enzyme UvrD (Orren and Sancar 1990). A DNA polymerase can now attach provided the sides of the newly created lesion have the appropriate hydroxyl or phosphate group and fill in the gap using the opposite strand as a template. Finally a DNA ligase enzyme fills in the remaining gap after the DNA polymerase has filled in the section and disassociated.

1.6 DNA Repair Methods *in vitro*

1.6.1 Simple ligation

Simple ligation is an *in vitro* method of DNA repair based on the BER *in vivo* repair pathway. Before PCR the samples are incubated with DNA Polymerase I (Pol I) which translates the nicks in the DNA strand, and the remaining gaps are closed by subsequent treatment with T4 DNA Ligase (Pusch et al. 1998). This simple method was performed by Pusch *et al.* (1998) on buried skeletal material between thirteen hundred and sixteen hundred years old excavated from an Alamannic burial site at Neresheim, Germany. The site's characteristics include a temperate climate with warm summers and cold winters, but prolonged periods of frost or snow are rare. Precipitation is recorded throughout the year. Work done later by Di Bernardo *et al.* (2002) again used this treatment on remains from the archaeological site of Pompeii with an additional step of a pre-denaturation to try and improve the method, based on the theory that the DNA would have accumulated cross-links over time. However the amplifiable regions of interest would have to be within these cross-linked regions for this method to be effective (Di Bernardo et al. 2002). The

Pompeii site characteristics included a soil/ash pH value slightly alkaline at 7.5 and low humic acid content (Cipollaro et al. 1999). The dynamics of the burial by the volcanic eruption of Vesuvius in AD 79 which buried the town rapidly in as much as thirty feet of hot ash also prevented microbial and fungal degradation and presumably an anaerobic environment with a stable temperature and relatively arid climate. The Di Bernardo team (Di Bernardo et al. 2002) increased the success rate of retrieval and amplification of DNA by 80% while the Pusch team (Pusch et al. 1998) was unable to amplify their target region through PCR. The difference between the samples could explain the differences in this repair method reported by these researchers.

1.6.2 Glycosylase with ligation

A proposed improvement to simple ligation includes an addition step of including a glycosylase as a pretreatment before the repair reaction. In this method a glycosylase that recognizes a variety of damage modifications, or a specific glycosylase if the modified damage product is known, is added to the damage DNA in the first step toward repair. It removes the aberrant base from the DNA backbone by hydrolyzing the N-glycosidic bond to produce an AP site (Ide and Kotera 2004). A proof reading enzyme usually belonging to the Pol I family can insert the correct base back into the apurinic site using the opposite strand as a template while the DNA is still double stranded. Finally just as in the simple ligation, a DNA ligase enzyme joins the strands at the nicks back together. The treated DNA is then ready for further amplifications after a short heat denaturation and inactivation of the repair enzymes but not high enough to denature the double stranded DNA.

1.7 Enzymes

DNA repair enzymes have the ability to search through vast tracts of DNA to find subtle anomalies in the structure. Each enzyme can have multiple functions such as a proofreading DNA polymerase or a very specific task such as the human repair enzyme 8-oxoguanine Glycosylase (hOGG1) which specifically removes 8-oxoguanine (oxoG), a damaged G with an extra oxygen atom but leaves all others (David 2005).

1.7.1 DNA Polymerase Klenow Fragment

The DNA Polymerase Klenow Fragment (Klenow) is the large fragment of DNA Polymerase I. It exhibits the 3'→ 5' exonuclease activity as well as 5'→ 3' polymerase activity but lacks the 5'→ 3' exonuclease activity of Pol I (Feinberg and Vogelstein 1983). It is most commonly used for nick translation and labeling of DNA, Filling in recessed 3' ends of DNA fragments and digesting away protruding 3' overhangs. A "fill-in" reaction is used to create blunt ends on fragments created by cleavage with restriction enzymes that leave 5' overhang while digesting 3' overhangs is another method for producing blunt ends on DNA generated from restriction enzymes that cleave to produce 3' overhangs (King et al. 1996). The ability to incorporate nucleotides at strand nicks and AP sites at a relatively low temperature makes it a good candidate for repairing damaged DNA *in vitro* before amplification.

1.7.2 T4 DNA Polymerase

The T4 DNA Polymerase is a bacteriophage of *E. coli*. The activities of T4 DNA Polymerase are very similar to Klenow fragment of Pol I. It catalyzes the synthesis of DNA in the 5′→ 3′ direction and requires the presence of template and primer. This enzyme has a 3′→ 5′ exonuclease activity which is much more active than that found in Pol I. Unlike Pol I, T4

DNA Polymerase does not have a 5′→ 3′ exonuclease function making it much like the Klenow Fragment of Pol I but has a higher fidelity and a more robust exonuclease ability (Gupta et al. 1984). Substituting T4 DNA polymerase for the Klenow in the simple ligation method should increase its efficiency.

1.7.3 T4 Ligase

The T4 DNA Ligase is an enzyme encoded by bacteriophage T4. It catalyzes a joining reaction between DNA molecules involving the 3' - hydroxy and the 5' - phosphate termini. It also catalyzes the covalent joining of two segments to one uninterrupted strand in double stranded DNA (dsDNA). This is very important for the repair of single stranded nicks that would cause the DNA to fragment when it is denatured in PCR. Bacteriophage T4 DNA Ligase is a single polypeptide with M.W. of 68,000 Daltons. Maximal activity is obtained at pH 7.5 - 8.0. At pH 6.9 and pH 8.3 the enzyme exhibits 40% and 65% of its full activity respectively. Mg^{2+} presence is required.

1.7.4 Endonuclease IV

Endonuclease IV (Endo IV) can act on a variety of oxidative damage in DNA. The enzyme is apurinic/apyrimidinic (AP) endonuclease that will hydrolyze intact AP sites in DNA. AP sites are cleaved at the first phosphodiester bond that is 5' to the lesion leaving at hydroxyl group at the 3' terminus and a deoxyribose 5'-phosphate at the 5' terminus which is important for ligation later on (Levin and Demple 1996). In addition Endo IV also has 3'-diesterase activity and can release phosphoglycoaldehyde, intact deoxyribose 5-phosphate and phosphate from the 3' end of DNA which will are blocking lesions to DNA polymerases and DNA ligase preventing further DNA repair (Sandigursky and Franklin 1993).

1.7.5 Phi 29

Phi 29 (alternatively Ψ29) is a DNA polymerase that is the product of a viral gene from the *Bacillus subtilis* phage 29. It is approximately 60 kDa in size and has a few properties that make it potentially useful in DNA repair and recovery. It has excellent strand displacement ability and processivity with an error incorporation rate 100 fold lower than *Taq* DNA Polymerase (Esteban et al. 1993). It is able to perform these functions at moderate temperatures so the DNA stands do not need to be heated to denature them and risk the fragmentation of the molecule as it is amplified so a thermocycler is not needed to amplify DNA and could be performed on site. In countries or locations in which sample removal is frowned upon this trait could be very useful. The less participants in the chain of custody also would greatly reduce the chance of contamination which is the greatest obstacle to degraded DNA and aDNA research The next trait it contains which makes it a potential repair enzyme is it possesses 3' → 5' exonuclease ability for proofreading and repairing damaged DNA template (Blanco and Salas 1996). It was reported to have successfully amplified full mitochondrial genomes of 6 felids samples from Thailand up to 19 years old that showed major degradation (Janecka 2006).

1.7.6 PreCR™ Repair Mix

The PreCR™ Repair Mix is an enzyme cocktail formulated to repair damaged template DNA prior to its use in the PCR, microarrays or other DNA technologies. PreCR™ claims to repair a broad range of DNA damage, including apurinic/apyrimidinic sites, thymine dimers, nicks, gaps, deaminated C and 8-oxo-guanine. In also claims to remove a variety of modifications from the 3′end of DNA which are major inhibitors to PCR and leaves a hydroxyl group. The PreCR™ Repair Mix does not repair all damage that inhibit/interfere with PCR which include many modified bases such as 8-oxo-7, 8-dihydro-2'deoxyadenosines or

fragmented DNA. The DNA ligase present in the mix is very active at sealing nicks in DNA but does not ligate blunt ends or nicks near a mismatch effectively. It consists of a mixture *Taq* DNA Ligase, Endonuclease IV, *Bst* DNA Polymerase, Formamidopyrimidine-DNA Glycosylase (Fpg), Uracil-DNA Glycosylase (UDG), T4 Pyrimidine Dimer Glycosylase (PDG) (alternatively T4 Endonuclease V), Endonuclease VIII (Endo VIII) which is proprietary and the relative concentrations of each are not disclosed.

Taq DNA Ligase catalyzes the formation of a phosphodiester bond between 5′ phosphate and 3′ hydroxyl termini of two adjacent oligonucleotides. The ligation will occur only if the oligonucleotides are perfectly paired to the complementary target DNA and have no gaps between them; therefore, a single-base substitution can be detected which allows for a very high fidelity without misincorporations. *Taq* DNA Ligase is active at elevated temperatures (Wu and Wallace 1989)

The Formamidopyrimidine-DNA Glycosylase (Fpg), also referred to as 8-oxoguanine DNA Glycosylase in some literature, acts as both a N-glycosylase and an AP-lyase. The N-glycosylase activity releases damaged purines from double stranded DNA, generating an apurinic site (AP site). The AP-lyase activity cleaves both 3′ and 5′ to the AP site thereby removing the AP site and leaving a 1 base gap. This enzyme repairs oxidative DNA damage by efficiently removing formamidopyrimidine lesions and 8-oxoguanine residues from DNA. Some of the damaged bases recognized and removed by Fpg include 7, 8-dihydro-8-oxoguanine (8-oxoguanine), 8-oxoadenine, Fapy-G, methyl-fapy-guanine, Fapy-A, aflatoxin B_1-fapy-guanine, 5-hydroxy-cytosine and 5-hydroxy-uracil (Tchou et al. 1994; Boiteux et al. 1992; Boiteux et al. 1990) . The *Bst* DNA Polymerase Large Fragment is the portion of the *Bacillus stearothermophilus* DNA polymerase protein that contains the 5′ → 3′ polymerase activity, but

lacks 5′ →3′ exonuclease activity similar function to the Klenow fragment of Pol I (Aliotta et al. 1996).

The *E. coli* Uracil-DNA Glycosylase (UDG) catalyses the release of free uracil from uracil-containing DNA. UDG efficiently hydrolyzes uracil from single-stranded or double-stranded DNA, but not from oligomers (6 or fewer bases) (Devchand et al. 1993).

The T4 Pyrimidine Dimer Glycosylase (PDG) has both DNA glycosylase and AP lyase activity. The 16 kDa protein recognizes cis-syn-cyclobutane pyrimidine dimers caused by UV irradiation. The enzyme cleaves the glycosyl bond of the 5′ end of the pyrimidine dimer and the endonucleolytic activity cleaves the phosphodiester bond at the AP site (Higgins and Lloyd 1987).

Endonuclease VIII from *E. coli* acts as both a N-glycosylase and an AP-lyase. The N-glycosylase activity releases damaged pyrimidines from double-stranded DNA, generating an apurinic site (AP site). The AP-lyase activity cleaves 3′ and 5′ to the AP site leaving a 5′ phosphate and a 3′ phosphate. Damaged bases recognized and removed by Endo VIII include urea, 5, 6- dihydroxythymine, thymine glycol, 5-hydroxy-5- methylhydantoin, uracil glycol, 6-hydroxy-5, 6-dihydrothymine (Dizdaroglu et al. 1993a; Hatahet et al. 1994).

1.8 Chemical

Several chemical additives have been reported to enhance recovery of DNA but most are effective only on cross-linking between DNA bases and covalently bonded heavy metals. An example of this is N-Phenacylthiazolium Bromide (PTB) which reportedly improves DNA retrieval from bones and coprolites by cleaving sugar-derived condensation products that otherwise may encapsulate nucleic acids becoming covalently cross-linked to them specifically in advanced stages of the Maillard reaction (Poinar et al. 1998; Hofreiter et al. 2000).

2.0 Methods and Procedures

2.1 Method optimization

DNA template was damaged, purified and repaired all within twenty four hours to prevent any unwanted damage products forming over time such as additional strand breaks which can spontaneously be generated from some forms of DNA damage. Certain oxidative products are unstable and could also convert to other modified bases which if not analyzed in a timely manner would give misleading information to the quantity and type of modified bases in the sample.

2.1.1 DNA extraction

The enzymatic extraction methods have been around since the 1970s and have been employed in many fields of molecular biology. The Proteinase K (PK) enzymatic method is a standard extraction method used in forensic science for its reliability. The method used in this research is a modified method from the PK method presented by Hansen (Hansen 1974). The PK extraction will be used to extract mitochondrial DNA from buccal mouth swabs. In 1.5mL centrifuge tubes extraction buffer (20% SDS, 0.9M tris base, 0.9M boric acid, 0.5M EDTA) is added to a up to a volume of 385 uL PK enzyme (20mg/mL) aliquot of 2μL is added and vortexed. A buccal swab of the inner cheek is added to the tube and quickly vortexed. The tubes are then incubated at 55°C for 3 hours.

2.1.2 DNA purification

2.1.2.1 Ethanol precipitation

DNA extracts to be purified and heat/acid treated samples are prepared in 1.5mL sterile centrifuge tubes. 10% v/v of 3M sodium acetate is added to each tube and vortexed for 1 minute.

A 2.5X volume of cold ethanol (95%) is added and tubes are placed on ice for 30 minutes to allow precipitation to occur. Tubes are the centrifuged in a table top centrifuge at 17,900 x g for 5 minutes. The supernatant is removed without dislodging pellet. The pellet is air dried for 1 hour. It is then resuspended in 150µL of ddH_2O and incubated at 37°C for 15 minutes. If the extracts were not being used immediately they can be stored at -20°C. If the extract is being used for the heat/acid treatment the pellet is resuspended in 50µL of ddH_2O and either used in a PCR or derivitized for GCMS.

2.1.2.2 QIAquick PCR purification bind/elute columns

QIAquick columns were used to purify DNA after damage and repair treatments to remove damaging agents and repair enzymes which could inhibit PCR or GCMS results. Buffer solutions were supplied from Qiagen with the columns. A 5X volume of PB Buffer was added to a 1X volume of sample to be purified and quickly vortexed. The mixture was pipetted to the centre of the membrane in the column and centrifuged for 1 minute at 17,900x g. The eluate is discarded. A volume of 750µL of PE Buffer was pipetted into the column and again centrifuged for 1 minute at 17,900x g. The eluate is again discarded. The column was transferred to a sterile 1.5mL centrifuge tube and 50µL of sterile ddH_2O was added to centre of the membrane within the column and allowed to incubate for 1 minute at room temperature (18°C). The column was centrifuged at 17,900x g to elute the DNA for 1 minute. Purified samples were then stored at -20° C.

2.1.3 DNA amplification and visualization

2.1.3.1 *Taq* DNA polymerase PCR amplification protocol

Thermostable DNA polymerase from *Thermus aquaticus* (*Taq*) was used to amplify DNA samples after extraction, after damage treatments and also after repair treatments to

evaluate effectiveness of damage and repair on the PCR reaction. Standard reactions were performed at 20μLvolumes in 0.2mL tubes and used mitochondrial DNA primers 14724F (5'-CGA AGC TTG ATA TGA AAA ACC ATC GTT G-3') and 15149R (5'-AAA CTG GAG CCC TCA GAA TGA TAT TTG-3') (Table 2). All reactions are performed on ice. The PCR reaction after optimization consisted of 200μM dNTPs, 0.2μM of each primer, 1.0mM $MgCl_2$, 1X PCR buffer (750mM tris-HCl [pH 8.8 at 25°C], 200mM $(NH_4)_2SO_4$, 0.1% tween 20), 0.5U *Taq* DNA Polymerase, 500ng of DNA template, the remaining volume was made up to 20μL using ddH_2O. Tubes were vortexed and spun down and placed in a 96 well Gradient Mastercycler (Eppendorf).

Table 2. Primers used in study

Primer	Sequence	Amplicon
MtF16210	TTT TCT ATT TTT AAC CTT TAG GAC	800bp
MtR408	CAG CAA TCA TCA ACC CTC AAC TAT	
Mt14724F	CGA AGC TTG ATA TGA AAA ACC ATC GTT G	425bp
Mt15149R	AAA CTG GAG CCC TCA GAA TGA TAT TTG	
Mt16 190F	CCC ATG CCT ACA AGC AAG TA	230bp
Mt16 420R	TGA TTT CAC GGA GGA TGG TG	
Amelogenin F	CCC TGG GCT CTG TAA AGA ATA GTG	106/112bp
(Nuclear DNA) R	AAT GGG CGC TTT TCA GCT TCT GTA	

Table 3. The primers used in each set of experiments within this study

Primer	DNase	Hydrogen Peroxide	Acid Buffer	Daklah Oasis	Copan	Çayönü Tepesi
Mt14724F	yes	yes	yes	Yes	yes	No
Mt15149R	yes	yes	yes	Yes	yes	No
Mt16 429R	no	no	no	Yes	No	No
Mt16 191F	no	no	no	Yes	No	No
Amelogenin (Nuclear DNA)	no	no	no	No	No	Yes

The cycling parameters included an initial denaturation at 94°C for 2 minutes followed by 25 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes. On completion the reaction was

placed at 4°C on hold. This PCR protocol was followed for all samples except the ancient samples. The 800bp amplicon was unable to recover a profile and was reduced to the 425bp amplicon during the strand break experiments. The amplicon was further reduced to 230bp for the Egyptian samples after profile was unable to be recovered from the 425bp amplicon.

2.1.3.2 Electrophoresis protocol

The detection of PCR products are applied to 2% gel electrophoresis (AGE) containing ethidium bromide for detection (EtBr) and viewed with a transilluminator under UV light. Load one well with molecular marker (5μL) and wells with 3μL of 6X loading buffer (Invitrogen) and 5μL sample. Gels were run for 30 minutes at 110 Volts. The gel is removed after it has run and viewed on the transiluminator (wavelength UV B) and photographed.

The ancient samples were run on a 6% polyacrylamide gel due to the small amplicon and the need for better separation to distinguish the sexing bands which differed by only several bases.

2.1.4 DNA quantification

DNA in this study was quantified with the use of very accurate fluorometric dyes which attach to dsDNA and are read by a special photometric analyzer. Only microlitres of sample are needed and the results very accurate. This method allowed damaged DNA to be measured along with viable DNA for accurate quantification.

2.1.4.1 Qubit fluorometer quantification

Quant-it was used to measure DNA concentrations in initial PK extractions and in purified PCR products to standardize reactions for final DNA concentrations of 500ng per 50μL reaction. Quant-it working solution is made by diluting the Quant-it reagent 1:200 in Quant-it

buffer in 5mL falcon tube (cannot be glass container). 200µL of working solution is required for each sample to be quantified and standards. Solutions are prepared as per table 4.

Table 4. Qubit standards / working solutions

	Standards	Samples
Working Solution	190µL	180-199µL
Standard	10µL	
Sample		1-20µL
Total Volume per tube	200µL	200µL

Vortex all tubes for 2-3 seconds and incubate for two minutes at room temperature avoiding direct light. Insert into Qubit Quantmeter and wait five seconds for each measurement. Five measurements are taken and averaged for a final nucleic acid concentration given in nanograms per microlitre (ng/µL).

2.1.5 DNA repair methods

All repair reactions were run with an undamaged DNA template positive control and a negative control containing only reagents and an aliquot of ddH_2O that was purified along with the damaged template to monitor contamination.

2.1.5.1 DNA Polymerase 1 Klenow Fragment repair method

In a 0.2mL microcentrifuge tube 2.5U of Klenow fragment in storage buffer (25mM tris-HCl [pH 7.5] 0.1mM EDTA, 1mM DTT and 50% [v/v] glycerol), 5µL 10X nick translation buffer (500mM tris-HCl [pH 8.0 at 25°C], 50mM $MgCl_2$, 10mM DTT), 500ng damaged DNA template, 0.4mM each dNTP and ddH_2O to a total volume of 50µL. The reaction was carried out for 60 minutes at 37°C, and terminated with a 20 minute incubation at 70°C.

2.1.5.2 T4 DNA Polymerase repair method

A volume of 2.5U T4 DNA Polymerase in storage buffer (20mM potassium phosphate [pH 7.5], 200mM KCl, 2mM DTT and 50% [v/v] glycerol), 10μL 5X nick translation buffer (335mM tris-HCl [pH 8.0 at 25°C], 33mM $MgCl_2$, 5mM DTT, 84mM $(NH_4)_2SO_4$), 500ng damaged DNA template, 0.4mM each dNTP and ddH_2O to a total volume of 50μL. The reaction was carried out for 60 minutes at 37°C, and terminated with a 20 minute incubation at 70°C

2.1.5.3 T4 DNA Ligase repair method

The T4 DNA Ligase was used in this study as a final treatment in the repair protocols to ligate the strand nicks left after the other repair methods were tested and to repair and spontaneous SSB created during the damaging treatments. T4 DNA Ligase was purchased from Fermentas Life Sciences at a concentration of 5U/μL. An aliquot of 200ng of treated DNA template was added to a 0.2mL centrifuge tube. A volume of 2μL of 10X ligase buffer (400mM tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP [pH 7.8] at 25°C) was added with 0.5U of T4 DNA Ligase to the tube and briefly vortexed. The reaction tubes were placed in a thermocycler (Eppendorf Mastercycler 96 well) for 1 hour with cycles between 10 and 30°C with a 10 second hold at each temperature per cycle. After enzymatic treatment DNA was purified with QIAquick purification columns and stored at -20°C for GCMS and PCR amplification.

2.1.5.4 Endonuclease IV reaction

Endonuclease IV (Endo IV) was used in this study as an additional repair step prior to simple ligation. It was purchased from New England BioLabs at a concentration of 10000U/mL in storage buffer (100mM NaCl, 50mM tris-HCl [pH 7.4] and 50% glycerol). All reactions were carried out on ice. An amount of 0.5μg of damaged template DNA was added to 0.2mL microcentrifuge tube along with 100U of Endo IV with 5μL of 10x reaction buffer (100mM

NaCl, 50mM tris-HCl, 10mM $MgCl_2$, 1mM DTT [ph7.9 at 25°C]). The volume was brought up to 50µL with ddH_2O. The volume of ddH_2O added is dependent on the concentration of DNA template added. Samples were incubated for 4 hours at 37°C which was followed by 20 minute incubation at 65°C to denature enzyme. Samples were cooled in ice then used in following repair steps.

2.1.5.5 Phi 29 helicase dependant DNA polymerase method

Phi 29 is usually used as a replicative enzyme for amplification of DNA template when a thermocycler is not available because it is very accurate and can replicate at moderate temperatures due to its high strand displacement ability. In this study phi 29 is used as a pretreatment to repair the DNA template before being amplified with *Taq* DNA Polymerase. Phi 29 DNA Polymerase was purchased from New England BioLabs at a concentration of 10000U/mL in storage buffer (100mM, KCl, 10mM tris-HCl [pH7.4], 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% NP-40 and 50% glycerol.). In 0.2mL microcentrifuge tube 500ng of damaged DNA template was added with 100U of phi 29 enzyme and 1X Reaction Buffer (50mM tris-HCl, 10mM $MgCl_2$, 10mM $(NH_4)_2 SO_4$, 4mM DTT [pH 7.7 at25°C]), 0.1mg/mL BSA and 0.2mM dNTP's. The tubes were vortexed briefly then spun down. The reactions were incubated for 4 hours at 37°C followed by a 10 minute heat inactivation at 65°C. Tubes were cooled on ice and used directly in following amplification.

2.1.5.6 PreCR™ Repair Mix

The protocol for the PreCR™ Repair kit (New England BioLabs Inc.) followed the manufacturer's instructions. This method summarized combined 1X ThermoPol Buffer, 100µM dNTP's, 1X NAD+, damaged template DNA and ddH_2O to 46µL at room temperature (18°C). Add 1µL of the PreCR™ Repair Mix to the reaction and mix gently. Incubate the reaction for

15-20 minutes at 37°C. Place the reactions on ice. Add the primers, a second aliquot of dNTP's (another 100μM) and the DNA polymerase directly to the repair reaction mix then proceed with the PCR amplification protocol.

2.1.6 Hydrolysis and derivatisation

DNA template from repaired and unrepaired as well as control samples with initial concentrations of 500ng were initially dried under vacuum in a 2mL autosampler vial, without heat, using an Eppendorf Concentrator 5301. The samples were then hydrolyzed with 0.5mL of 60% formic acid in evacuated tubes filled with nitrogen and heated to 140° C for 30 minutes. The samples were then lyophilized for 48 hours following the methods of Senturker *et al.* (Senturker and Dizdaroglu 1999). The samples were then derivitized with 0.4mL of BSTFA with 1% trimethlychlorosilane (Sigma-Aldrich) which is a trimethylsilyl donator that allows compounds to be derivitized into thermostable compounds for GCMS analysis and 0.1mL acetonitrile (Sigma-Aldrich) which acts as the inorganic solvent for the nucleic acids. The tubes were purged with nitrogen and then sealed with teflon-coated septa. The derivitization was carried out at 120°C for 30 minutes. A final dilution of 2mL of acetonitrile were then injected into the sealed tubes and immediately brought to be analyzed by GCMS.

2.1.7 Gas chromatography mass spectroscopy

The derivitized samples were analyzed by Varion model 450 gas chromatograph coupled with a Varion model 300-MS quadrupole GCMS mass spectrometer equipped with factor four capillary column (VF-5ms, 30m x 0.25mm ID, DF=0.25um). Helium was used for the carrier gas at a flow rate of 1.0 mL/min. Samples were introduced via split mode with a one in ten split by an autosampler with the injection port at a temperature of 280°C. The oven temperature was

initially 50°C for 1 minute then ramped up to 280°C at a rate of 10°C/min and then held for 6 minutes. Under electrospray ionization (EI) conditions, with ionization energy 70eV, ion source set at 200°C. The scan range was from 70 to 600amu. The GCMS interface temperature was set at 270°C. The quantitative analysis of major oxidative products was performed with GCMS

Table 5. Modified Bases and Molecular Weights Searched For in Study

Modified Base	Undamaged Base	Molecular Weight	Derivitized Molecular weights
8-hydroxyadenine	Adenine	FW 279.5	FW 367.7
4,6-diamino-5-formamidopyrimidine	Adenine	FW 153.1	FW 369.1
N6 methyladenine	Adenine	FW 149.2	FW 355.1
2-hydroxyadenine	Adenine	FW 151.1	FW 367.7
1-methyladenine	Adenine	FW 148.2	FW 364.2
2,6-diamino-4-hydroxy-5-formanodopyridimine	Guanine	FW 169.1	FW 457.9
7-methylguanine	Guanine	FW 171.2	FW 387.2
Oxazolone	Guanine	FW 130.1	FW 420.8
1-hydroxyguanine	Guanine	FW 167.1	FW 383.8
8-hydroxyguanine	Guanine	FW 167.1	FW 455.8
5-Formyluracil	Thymine	FW 140.1	FW 358.7
5-hydroxyhydantoin	Thymine	FW 115.1	FW 316.1
5-hydroxy-5-methyhydantoin	Thymine	FW 130.1	FW 346.6
5-Hydroxy-6-Hydrothymine	Thymine	FW 144.1	FW 360.7
5-hydroxymethyluracil	Thymine	FW 142.1	FW 358.7
Uracil	Thymine/Cytosine	FW 113.1	FW 257.1
Thymineglycol	Thymine	FW 160.1	FW 448.9
5-OH-6-hydroperoxide	Cytosine	FW 161.1	FW 449.8
5,6-dihydrocytosine	Cytosine	FW 143.1	FW 431.8
5-hydroxy-6-hydrouracil	Cytosine	FW 130.1	FW 346.6
Uracil glycol	Cytosine	FW 146.0	FW 434.8
Cytosine glycol	Cytosine	FW 145.1	FW 433.8
5-hydroxy-6-hydrocytosine	Cytosine	FW 129.1	FW 345.7
5-hydroxycytosine	Cytosine	FW 127.1	FW 343.6
5-hydroxymethylcytosine	Cytosine	FW 141.1	FW 358.6
5,6-dihydrouracil	Cytosine	FW 114.1	FW 284.6
5-hydroxyuracil	Cytosine	FW 128.1	FW 344.6

selected ion monitoring (SIM) mode. The selected ions of major oxidative products were their corresponding base peaks and /or [M-15+] ions in SIM mode. The dwell time for each ion was set at 80ms. Output files were analyzed using Varion MS workstation version 6 and the NIST98 Mass Spectral Database.

2.2 Experimental formation of Strand breaks

To artificially induce strand nicks without causing other forms of damage the enzyme DNase was used because of its ability to remove bases while leaving the 3' and 5' ends of DNA intact. A series of concentration gradient solutions was tested over time intervals on DNA template to induce damage to the point of *Taq* DNA Polymerase inhibition but not to completely degrade the molecule to single base pairs which is its intended commercial purpose.

2.2.1 DNase treatment

The creation of SSB was achieved using DNase enzyme with Mg^{2+} ions which cleaves each strand of dsDNA independently in a statistically random fashion (Sambrook and Russell 2001). DNase I, RNase-free was purchased from Fermentas Life Sciences at a concentration of 1U/μL. (1U is defined as 1 unit of the enzyme which completely degrades 1μg of plasmid DNA in 10 minutes at 37°C). An amount of 1μg template DNA was added to 1.5mL centrifuge tubes and combined with 1.0U, 0.5U or 0.1U of DNase in storage buffer (50mM tris-acetate [pH 7.5], 10mM $CaCl_2$ and 50% [v/v] glycerol). Add 1μL of 10X reaction buffer with $MgCl_2$ (100mM, tris-HCl [pH 7.5 at 25°C], 25mM $MgCl_2$, 1mM $CaCl_2$). Then ddH_20 was added to the centrifuge tubes to bring the volume of the reaction up to 50μL. Reactions are incubated for various time intervals at 37°C followed by a 10 minute heat inactivation at 65°C. Samples were cooled on ice and purified using QIAquick PCR purification columns.

2.3 Experimental formation of Oxidative damage

Oxidative processes modify DNA and are generated from reactions of DNA with reactive oxygen species. In this study oxidative damage was generated through the addition of various concentrations of H_2O_2 to the DNA template until the *Taq* DNA Polymerase used for all amplifications was inhibited. H_2O_2 was chosen because of its reliable and well studied induction of oxidative damage on DNA.

2.2.1 Hydrogen peroxide

DNA template at a mass of approximately 0.5μg was added to a 1.5mL microcentrifuge tube along with 10μL of 5% H_2O_2 and ddH_2O to achieve a final volume of 50μL. The volume of ddH_2O added is dependent on the initial concentration of the PCR template added. The samples are vortexed for 15 seconds, quick spin (approximately 30 seconds) and incubated at 37°C for interval time periods. The samples are cleaned using QIAquick PCR kit spin columns.

2.4 Experimental formation of hydrolytic damage

Hydrolytic damaged occurs through spontaneous chemical reactions over time in the presence of water, heat, acidic environments or various combinations of each. Strand breaks, abasic sites and deamination are common damage types accumulated through hydrolytic damage. The deamination of DNA bases occurs more frequently in pyrimidines than in purines but both are equally mutagenic (Mol et al. 1999) In this study a heat treatment combined with an acid buffer was used to induce hydrolytic damage on DNA within a short experimental time.

2.4.1 Heat/acid buffer

Following the methods of ,Nakamura and Swenberg (Nakamura and Swenberg 1999) an acid buffer solution was prepared consisting of 10mM sodium citrate, 10mM NaH_2PO_4, and

10mM NaCl at pH5.0. Template DNA of approximately 0.5μg of DNA is placed in a 1.5mL microcentrifuge tube and dried down in concentrator. The DNA was suspended in 100μL of the acid buffer, vortexed for 30 seconds and quick spin. The samples were incubated at 70°C at a mix speed of 500rpm for various lengths of time. Once incubation was completed the samples were immediately placed on ice to stop the reaction. Once cooled, the samples were then purified via ethanol precipitation and suspended in 50μL ddH_2O. A temperature /time control containing sample and 100μL of ddH_2O was ran alongside the samples for the maximum reaction time to ensure results were from damaging agent and not time or temperature factors. A negative control containing only acid buffer was also ran for maximum reaction time.

2.5 Ancient samples.

The previous methods were modified slightly when used on actual ancient degraded DNA samples which had been previously attempted to be amplified and failed to produce any viable template. This research was conducted at the Paleo-DNA Laboratory at Lakehead University, Thunder Bay, Canada. The Paleo-DNA Laboratory is a research facility for the extraction, amplification and analysis of forensic DNA, ancient or degraded DNA.

The Paleo-DNA laboratory is divided in three sections: the pre-PCR area (clean laboratory); the amplification area (PCR room); and the post-PCR area. This physical separation conforms to all of the guidelines for ancient DNA analysis and for a forensic laboratory. There is a separate air system for the clean laboratory area with its own ducting, venting and filtering, along with an independent vacuum air system removing air from the sample work stations and another air system with the air shower entry. There is an exhaust air system with the PCR room to remove build-up of amplified DNA products. The final air system is in the post-PCR area. The main clean laboratory is a sealed containment facility with air pressured double-door entry.

Samples enter the clean laboratory by way of a UV irradiated wall pass-through. There is a pass-through from the sample storage room to the clean laboratory corridor to be used for sample entry. Samples are received to the laboratory double bagged and contained in a box. It is removed from the box and the outside bag. The inside bag is cleaned with bleach, ethanol and then placed into the pass-through. The pass-through is sprayed with water and then UV irradiated. UV irradiation is ineffective on dry material. These pass-throughs are uni-directional, only one door can be opened at one time. Once the sample is placed into the pass-through it is UV irradiated for at least 20 minutes. The sample can then be removed from the pass-through from the inside of the clean laboratory. The bag containing the sample is again washed with bleach and ethanol before being taken into the sample preparation room. There are also pass-through between reagent preparation and sample preparation for the passing of reagents and sterilized consumables for use in the laboratory. Another pass-through exists between samples preparation and PCR preparation for the passing of the purified DNA extracts. Once the experiments are prepared they are placed into another pass-through that allows them to be moved into the PCR room. The strict conditions under which the pass-through operates is critical to ensure back-contamination does not occur. The pass-through is decontaminated from the outside after each time it has been used with water spray and UV irradiation. Prior to use it is again UV irradiated for no less that 20 minutes. The pass through is then cleaned with bleach and ethanol from the inside and the experiments are placed into the pass through. There is little chance of back-contamination except from contaminated air as the pass-through doors have a locking mechanism that prevents doors on both side being opened simultaneously. It is only the air that enters the pass-through from the outside when the experimental samples are removed that

requires decontamination before opening the pass-through from the inside of the clean laboratory.

All laboratory personnel who are entering the clean laboratory are fitted with full body tyvek suits (primary barrier) that have hoods, enclosed boots and sleeves. A second pair of tyvek boots are worn over the top of the tyvek boots attached to the suit to ensure the attached boots do not perish and cause a rip in the primary barrier. This second pair of boots provides a secondary barrier. A pair of gloves is worn under the tyvek suits (primary barrier). A second set of sleeves and gloves (secondary barrier) are worn over top of the suit and first pair of gloves. These sleeves can be changed between each sample being prepared but can only be removed and changed in the suit-up room outside the clean laboratory. A third pair of gloves can be worn over the top of the first two pairs (tertiary barrier) and can be changed between the preparation of each sample or between each procedure in the laboratory. This is to ensure no carry-over contamination when preparing reactions and in all functions within the laboratory. Eyewear and face masks are also worn to enclose the face (primary barrier). The items of the primary barrier (suits, masks and sleeves) are wiped down with bleach and ethanol every use and are changed regularly to prevent potential contamination.

In the other areas of the laboratory there are designated laboratory coats to be used only within that area. So there are separate lab coats for the PCR room and separate coats for the gel documentation room, the general analysis laboratory, the sample storage room and the forensic comparisons room. These laboratory coats stay in that designated area to prevent transfer of potential contamination from one location to another. Gloves are worn in all other areas at all times and in some procedures additional sleeves are worn over the top of the lab coats to enable their change between procedures to reduce the potential for contamination.

Once the sample is within the facility it is taken to the sample preparation room where it is removed from its packaging (under a negative displacement hood or biosafety cabinet) and exposed with UV irradiation for a period of 12 to 48 hours (being sprayed with water and turned over periodically throughout this period). This will UV damage any DNA that might be on the external surface of the sample whether from the handlers of the sample (both past and present), the soil, the archaeologist, or from other samples within the archaeological record, previous storage facility or packaging. It will also destroy a small amount of the endogenous DNA depending on the length of time of exposure. The sample is cleaned by brushing or washing within this irradiation step and re-exposed to the UV. The sample can also be wiped with bleach to oxidatively damage any DNA that might be on the external surface of the sample. Caution must be taken to ensure no bleach residue is left on the sample that may damage the DNA during processing. The sample can also be wiped with ethanol. The sample is then prepared for extraction and depending on the size of the sample it will either be ground up, or biological material will be removed from the internal areas of the sample. The removal of biological material from inside the sample is a further step that prevents contamination from any of the handlers if the UV irradiation has not been successful and further irradiation is not practical (as the endogenous DNA would be further damaged on the surface). The material removed from the inside of the sample can be treated (by a pre-wash, decalcification or demineralization step) or ground into a fine powder. Once the prepared sample is ready for extraction the appropriate extraction solutions are added and it is left to incubate from 8 hours to overnight. The extract is then purified and available for further analysis.

The extraction and purification stages are performed in the designated sample preparation room. There is a dedicated biosafety cabinet for sample preparation. There is also a dedicated

hood for each and every step of the extraction and purification procedure. Each hood had dedicated equipment for that one step only.

In the PCR preparation room there are dedicated hoods for the preparation of the PCRs. No DNA samples or extracts have entered these self-contained work areas. Once the reaction mix is made and aliquots are placed in the reaction tubes, the tubes are transferred to another hood for the addition of the extracted DNA from the samples. No positive control is kept in the clean laboratory, as it could become a source of contamination due to the DNA being more intact, robust and in higher concentration.

Once the PCR is prepared, it is passed out of the containment laboratory to a PCR room through a UV irradiated pass through. Here, the positive control is added if required. None of the other tubes are opened within this room before or after the PCR cycling to ensure that this potential contamination area is contained; and strict decontamination of this area is performed regularly. Once the PCR is complete, the reaction is moved into one of the general analysis laboratories for gel electrophoresis and analysis.

All work areas are cleaned before and after use. All equipment is cleaned before and after each use. The cleaning that is performed before and after use involves bleach washing, ethanol washing and UV irradiation. The clean laboratory is cleaned routinely according to the cleaning SOP's of the Paleo-DNA Laboratory. All hoods are equipped with UV lighting and irradiated on a regular basis; before and after every procedure. The cleaning solutions used include 70% ethanol, bleach and Terg-a-zyme™. All consumables are sterilized before use by autoclaving and cross-linking. All re-used items are first soaked in Terg-a-zyme™, followed by bleach then rinsed in ethanol. If they are metal they are sonicated, baked, cross-linked and autoclaved in

sterile pouches. For plastics they are cross-linked and autoclaved in autoclave paper or sterile pouches.

The Paleo-DNA laboratory attempts to maintain a sterile environment. To ensure this the work areas are cleaned before and after every use by the analyst. There are also very strict standard operating procedures that regulate what type of experiment can be scheduled and operated. This ensures that while the laboratory is being used for sample preparation all other experiments are postponed until the next routine and thorough clean of that area. There is unidirectional flow of the samples and of the analysts within the clean laboratory to prevent the possibility of carry-over contamination being passed by the analyst themselves.

There are many controls that are employed in the analytical process. These begin with an extraction procedural control. This is where a tube is placed out throughout the process of sample preparation. This tube is extracted as if there was a sample in within it and tested for the presence of DNA. There is also an extraction negative control. This is a negative control containing the reagents of the extraction. This is to ensure that there is not any contamination in the reagents. Once the extracted sample is passed into the PCR preparation room there are additional controls. These include a PCR reagent negative control to ensure the reagents are not contaminated. There is a PCR procedural control which is a tube that is left open during the preparation of the PCR reaction.

2.5.1 Samples

There are 6 samples that were used to evaluate the efficiency of the assessed *in vitro* DNA repair methods on ancient samples. These samples came from three archaeological sites 1) Daklah Oasis, Egypt; 2) Copan, Honduras; and 3) Çayönü Tepesi, Turkey. The Daklah Oasis samples date to approximately AD300-600, they are recovered from the Kellis 2 cemetery and

are from naturally mummified human remains (Graver et al. 2000; Lamers et al. 2009). The environment is in the western desert of Egypt and extremely dry and arid. The samples from the Copan site are dated approximately to between AD400-800 (Matheson et al. 2003). Copan is a classical Mayan site in western Honduras. The Çayönü Tepesi sample dates to approximately 7,500-7,800BC from the "Skull Building" within the site (Matheson and Loy, 2001). All of these samples have previously been analyzed but the analysis had failed due to DNA damage or quantity.

2.5.2 Extraction

The ancient samples required a different extraction procedure than the modern DNA samples to enhance the recovery of minute quantities of DNA. A 1500μL volume of 0.5M EDTA was added per sample, then 20% sarkosyl (75μL per reaction) was added. A 120μL volume of PK was added to the tubes and they were vortexed briefly and spun down with a quick centrifugation. The tubes were then incubated at 56°C for 12 hours.

2.5.3 Purification

2.5.3.1 Silica bead purification

After the incubation was complete a volume of 3000μL guanidinium thiocyanate (GuSCN) solution was added to the sample extract. This was followed by 15μL of a silica bead (Sigma) slurry that was added to the guanidinium/extract solution. The solution was placed in an ice bath for 1 hour then centrifuged at 12,000rpm for 5 minutes. The supernatant was removed carefully not to disturb the silica bead pellet and discarded. A volume of 1000μL of working wash buffer was added to silica bead pellet. The solution was again centrifuged at 12,000rpm for 2 minutes and the supernatant removed and discarded. A volume of 250μL of 100% ethanol was

added then centrifuged at 12,000rpm for 2 minutes. The supernatant was again removed and discarded while the pellet was allowed to air dry at room temperature (18°C). A volume of 100μL of ddH_20 was added to elute the DNA for further purification.

2.5.3.2 Size exclusion chromatography purification protocol

The P-30 Micro Bio-Spin size exclusion chromatography columns purification system was used following the extraction and purification to remove any possible excess inhibition that may have co extracted and purified especially heavy metals and humic acids often found in ancient materials.

Invert the column sharply several times to resuspend the settled gel and remove any bubbles, next snap off the tip and place the column in a 2.0mL microcentrifuge tube and centrifuge at 4500rpms for1.5 minutes, discard the collection tube with packing buffer, add 50μL of sample to top of column and centrifuge for 4500rpm for 4 minutes, discard the column and keep the tube.

2.5.4 Amplification

2.5.4.1 Mitochondrial DNA amplification

The conditions of the PCR were different for the ancient samples. These PCRs were conducted in 25μL reaction volumes with final concentrations of 200μM dNTPs, 0.2μM of each primer, 2.0mM $MgCl_2$, 1X PCR buffer minus Mg and 1U Platinum *Taq* DNA Polymerase (Invitrogen). The remaining volume was completed with ddH_2O and DNA template. The cycling parameters included an initial denaturation at 94°C for 2 minutes followed by 50 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes. On completion the reaction was placed at 4°C on hold.

2.5.4.2 PreCR™ on Daklah Oasis samples with smaller amplicon

The PreCR™ Repair Mix was retested with primers targeting a smaller amplicon of 210bp when the 425bp target region failed to amplify for the Daklah Oasis samples. The Daklah Oasis samples were from the original unrepaired extract already tested for DNA amplification. Method was rerun in the clean room at the Paleo-DNA Lab Lakehead University with exact same contamination protocols as before with the only deviation from before was the substitution of the smaller amplicon primers but were of the same concentration and were already tested for contamination. The PCR conditions and cycling parameters were the same as section 2.5.4.1 with the only difference being the primers. Mitochondrial primers targeting a small section of the hypervariable region 1 (HVR1) within the mitochondrial control region were used. These were mt16190F (5'- CCC ATG CCT ACA AGC AAG TA -3') and mt16420R (5'- TGA TTT CAC GGA GGA TGG TG -3'). These primers were chosen because of previous studies use of this primer region on the ancient samples in this study and could then be used for comparison analysis.

2.5.4.3 Sexing amplification of the Çayönü Tepesi samples

The Çayönü Tepesi sample 9 was tested with amelogenin primers to identify the sex of the remains (Sullivan et al. 1993). If two bands are present in the target region the result is interpreted as male. the Y chromosome has a small deletion in the 6bps which shows up as two bands as a male has an X and a Y chromosome while the female has only the X so would show up as only one band in the target region. The Çayönü Tepesi sample 9 underwent same repair methods simultaneous to the other samples. The Çayönü Tepesi sample 9 had only different PCR concentration and thermocycler parameters for amplification after repair. These PCRs were conducted in 25μL reaction volumes with final concentrations of 200μM dNTPs, 0.2μM of each

primer, 2.0mM $MgCl_2$, 1X PCR buffer minus Mg and 1U Platinum *Taq* DNA Polymerase (Invitrogen). The remaining volume was completed with ddH_2O and DNA template. The cycling parameters included an initial denaturation at 94°C for 2 minutes followed by 45 cycles of 94°C for 30 seconds, 60°C for 1 minute and 72°C for 2 minutes. On completion the reaction was placed at 4°C on hold.

2.5.4 Gel electrophoresis

Following amplification, PCR products were analyzed using 6% polyacrylamide gel electrophoresis (PAGE) and a 100bp molecular marker (2.0μL) (Fermentas) for size approximation. The samples were loaded with 5.0μL of PCR product and 3.0μL of 6X loading dye (2.5% xylene cyanol, 2.5% bromophenol blue, 35% ficoll and 544.0μL of ddH_2O) and were run at 118v for 45 minutes. The results were stained with EtBr solution and visualized with a UV transilluminator.

3.0 Results

3.1 Optimization

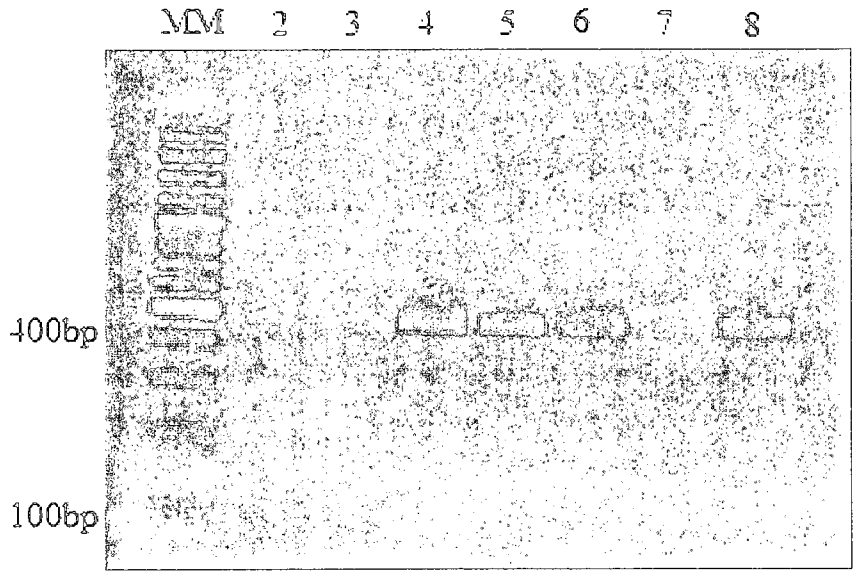


Figure 4. Optimization of Detection PCR
Lane 1. 100bp molecular marker; Lane 2. Empty; Lane 3. 20 cycles; Lane 4. 25 cycles; Lane 5. 30 cycles; Lane 6. 35 cycles; Lane 7. negative control; Lane 8. positive control at 25 cycles.

PCR was optimized for 50μL reactions with 25 PCR cycles (Figure 4). Twenty five cycles were used as the standard for the rest of the project for both the damaged and repaired template. The least amount of cycles was used to avoid as much as possible false positives from possible contamination except in the ancient samples due to the tiny fragments and minute quantities present of highly degraded DNA where 50 cycles were used have the highest chance of success.

3.2 Strand breaks and repair

3.2.1 Strand break DNA damage using DNase treatments

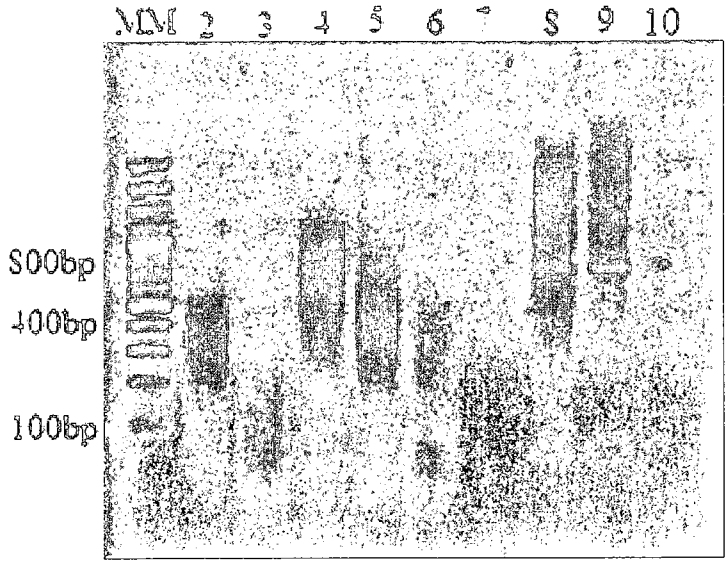


Figure 5. Induction of DNA strand breaks using DNAse digestion of template DNA 1μL to 0.5μL over time intervals

Smears generated by digestion of an 800bp PCR product. Lane 1. 100bp molecular ladder. Lanes 2 to 6 shows the DNase digested DNA after PCR at 1U concentration over 15 seconds for lane 2, 30 seconds for lane 3, 1 minute for lane 4, 2 minutes for lane 5 and 5 minutes for lane 6. Lane 7 is empty. Lanes 8 to 10 contain digested DNA with DNase concentration of 0.5U. Lane 8 shows 15 seconds time exposure, Lane 9 30 seconds exposure and lane 10 at 1 minute exposure.

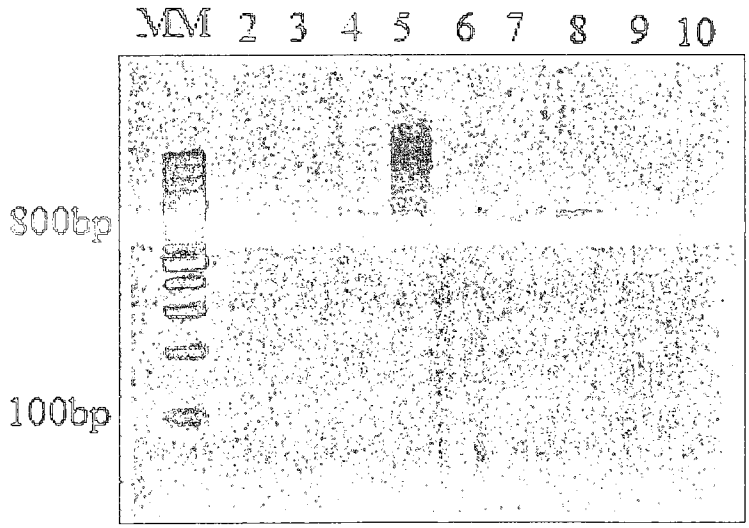


Figure 6. Induction of DNA strand breaks using DNAse digestion of 0.1μL of template DNA over time intervals

Lane 1. 100bp ladder; Lanes 2. and 3. the DNase digested DNA after PCR at 0.5U over 2 minutes and 5 minutes. Lanes 4 to 8 shows DNase digested DNA after PCR at 0.1U over time intervals. Lane 4. 15 seconds; lane 5. 30 seconds; Lane 6. 1 minute, Lane 7. 2 minutes Lane 8. 5 minutes.

3.2.2 Repair of DNase strand break damage

Table 6. Strand Break Repair amplification results

DNAse damaged template concentration and exposure time	T4 DNA Ligase	Klenow	T4 DNA Polymerase	PreCR™
15 seconds at 1 U	negative	negative	negative	Negative
30 seconds at 1 U	negative	negative	negative	Negative
1 minute at 1U	positive	negative	negative	Negative
2 minutes at 1 U	negative	negative	negative	Negative
5 minutes at 1 U	positive	negative	negative	Negative
15 seconds at 0.5 U	positive	negative	negative	Negative
30 seconds at 0.5 U	positive	negative	negative	Negative
1 minute at 0.5 U	negative	negative	negative	Negative
2 minutes at 0.5 U	positive	negative	negative	Negative
5 minutes at 0.5 U	positive	negative	negative	Negative

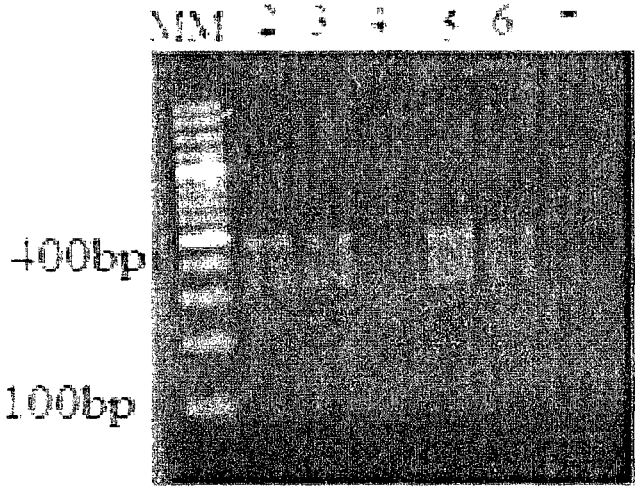


Figure 7. Repaired DNase damaged template with T4 DNA Ligase at 0.5U DNase.
Lane 1. 100 bp ladder; Lanes 2 to 6 contain the repaired DNA damaged with the 0.5U of DNase over time intervals Lane 2. 15 seconds; Lane 3. 30 seconds; Lane 4. 1 minute; Lane 5. 2 minutes; Lane 6. 5 minutes; Lane 7. PCR negative.

Optimization of the conditions for the experimental degradation of DNA using DNase to generate DNA fragmentation identified the optimal digestion time (Figure 5 and 6). The DNA ligase treatment was able repair the DNA to generate an amplifiable product which is shown by the bands at slightly above the 400bp molecular marker which corresponds to the 425bp primers used (Figure 7). There was failure in lane 4 which was the middle treatment time with the

DNAse while the higher damaged template and lower damaged template both had successful amplifiable products after repair treatment when only smears were visible as shown in Figure 5- lanes 2 to 6. There was also smearing from the 300bp to 500bp in lanes 2, 3, 5, 6 in figure 6. showing significant unspecific product. The size of the amplicon had to be reduced from 800bp to 425bp in order to recover bands in gel after excessive fragmentation induced through the DNAse digestion.

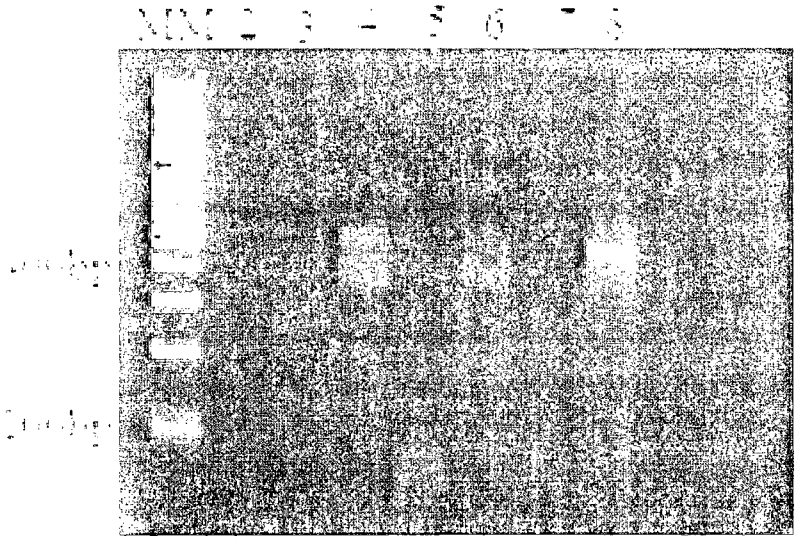


Figure 8. Repaired DNase damaged template withT4 DNA Ligase at 1.0 U of DNase. Lane 1. 100bp ladder; Lane 2. empty; Lanes 3 to 7 damaged DNA template repaired. Lane 3. 15 seconds; Lane 4. 30 seconds; Lane 5. 1 minute; Lane 6. 2 minutes; Lane 7. 5 minutes. Lane 9.negative Contol. Lane 10 Positive Undamaged Control.

Successful amplification of the DNase treated template at the 1U concentration after T4 DNA Ligase treatment is shown in figure 8. Lane 4 which is the 30 second exposure and has a band slightly above the 400bp molecular marker which corresponds to the 425bp target fragment, lane 5 which is the 2 minute exposure time had a band at approximate 425bp target

region. Lanes 5 had a faint smearing within the 300 to 500bp region as well as unspecific product under the 100bp region. Lane 7 which was the 5 minute exposure mark had a small smear of unspecific product under the 100bp molecular marker region. Not shown are the T4 DNA Polymerase and Klenow enzyme treatment on the DNase damaged template results which failed to amplify without the addition of the T4 DNA Ligase treatment and had far weaker bands than the DNA ligase treatment alone.

3.3 Oxidative damage and repair

3.3.1 Oxidative DNA damage using hydrogen peroxide treatments

Table 7. Time treatments and volumes of experimentally damaged DNA samples

DNA sample exposure times	3% H_2O_2	6% H_2O_2	10% H_2O_2	35% H_2O_2
30 minutes	10µl	10µl	10µl	10µl
1 hour	10µl	10µl	10µl	10µl
2 hours	10µl	10µl	10µl	10µl
4 hours	10µl	10µl	10µl	10µl
6 hours	10µl	10µl	10µl	10µl
8 hours	10µl	10µl	10µl	10µl
10 hours	10µl	10µl	10µl	10µl
12 hours	10µl	10µl	10µl	10µl
14 hours	10µl	10µl	10µl	
16 hours	10µl	10µl	10µl	
18 hours	10µl	10µl	10µl	
20 hours	10µl	10µl	10µl	
22 hours	10µl	10µl	10µl	
24 hours	10µl	10µl	10µl	
26 hours	10µl	10µl		
28 hours	10µl	10µl		
30 hours	10µl	10µl		
32 hours	10µl	10µl		
36 hours	10µl	10µl		
40 hours	10µl	10µl		
44 hours	10µl	10µl		
48 hours	10µl	10µl		

The concentration of 6% H_2O_2 was chosen to perform repair reactions on because it was able to induce the damage in an appropriate amount of time while 10% and 35% caused no amplification.

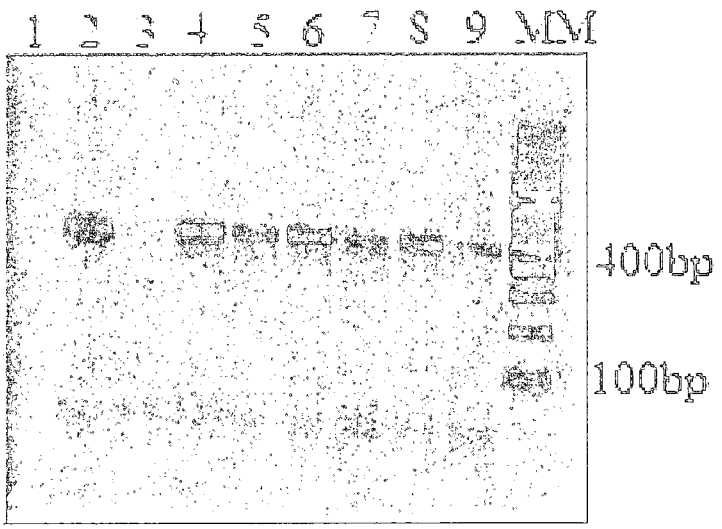


Figure 9. Oxidative damage induced using H_2O_2 treatments over time.
Lane 1 Empty. Lane 2 A 2% agarose gel showing amplification of DNA after 3% H_2O_2 treatments and 6% H_2O_2 treatments. Lane 10. 100bp molecular ladder; Lanes 8. and 9. are 3% and 6% H_2O_2 treatments at 15 minute time intervals. Lanes 6. and 7. are 3% and 6% H_2O_2 treatments at 1 hour. Lanes 4. and 5. are 3% and 6% H_2O_2 treatments at 2 hours. Lanes 2. and 3. are 3% and 6% H_2O_2 treatments at 4 hours.

Failure to successfully amplify came at the 4 hour treatment mark with the 6% concentration of H_2O_2 (Figure 9) and the 3% treatment caused failure at the 8 hour mark (not shown). All lanes showed slight smearing and product below the 100bp molecular marker region.

3.3.2 Oxidative damage repair using DNA polymerases and DNA ligases

The additional step of ligation with the T4 DNA Ligase demonstrated successful repair of the damaged DNA from the H_2O_2 incubated DNA up to 8 hours (Figure 10). T4 DNA Polymerase without T4 DNA Ligase greatly increased the amplification of the damaged DNA template doubling the damage treatment time for successful amplification from 4 hours to 8

hours. Both the Klenow and T4 DNA Polymerase failed to amplify after the 8 hour H_2O_2 damage treatments.

Table 8. Klenow Ligase treatments and T4 DNA Polymerase treatments over time intervals

Exposure time	Klenow and DNA ligase amplification result	T4 Polymerase and DNA ligase amplification result
30 min	Positive	Positive
1 hour	Positive	Positive
2 hours	Positive	Positive
4 hours	Positive	Positive
6 hours	Positive	Positive
8 hours	Positive	Positive
10 hours	Negative	negative
12 hours	Negative	Negative

Table 9. Oxidative products measured in the T4 DNA Polymerase - T4 DNA Ligase treatment

Modified Base	Retention time	Damaged Area	Repaired T4 area	Difference in Area	M/Z1	M/Z2	M/Z3
5,6-dihydroxycytosine	5.313	8.490e+8	7.728e+7	9%	257	416	431
5,6-dihydroxyuracil	5.432	5.088e+9	4.436e+7	17%	343	417	432
Uracil	5.457	2.816e+10	9.510e+7	86%*	147	256	257
4,6-diamino-5-formamidopyrimidine	5.611	8.470e+8	4.261e+7	15%	280	354	369
Uracil glycol	5.632	5.041e+9	1.354e+9	44%	245	419	434
cytosine glycol	5.634	2.834e+10	1.158e+9	69%	245	419	434
5-hydroxyhydantoin	16.671	8.887e+7	2.756e+8	36%		310	316
5-hydroxy-5-methylhydantoin	16.683	8.007e+8	2.206e+8	47%		331	346
Thymine glycol	16.690	8.007e+8	6.944e+7	13%		433	448
5-hydroxycytosine	19.038	9.974e+7	1.962e+7	63%		328	343
8-hydroxyadenine	19.803	3.009e+9	#74,568	99%		352	367
5-hydroxymethyluracil	20.843	3.141e+7	3.185e+7	>1%		343	358
2,6-diamino-4-hydroxy-5-formamidopyrrimidine	23.311	4.091e+10	6.323e+6	7%		442	457
8-hydroxyguanine	23.340	4.847e+10	#112,929	99%	440	442	455
1-hydroxyguanine	N/D	4.847e+10	N/D	N/D	367	368	383
5-hydroxyuracil	N/D	1.190e+9	N/D	N/D	325	344	357

* Indicates an increase

Individual ion count if under 1 million

N/D ion showed up in multiple peaks or could not be detected from background due to low level

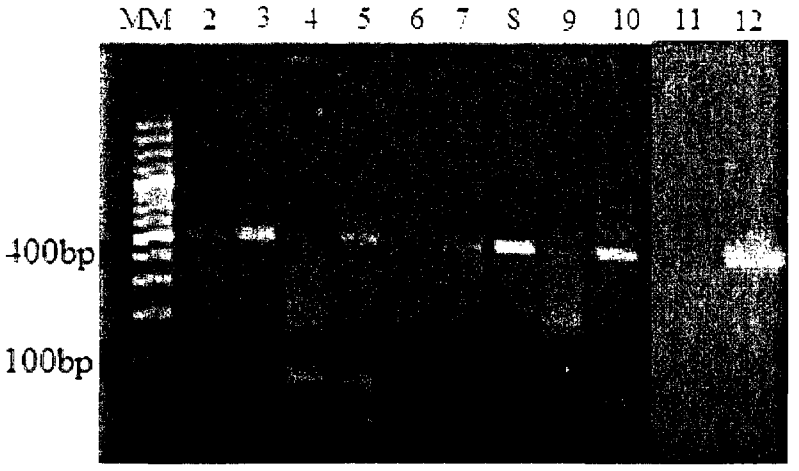


Figure 10. Klenow - T4 DNA Ligase repaired and T4 DNA Polymerase - T4 DNA Ligase repaired on 4 hours and 8 hours of DNA damage using H_2O_2 treatments.
Lane 1. 100bp molecular marker; Lane 2. Klenow 4 hours repaired; Lane 3. Klenow and T4 DNA Ligase; Lane 4. T4 DNA Polymerase 4 hours repaired; Lane 5. T4 DNA Polymerase and T4 DNA Ligase 4 hours repaired; Lane 6. Empty; Lane 7. Klenow 8 hours repaired; Lane 8. Klenow and T4 DNA Ligase 8 hours; Lane 9. T4 DNA Polymerase 8 hours; Lane 10. T4 DNA Polymerase and T4 DNA Ligase 8 hours. Lane 11 negative control. Lane 12 positive control.

Table 10. Oxidative products measured in the Klenow with T4 DNA Ligase treatment

Modified Base	Retention time	Damaged Area	Repaired Klenow Area	Difference in Area	M/Z1	M/Z2	M/Z3
5,6-dihydroxycytosine	5.317	8.490e+8	7.798e+7	9%	257	416	431
Uracil	5.439	2.816+10	7.133e+7	49%*	147	256	257
5,6-dihydroxyuracil	5.446	5.088e+9	5.837e+7	>1%	343	417	432
cytosine glycol	5.614	2.834e+10	6.614e+8	47%*	245	419	434
4,6-diamino-5-formamidopyrimidine	5.619	8.470e+8	9.10e+8	5%	280	354	369
Uracil glycol	5.623	5.041e+9	1.429e+9	43%	245	419	434
5-hydroxyhydantoin	16.671	8.887e+7	2.811e+8	35%		310	316
Thymine glycol	N/D	8.007e+8	N/D	N/D		433	448
5-hydroxy-5-methylhydantoin	16.677	9.610e+7	8.007e+8	11%*		331	346
5-hydroxycytosine	19.043	9.974e+7	2.598e+7	59%		328	343
5-hydroxymethyluracil	20.836	3.141e+7	1.369e+7	31%		343	358
2,6-diamino-4-hydroxy-5-formamidopyrrimidine	23.303	4.091e+10	7.573e+6	26%*		442	457
8-hydroxyguanine	23.325	4.847e+9	#435,939	99%	440	442	455
1-hydroxyguanine	N/D	4.847e+10	N/D	N/D	367	368	383
8-hydroxyadenine	N/D	3.009e+9	N/D	N/D		352	367
5-hydroxyuracil	N/D	1.190e+9	N/D	N/D	325	344	357

* Indicates an increase
# Individual Ion count if under 1 million
N/D ion showed up in multiple peaks or could not be detected from background due to low level

The T4 DNA Polymerase - T4 DNA Ligase repair method showed an increase of 86% in uracil the post repair sample to the H_2O_2 damaged sample (Table 7). Uracil oxidative products such as 5,6-dihydroxyuracil and uracil glycol showed a decrease of 17% and 44% respectively while the oxidative uracil product 5-hydroxymethyluracil showed measured virtually no change between damaged and repaired samples. The oxidative uracil product 5-hydroxyuracil was not detectable in any peaks possibly being removed close to 100% to a level that the GCMS could not measure from background noise. 4,6-diamino-5-formamidopyrimidine and 2,6-diamino-4-hydroxy-5-formamidopyrimidine are the purine oxidative damage products of A and G commonly called Fapy A and Fapy G. These PCR blocking lesions showed a decrease of only 15% (Fapy A) and 7% (Fapy G) between damaged and repaired samples. The T4 DNA Polymerase - T4 DNA Ligase showed a more significant decrease of 47% and 36% for the pyrimidine blocking lesions 5-hydroxyhydantoin (C) and 5-hydroxyhydantoin (G). Two common G oxidative damage products 8-hydroxyguanine and 1-hydroxyguanine had a 99% reduction for the 8-hydroxyguanine while the 1-hydroxyguanine was undetectable from the base line background noise in the repaired sample.

3.3.3 Oxidative damage repair using Glycosylases, DNA polymerases and DNA ligases

Klenow - T4 DNA Ligase repaired has a substantial reduction in the uracil oxidative products of 5-hydroxymethyluracil of 31% and uracil glycol of 43%. Uracil content increased 49% in the repaired sample versus the damaged sample and the oxidative uracil product 5,6-dihdroxyuracil had virtually no change with under a 1% difference between treated and damaged (Table 9). The 5-hydroxyuracil was not able to be detected with a certainty in any of the peaks either because ion count was to low or not present. The purine blocking lesions Fapy A and Fapy G had a slight decrease of 5% decrease in Fapy A and an increase of 26% for Fapy G in treated

versus damaged. The hydantoins the pyrimidine blocking lesions 5-hydroxyhydantoin had a significant 35% decrease in treated versus damaged while 5-hydroxy-5-methlyhydantoin had an increase of 11% in treated versus damaged. The major oxidative damage products 8-hydroxyguanine, 1-hydroxyguanine, 8-hydroxyadenine, 5 hydroxycytosine all had major decreases in detectable products in the repaired versus damaged with the hydroxyguanines and hydroxyadenines being almost completely absent from the treated in comparison to the damaged sample. The 5-hydroxycytosine had 5the lowest decrease of 59% of the hydroxy oxidative damage products but was still very significant.

Table 11. Repair Methods relative increases

Repair Method	% increase in time recovery
Klenow	0%
T4 DNA Polymerase	0%
Klenow with DNA ligase	100%
T4 Polymerase with DNA ligase	100%
Endo IV with Klenow and DNA ligase	350%
Endo IV with T4 DNA Polymerase and DNA ligase	400%
Phi 29	450%
PreCR™	650%

Table 12. Results for amplification of Endo IV added to Klenow - DNA Ligase method and T4 DNA polymerase ligase method

Exposure time	Endo IV added to Klenow - DNA ligase amplification result	Endo IV added toT4 DNA Polymerase - DNA ligase amplification result
30 min	Positive	Positive
1 hour	Positive	Positive
2 hours	Positive	Positive
4 hours	Positive	Positive
6 hours	Positive	Positive
8 hours	Positive	Positive
10 hours	Positive	Positive
12 hours	Positive	Positive
14 hours	Positive	Positive
16 hours	Negative	Positive
18 hours	Negative	Negative
20 hours	Negative	Negative

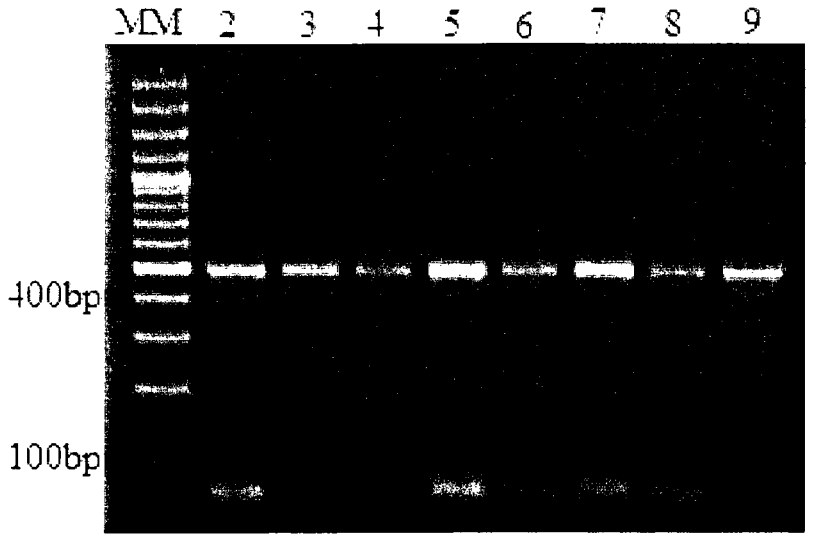


Figure 11. Endo IV - Klenow - T4 DNA Polymerase - T4 DNA Ligase repair treatments H_2O_2 damage 30 minutes to 4 hours.
Lane 1. 100bp molecular marker; Lane 2. Endo IV-Klenow-T4 DNA Ligase repair treatment (E/K/L) 30 minutes; Lane 3. Endo IV-T4 DNA Polymerase-T4 DNA Ligase repair treatment (E/T4/L) 30 minutes; Lane 4. E/K/L repair treatment 1 hour; Lane 5. E/T4/L repair treatment 1 hour; Lane 6. E/K/L repair treatment 2 hours; Lane 7. E/T4/L repair treatment 2 hours; Lane 8. E/K/L repair treatment 4 hours; Lane 9. E/T4/L repair treatment 4 hours.

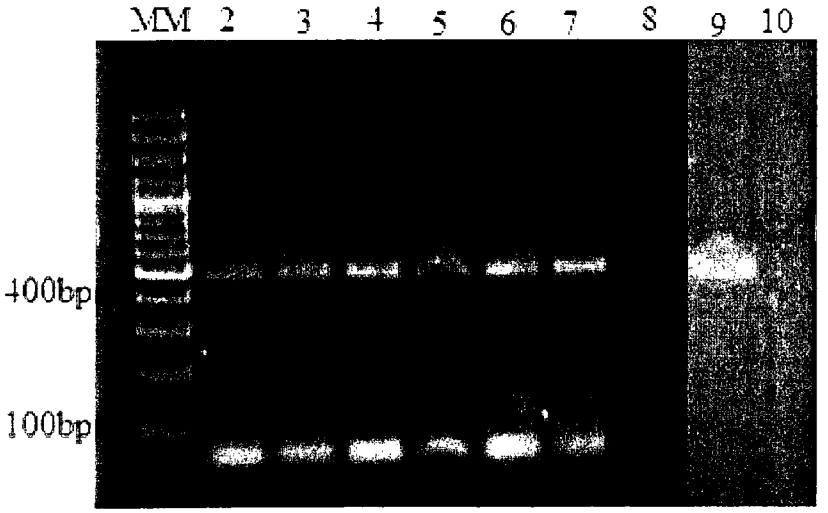


Figure 12. Endo IV - Klenow - T4 DNA Polymerase - T4 DNA Ligase repair treatment H_2O_2 damage 8 hours to 16 hours
Lane 1. 100bp molecular marker; Lane 2. Endo IV-Klenow-T4 DNA Ligase (E/K/L) repair treatment 8 hours; Lane 3. Endo IV-T4 DNA Polymerase-T4 DNA Ligase (E/T4/L) repair treatment 8 hours; Lane 4. E/K/L repair treatment 12 hours; Lane 5. E/T4/L repair treatment 12 hours; Lane 6. E/K/L repair treatment 16 hours; Lane 7. E/T4/L repair treatment 16 hours. Lane 8 empty. Lane 9 positive control. Lane 10 negative control

Pretreatment with the glycosylase Endo IV increased the repair of H_2O_2 damaged DNA template repair methods significantly (Figure 11 and 12). With the additional enzymatic pretreatment the time period for recoverable H_2O_2 damage DNA template was increased from 4 hours initially to 16 hours. The addition of the Endo IV pretreatment doubled the recovery time from 8 to 16 hours for the Klenow - T4 DNA Ligase repair method and the T4 DNA Polymerase - T4 DNA Ligase method. The T4 DNA Polymerase had slightly less unspecific product in its time intervals.

Table 13. Oxidative products measured in three stages Endo IV - T4 DNA Polymerase - T4 DNA Ligase treatments

Modified Base	Retention time	Damaged Area	Repaired Area	Difference in Area	M/Z1	M/Z2	M/Z3
5,6-dihydroxycytosine	5.437	8.490e+8	2.080e+8	56%	257	416	431
Uracil	5.552	2816e+10	7.617e+7	57%*	241	255	256
Uracil glycol	5.558	5.041e+9	5.548e+7	3%	245	419	434
5,6-dihydroxyuracil	5.561	5.088e+9	7.163e+7	16%*	343	417	432
cytosine glycol	5.561	2.834e+10	7.163e+7	50%*	245	419	434
4,6-diamino-5-formamidopyrimidine	5.727	8.470e+8	8.530e+8	>1%	280	354	369
5-hydroxymethyluracil	10.461	3.141e+7	1.991e+7	20%		343	358
5-hydroxyuracil	10.461	1.190e+9	1.991e+7	>1%	325	344	357
5-hydroxycytosine	12.719	9.974e+7	1.760e+7	65%	269	328	343
5-hydroxyhydantoin	16.261	8.887e+7	5.232e+6	16%		310	316
2,6-diamino-4-hydroxy-5-formamidopyrrimidine	23.422	4.091e+10	2.573e+7	33%		442	457
Thymine glycol	N/D	8007e+8	N/D	N/D		433	448
8-hydroxyadenine	N/D	3.009e+9	N/D	N/D		352	367
8-hydroxyguanine	N/D	4.847e+10	N/D	N/D	440	442	455
1-hydroxyguanine	N/D	4.847e+10	N/D	N/D		367	383
5-hydroxy-5-methylhydantoin	N/D	8.007e+8	N/D	N/D		331	346

* Indicates an increase

\# Individual Ion count if under 1 million

N/D ion showed up in multiple peaks or could not be detected from background due to low level

The three step repair treatment Endo IV - T4 DNA Polymerase - T4 DNA Ligase (Table 4) has a repair of uracil oxidative product 5-hydroxymethyluracil of 20% but an increase of uracil by 57% and 5,6-dihydroxyuracil by 16%. Other uracil products such as uracil glycol and 5-hydroxyuracil had no significant difference between treated and damaged samples showing only a 3% and less than 1% difference. 5,6 –dihydroxyuracil and cytosine glycol both had retention times of 5.561 minutes but both ion profiles were present in the peak but the percentage of the peak each product contained was unknown.

The purine blocking lesion Fapy A showed no significant difference between treated versus damaged but the Frapy G lesion had a 33% reduction in treated versus damaged. The pyrimidine blocking lesion 5-hydroxyhydantoin had a slight decrease of 16% between treated versus damaged but the pyrimidine blocking lesion 5-hydroxy-5-methlyhydantoin ion was undetectable from background ions in the treated versus damaged. The hydroxy oxidative products 8-hydroxyguanine, 1-hydroxyguanine, 8-hydroxyadenine were had ion counts undetectable in treated versus damaged. While the hydroxy product 5-hydroxycytosine had a significant reduction of 65% between treated versus damaged. 5-hydroxymethyluracil and 5-hydroxyuracil both were found in the peak at retention time 10.461 minutes but both ion profiles were clearly present and able to be identified.

3.3.4 Oxidative damage repair using the PreCR™ repair treatment

The PreCR™ repair enzyme mix was able to recover an amplifiable profile up to the 26 hours of H_2O_2 damage mark but failed for subsequent intervals after 26 hours (Figure 13). There is a decreasing amount of amplified product in the target region as the damage treatments increased and the 22 hours treatment had only unspecific product and none in the target region.

Table 14. PreCR™ amplification results versus exposure times

Exposure time	PreCR™ amplification result
30 min	Positive
1 hour	Positive
2 hours	Positive
4 hours	Positive
6 hours	Positive
8 hours	Positive
10 hours	Positive
12 hours	Positive
14 hours	Positive
16 hours	Positive
18 hours	Positive
20 hours	Positive
22 hours	Negative
24 hours	Positive
26 hours	Positive
28 hours	Negative
30 hours	Negative

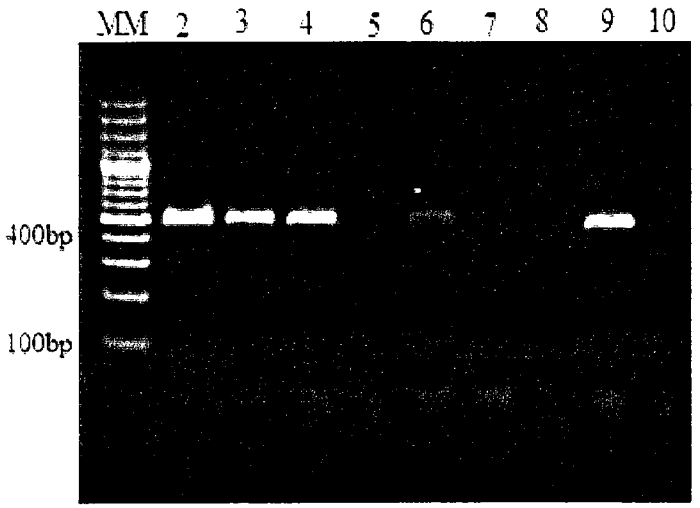


Figure 13. DNA damage using H_2O_2 and repaired with PreCR™ between 16 hours to 26 hours treatments

Lane 1. 100bp molecular marker; Lane 2. repaired 16 hours treatment; Lane 3. repaired 18 hours treatment; Lane 4. repaired 20 hours treatment; Lane 5. repaired 22 hours treatment; Lane 6. repaired 24 hours treatment; Lane 7. repaired 26 hours treatment. Lane 8 empty. Lane 9 positive control. Lane 10 negative control

This was relatively the same amount of unspecific product present in all lanes shown by slight smearing and a wide band under the 100bp molecular marker in all lanes.

Table 15. Oxidative products measured in the PreCR™ treated sample

Modified Base	Retention time	Damaged Area	Repaired Area	Difference in Area	M/Z1	M/Z2	M/Z3
5,6-dihydroxycytosine	5.587	8.490e+8	6.666e+8	16%	257	416	431
4,6-diamino-5-formamidopyrimidine	8.168	8.469e+8	1.010e+7	59%	280	354	369
Uracil	9.069	2.816e+10	1.829e+9	21%	147	255	256
5,6-dihydroxyuracil	9.753	5.088e+9	6.236e+9	13%*	343	417	432
Uracil glycol	9.76	5.041e+9	3.960e+9	13%	245	419	434
cytosine glycol	9.819	2.834e+10	4.289e+9	17%*	245	419	434
5-hydroxyhydantoin	13.159	8.887e+7	2.002e+9	40%	310	316	331
Thymine glycol	13.565	8.007e+8	4.200e+7	38%	259	433	448
5-hydroxycytosine	N/D	8.469e+8	N/D	N/D	241	255	256
5-hydroxyuracil	14.496	1.190e+9	#55 326	99%	325	344	359
5-hydroxy-5-methylhydantoin	15.770	8.007e+8	#816 210	99%		331	346
8-hydroxyguanine	19.290	4.847e+10	1.138e+10	56%	440	442	455
1-hydroxyguanine	19.290	4.847e+10	1.138e+10	56%	367	368	383
8-hydroxyadenine	19.796	3.009e+9	#260 000	99%		352	367
5-hydroxymethyluracil	20.947	3.141e+7	2.401e+8	7%		343	358
2,6-diamino-4-hydroxy-5-formamidopyrrimidine	23.354	4.091e+10	1.526e+7	47%		442	457

* Indicates an increase
\# Individual ion count if under 1 million
N/D ion showed up in multiple peaks or could not be detected from background due to low level

The PreCR™ repaired template shows that for uracil and its oxidative products there was a decrease in all except 5,6-dihydroxyuracil that had a 13% increase (Table 15). Uracil, uracil glycol, 5-hydroxymethlyuracil all had slight reductions with 21%, 13%, and 7% respectively in products between treated vs. damaged the purine blocking lesions Fapy A and Fapy G both had significant reduction (Table 15). Fapy A had a 59% reduction in product from treated versus damaged while Fapy G had a 47% reduction from treated versus damaged (Table 15). The pyrimidine blocking lesions also had significant reductions with 5-hydroxyhydantoin having a

40% reduction and 5-hydroxy-5-methlyhydantoin being virtually eliminated from the treated versus repaired sample with a change of over 99%. The hydroxy products 1-hydroxyguanine and 8-hydroxyguanine both showed up in the peak at 19.290 with clear ion profiles but both had the exact reduction in ion between treated and damaged with a reduction of 56% (Table 15). 8-hydroxyadenine was virtually absent from the treated sample compared to the damaged with over 99% reduction in ions. The C oxidative product 5-hydroxycytosine ion was at such low levels that it could not be detected from the background noise.

3.3.5 Oxidative damage repair using Phi 29 repair treatment

The Phi 29 was able increase the recovery time for the experimentally H_2O_2 damaged DNA template by four times (Figure 14). The bands were very faint and in all lanes with the

Table 16. Phi 29 amplification results vs treatment times

Exposure time	Phi 29 amplification result
30 min	Positive
1 hour	Positive
2 hours	Positive
4 hours	Positive
6 hours	Positive
8 hours	Positive
10 hours	Positive
12 hours	Positive
14 hours	Positive
16 hours	Positive
18 hours	Positive
20 hours	Negative
22 hours	Negative

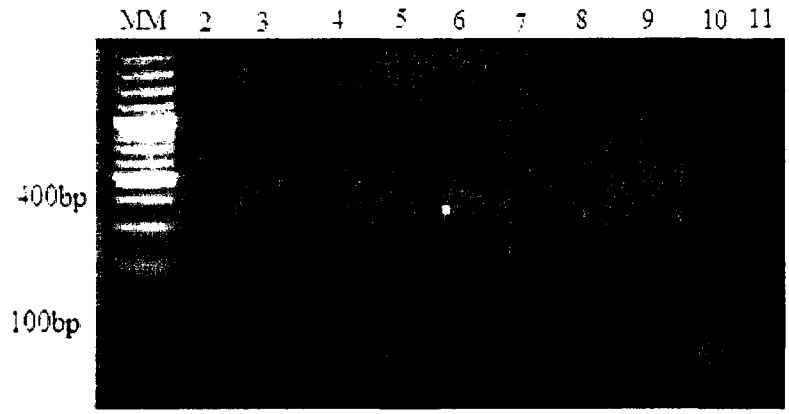


Figure 14. DNA damaged using H_2O_2 and repaired with Phi 29 between 8 to 20 hours . Lane 1. 100bp molecular marker; Lane 2. Empty; Lane 3. 8 hours; Lane 4. 10 hours; Lane 5. 12 hours; Lane 6. 14 hours; Lane 7. 16 hours; Lane 8. 18 hours; Lane 9. 20 hours. Lane 10 positive control. Lane 11 negative control.

most prominent being the 14 hours interval but it just slightly more than the others. The phi 29, the Endo IV - T4 DNA Polymerase - T4 DNA Ligase, Endo IV - Klenow - T4 DNA Ligase, T4 DNA Polymerase - T4 DNA Ligase, Klenow - T4 DNA Ligase all had a fourfold increase but the Endo IV- T4 DNA Polymerase - T4 DNA Ligase and the T4 DNA Polymerase - T4 DNA Ligase methods had the strongest bands. The three stage Endo IV - T4 DNA Polymerase - T4 DNA Ligase had the least amount of unspecific product in the lanes but still contained primer dimerization, small unspecific products and slight smearing.

3.4 Heat / acid damage and repair

3.4.1 Hydrolytic damage induced to DNA sample template

The increasing damage treatment on PCR amplification showed band intensity decreases as time treatment increases with total failure past the 8 hours mark (Figure 15). Unspecific product increases as treatment time increases then also disappears after the eight hour mark. There is a substantial reduction in band intensity after the 1 hour treatment time.

Table 17. DNA sample exposure times to Acid Buffer

DNA sample exposure time	Acid buffer	Amplification result
15 min	10µl	Positive
30 min	10µl	Positive
1 hour	10µl	Positive
2 hours	10µl	Positive
4 hours	10µl	Positive
6 hours	10µl	Positive
8 hours	10µl	Positive
10 hours	10µl	Negative
12 hours	10µl	Negative
14 hours	10µl	Negative
16 hours	10µl	Negative
18 hours	10µl	Negative
20 hours	10µl	Negative
22 hours	10µl	Negative
24 hours	10µl	Negative
26 hours	10µl	Negative
28 hours	10µl	Negative
30 hours	10µl	Negative
32 hours	10µl	Negative

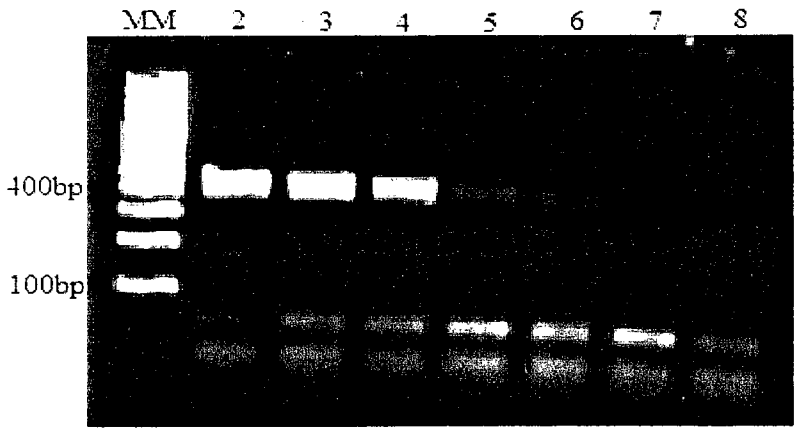


Figure 15. Heat/acid Damage from 15 minutes to 8 hours treatments
Lane 1. 100bp molecular marker; Lane 2. H/A treatment 15 minutes; Lane 3. H/A treatment 30 minutes; Lane 4. H/A treatment 1 hour; Lane 5. H/A treatment 2 hours; Lane 6. H/A treatment 4 hours; Lane 7. H/A treatment 6 hours; Lane 8. H/A treatment 8 hours.

3.4.2 Hydrolytic damage repair using DNA polymerases and DNA ligases

Table 18. Klenow - DNA Ligase treatment and T4 DNA Polymerase treatment versus exposure time amplification results

Exposure time	Klenow - DNA Ligase amplification result	T4 Polymerase - DNA Ligase amplification result
15 min	Positive	Positive
30 min	Positive	Positive
1 hour	Positive	Positive
2 hours	Positive	Positive
4 hours	Positive	Positive
6 hours	Positive	Positive
8 hours	Positive	Positive
10 hours	Positive	Positive
12 hours	Positive	Positive
14 hours	Positive	Positive
16 hours	Negative	Positive
18 hours	Negative	Negative
20 hours	Negative	Negative

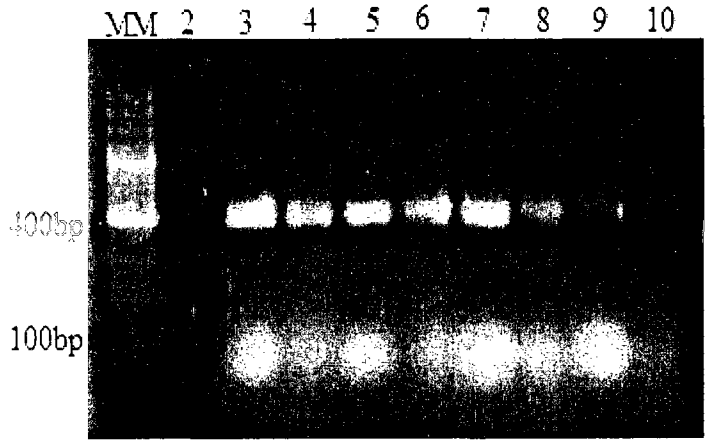


Figure 16. Klenow - T4 DNA Polymerase - T4 DNA Ligase repaired between 15 minutes to 2 hours

Lane 1. 100bp molecular marker; Lane 2. Empty; Lane 3. Klenow and T4 DNA Ligase 15 minutes; Lane 4. T4 DNA Polymerase and T4 DNA Ligase 15 minutes; Lane 5. Klenow and T4 DNA Ligase 30 minutes; Lane 6. T4 DNA Polymerase and T4 DNA Ligase 30 minutes; Lane 7. Klenow and T4 DNA Ligase 1 hour; Lane 8. T4 DNA Polymerase and T4 DNA Ligase 1 hour; Lane 9. Klenow and T4 DNA Ligase 2 hours; Lane 10. T4 DNA Polymerase and T4 DNA Ligase 2 hours.

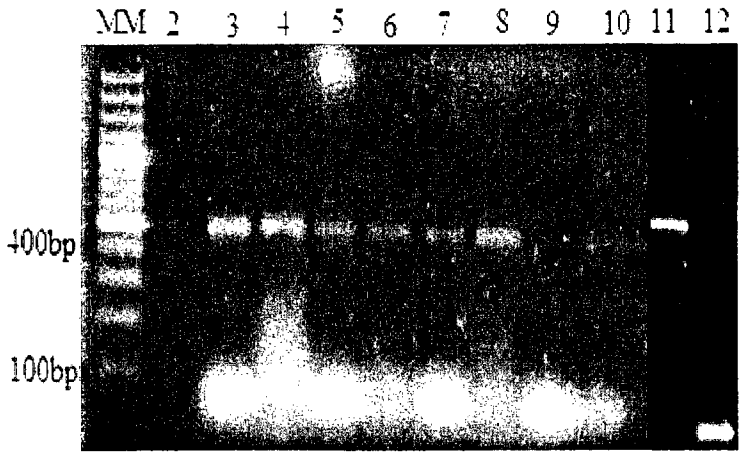


Figure 17. Klenow - T4 DNA Ligase - T4 DNA Polymerase repaired hydrolytic damage treated between 4 to16 hours
Lane 1. 100bp molecular marker; Lane 2. Empty; Lane 3. Klenow and T4 DNA Ligase 4 hours; Lane 4. T4 DNA Polymerase and T4 DNA Ligase 4 hours; Lane 5. Klenow and T4 DNA Ligase 8 hours; Lane 6. T4 DNA Polymerase and T4 DNA Ligase 8 hours; Lane 7. Klenow and T4 DNA Ligase 12 hours; Lane 8. T4 DNA Polymerase and T4 DNA Ligase 12 hours; Lane 9. Klenow and T4 DNA Ligase 16 hours; Lane 10. T4 DNA Polymerase and T4 DNA Ligase 16 hours. Lane 11 positive control. Lane 12 negative control.

The Klenow - T4 DNA Ligase treatment was able to increase the recovery time from 8 hours to 14 hours before the PCR failed on heat/acid damaged DNA template (Figure 16 and 17). T4 DNA Polymerase - T4 DNA Ligase treatment was slightly better with a doubling of recovery time to 16 hours in comparison to untreated heat/acid damaged DNA template. The Klenow lanes contained very bright bands located just under the 100bp molecular marker region which increased in intensity with an increase in damage time intervals. The T4 DNA Polymerase lanes had more smearing throughout the lanes but much less intense bands under the 100bp molecular marker regions.

3.4.3 Hydrolytic damage repair using glycosylases, DNA polymerases and DNA ligases

Table 19. Endo IV added to Klenow Ligase method and T4 DNA Polymerae method amplification results versus treatment times

Exposure time	Endo IV added to Klenow - DNA Ligase amplification result	Endo IV added toT4 Polymerase - DNA Ligase amplification result
30 min	Positive	Positive
1 hour	Positive	Positive
2 hours	Positive	Positive
4 hours	Positive	Positive
6 hours	Positive	Positive
8 hours	Positive	Positive
10 hours	Positive	Positive
12 hours	Positive	Positive
14 hours	Positive	Positive
16 hours	Negative	Positive
18 hours	Negative	Negative
20 hours	Negative	Negative

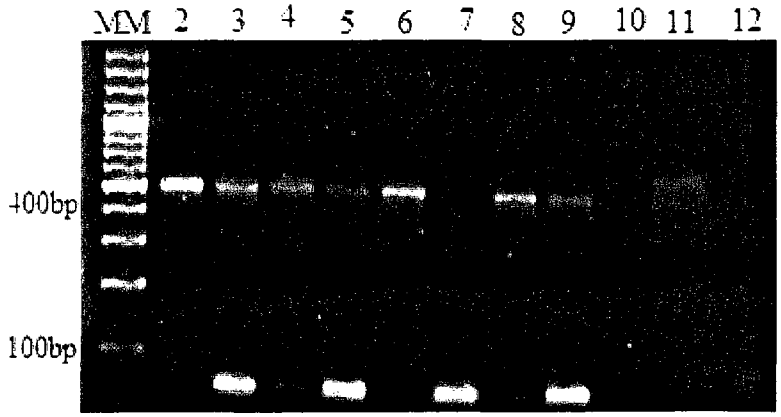


Figure 18. Endo IV -Klenow -T4 DNA Polymerase - T4 DNA Ligase repair treatment of hydrolytic damage treated between 4 to 16 hours.
Lane 1. 100bp molecular marker; Lane 2. Endo IV-T4 DNA Polymerase-T4 DNA Ligase (E/T4/L) repair treatment at 4 hours; Lane 3. Endo IV-Klenow-T4 DNA Ligase (E/K/L) repair treatment at 4 hours; Lane 4. E/T4/L repair treatment at 8 hours; Lane 5. E/K/L repair treatment at 8 hours; Lane 6. E/T4/L repair treatment at 12 hours; Lane 7. E/K/L repair treatment at 12 hours; Lane 8. E/T4/L repair treatment 16 hours; Lane 9. E/K/L repair treatment at 16 hours. Lane 10 empty. Lane 11 positive control. Lane 12 negative control.

The additional step using the glycosylase Endo IV has not extended the ability to amplify damaged DNA template from increased treatment times but has increased the band intensity and decreased the unspecific product and smearing (Figure 18). The T4 DNA Polymerase has less unspecific product in its lanes relative to the Klenow samples with less smearing and bands under the 100bp molecular ladder. Band intensity decreases over time treatments but has doubled the time to 16hrs from the unrepaired heat/acid damaged template. The T4 DNA Polymerase has brighter sharper bands over all time intervals relative to the Klenow samples.

3.4.4 Hydrolytic damage repair using the PreCR™ repair kit

Table 20. PreCR™ amplification results vs treatment times

Exposure time	PreCR™ amplification result
30 min	Positive
1 hour	Positive
2 hours	Positive
4 hours	Positive
6 hours	Positive
8 hours	Positive
10 hours	Positive
12 hours	Positive
14 hours	Positive
16 hours	Positive
18 hours	Positive
20 hours	Negative
22 hours	Negative

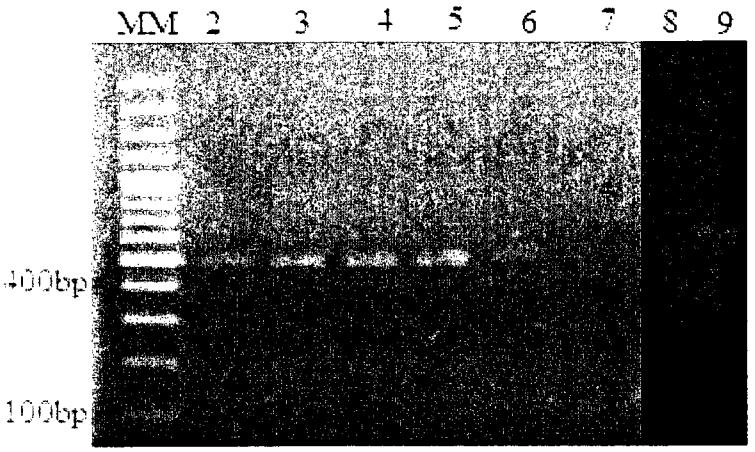


Figure 19. PreCR™ Enzyme Repair Mix used on heat/acid hydrolytic damage treated samples between 8 to 22 hours
Lane 1. 100bp molecular marker; Lane 2. PreCR™ repair treatment 8 hours; Lane 3. PreCR™ repair treatments 10 hours; Lane 4. PreCR™ repair treatment 12 hours; Lane 5. PreCR™ repair treatment 14 hours; Lane 6. PreCR™ treatment 16 hours; Lane 7. treatment 18 hours; Lanes 8 negative control. Lane 9 positive control.

The PreCR™ repair mix time treatment for recovery of amplified product with a faint band at the 18 hours mark (Figure 19). The lanes were free of unspecific product either smearing or bands under the 100bp mark seen in the unrepaired heat/acid damaged DNA template. The PreCR™ mix increased the amplification and recovery time versus untreated damaged product from 8 hours maximum to 18 hours but the 18 hours time interval band is very weak. There was approximately a doubling of time for damaged treatment from damaged unrepaired to damage PreCR™ treated template viability. The bands are of less intensity than the three step Endo IV - T4 DNA Polymerase - T4 DNA Ligase but have the two methods have approximately the same recovery time intervals for the heat/acid damaged template.

3.4.5 Hydrolytic damage repair using Phi 29

Phi 29 treatment on the heat acid damaged template was the highest with a threefold increase in recovered viable template over the longest damage intervals (Figure 20). A band was

clearly visible in the 24 hours exposure time shown in lane 5 but there was a rapid decline in band intensity from the 20 hours damaged template in lane 3 to the last visible band in lane 5. Unspecific product began to accumulate as the damage time intervals increased.

Table 21. Phi 29 treatment amplification results versus treatment times

Exposure time	Phi 29 amplification result
15 min	Positive
30 min	Positive
1 hour	Positive
2 hours	Positive
4 hours	Positive
6 hours	Positive
8 hours	Positive
10 hours	Positive
12 hours	Positive
14 hours	Positive
16 hours	Positive
18 hours	Positive
20 hours	Positive
22 hours	Positive
24 hours	Positive
26 hours	Negative
28 hours	Negative

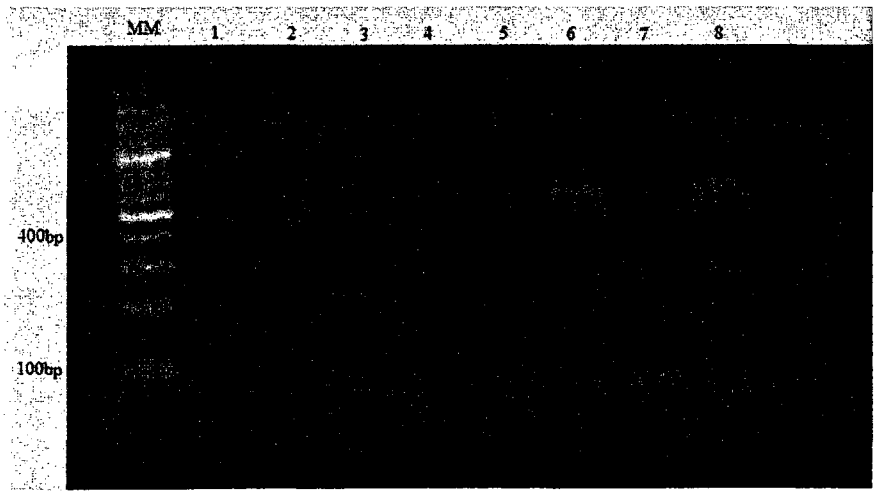


Figure 20. Heat/acid hydrolytic damage treated DNA between 16 to 24 hours repair using Phi 29

MM. 100bp molecular marker; Lane 1. 16 hours treatment; Lane 2. 18 hours treatment; Lane 3. 20 hours treatment; Lane 4. 22 hours treatment; Lane 5. 24 hours treatment; Lane 6. PCR positive control; Lane 7. PCR negative, undamaged, unrepaired template

3.5 Ancient samples

Molecular marker with a maximum upper range of 300bp was mistakenly run alongside the samples when the target amplicon of 425bp was outside the range. Due to the nature of the samples they were not able be run again with the appropriate molecular marker. The results were not deemed to be effected by this factor. The PreCR™ negative (lane 2) and the extraction negative (lane 3) both had no bands in the target region but a small band of unspecific product was observed in the PCR negative. Lane 3 to lane 7 are the unrepaired samples. In lane 3 the DKT380 sample had a small band significantly lower than the target

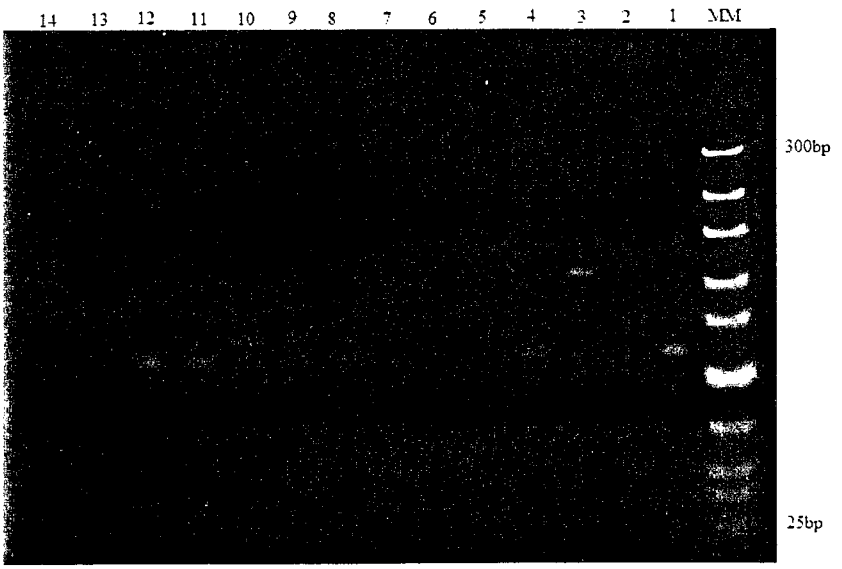


Figure 21. Unrepaired Ancient Samples and PreCR™ treated Daklah Oasis samples. MM- Ultra Low Range DNA Ladder; Lane 1. PCR Negative; Lane 2. Extraction Negative; Lane 3. DKT380; Lane 4. DKT388; Lane 5. DKT 426; Lane 6. COP26; Lane 7. COP30; Lane 8. PreCR™ Neg; Lane 9. PreCR™ treated DKT380(1); Lane 10. PreCR™ treated DKT380(2); Lane 11. PreCR™ treated DKT388(1); Lane 12. PreCR™ treated DKT388(2); Lane 13. PreCR™ treated DKT 426(1); Lane 14. PreCR™ treated DKT426(2).

region indicating some amplification but fragmentation or damage was believed to be inhibiting. Another smaller band was observed lane 4 the DKT388 sample around the 100bp molecular marker which also indicating unspecific product much smaller than the 425bp amplicon targeted. The COP26 and COP30 samples in lanes 6 and 7 had no visible signs of amplification. The PreCR™ treated DKT380 sample and the DKT388 sample in lanes 9 to 12 had several smaller bands around the 100 to MM and some light smearing well under the 425bp amplicon targeted. The DKT426 in lane 13 and 14 had no visible signs of amplification.

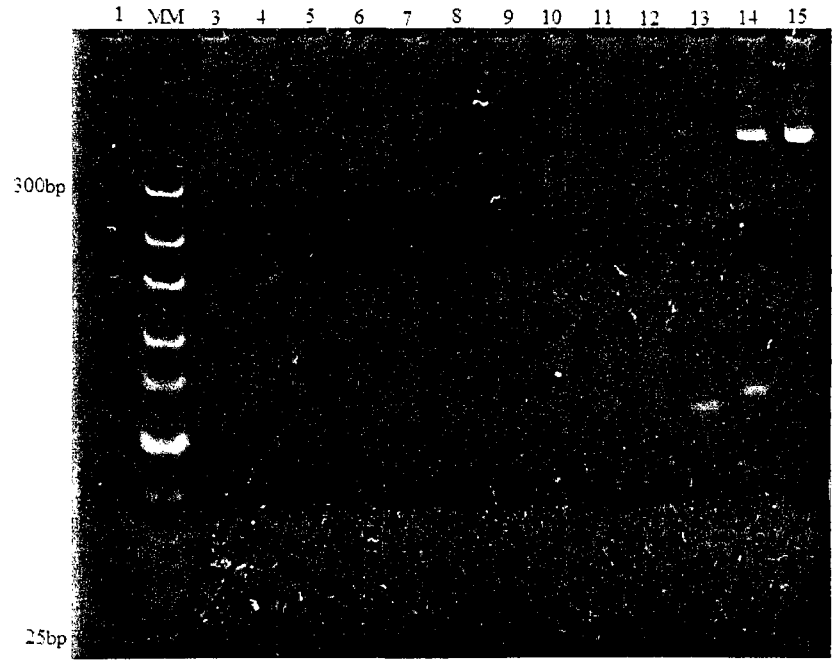


Figure 22. PreCR™ treated Copan samples and Phi 29 treated Ancient samples
Lane 1. PreCR™ treated COP26(1); MM- Ultra Low Range DNA Ladder; Lane 3. PreCR™ treated COP26(2); Lane 4. PreCR™ treated COP30(1); Lane 5. PreCR™ treated COP30(2); Lane 6. Phi 29 Negative Control; Lane 7. Phi 29 treated DKT380(1); Lane 8. Phi 29 treated DKT380(2); Lane 9. Phi 29 treated DKT388(1); Lane 10. Phi 29 treated DKT388(2); Lane 11. Phi 29 treated DKT 426(1); Lane 12. Phi 29 treated DKT 426(2); Lane 13. Phi 29 treated COP26(1); Lane 14. Phi 29 treated COP26(2); Lane 15. Phi 29 treated COP30(1).

The Copan samples results for the PreCR™ treated samples in lanes 1, 3, 4 and 5 all had no amplification indicating no repair (Figure 22). Lane 6 had the Phi 29 negative PCR control

which had no amplification indicating reagent purity. Lanes 7 to 12 showed the Daklah Oasis samples treated with Phi 29 had no amplification indicating no repair. The Phi treated COP26(1) did have a visible band but it was far below the target region indicating unspecific product. The second replicate COP26(2) in lane 14 had an extremely thick bright band in the target region indicating a very successful repair. Lane 15 also indicated a successful repair had taken place which was the Phi 29 treated COP30(1) showing the bright thick band in the target region.

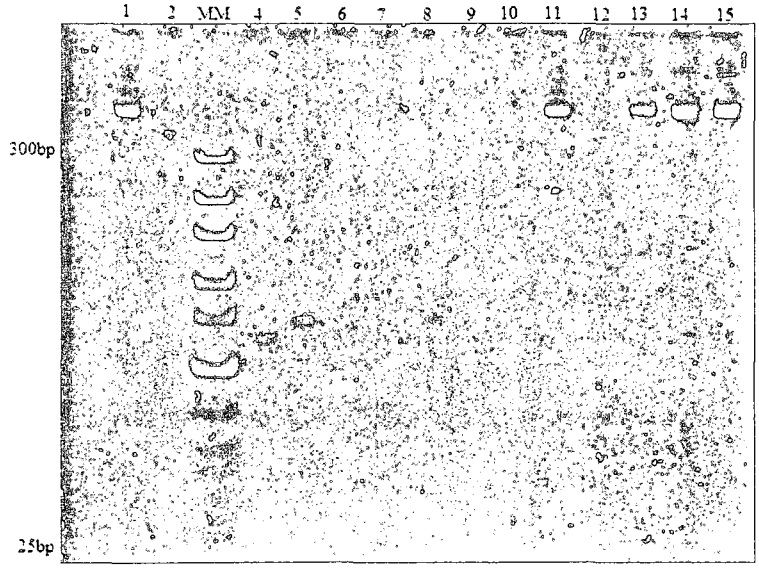


Figure 23. PHI 29 treated Copan samples and T4 DNA Polymerase - T4 DNA Ligase treated Ancient samples
Lane 1. Phi 29 treated COP30(2); Lane 2. T4 Negative; MM Ultra Low Range DNA Ladder; Lane 4. T4 treated DKT380(1); Lane 5. T4 treated DKT380(2); Lane 6. T4 treated DKT388(1); Lane 7. T4 treated DKT388(2); Lane 8. T4 treated DKT426(1); Lane 9. T4 treated DKT 426(2); Lane 10. T4 treated COP26(1); Lane 11. T4 treated COP26(2); Lane 12. T4 COP30(1); Lane 13 T4 treated COP30(2); Lanes 14. and 15. Positive Controls.

Lane one shows the successful repair of the second replicate of COP30 (Figure 23). The band is extremely bright indicating a large amount of DNA present with no unspecific products or smearing visible in the lane. Lane 2 shows the negative PCR for the T4 DNA polymerase for reagent purity which is confirmed by no visible amplified product. Lanes 4 and 5 are the

replicates of DKT 380 treated with the T4 DNA Polymerase method and have visible bands well under the target region but are of intermediate brightness indicating partial recovery. No repair was visible on treated DKT 388 in either replicate using T4 DNA Polymerase method. Lane 8 containing the T4 DNA Polymerase treated DKT426 replicate 1 did have a small weak band in the approximate area as the DKT 380 samples but replicate two had no visible products. The COP26 replicate 1 (lane 11) and the COP30 replicate 2 that were treated with the T4 DNA Polymerase method both had bright strong bands in the target region with no unspecific product visible indicating successful repair. The bands from the T4 DNA Polymerase method although of high quality were slightly less intensity than the Phi 29 treated Copan samples.

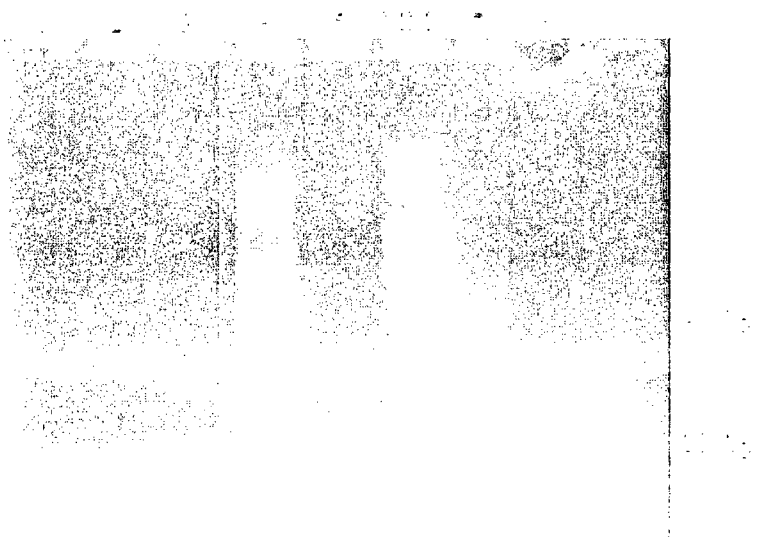


Figure 24. Daklah Oasis untreated samples PCR with primers 16 190F and 16420R
Lane 1. Empty; Lane 2. DKT426; Lane 3. Extraction NEG; Lane 4. DKT380; Lane 5. DKT388; Lane 6.; MM 100bp molecular marker; Lane 7. Positive Control.

Following the unsuccessful attempt to recover a viable amplicon from the DKT samples severe fragmentation was suspected so new primers were used to amplify a smaller amplicon. Lane 3 was the extraction negative which tested positive for DNA for the smaller amplicon

(Figure 24) but was negative in the previous PCRs with the 425bp amplicon. The DKT sample 380 also had some smearing but had no specific band or bands within the smear. The other samples tested negative for viable DNA using the standard *Taq* DNA Polymerase amplification protocol. The positive control had the specific band in the target region indicating successful amplification.

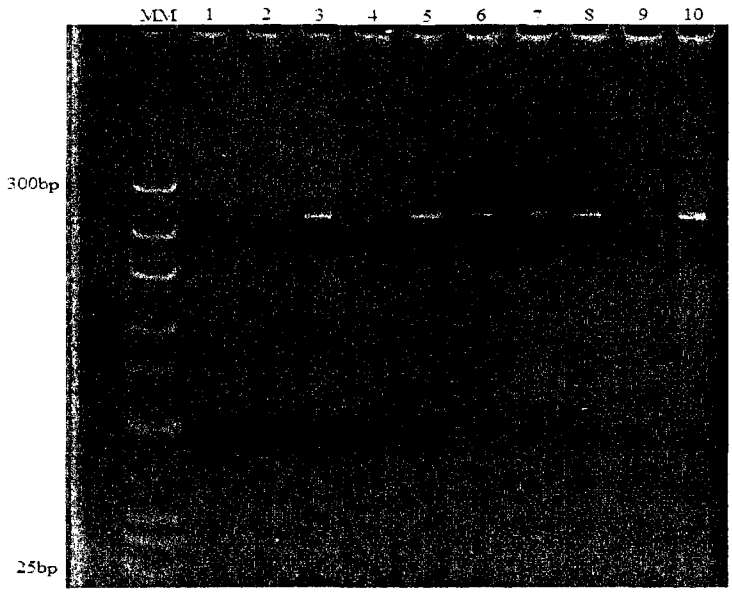


Figure 25. PreCR™ treated Daklah Oasis samples with Primers 16190F and 16420R
MM Ultra Low Range DNA Ladder; Lane 1. PCR NEG; Lane 2. PreCR™ NEG; Lane 3. Extraction NEG; Lane 4. DKT380(1); Lane 5. DKT380(2); Lane 6. DKT388(1); Lane 7. DKT388(2); Lane 8. DKT426(1); Lane 9. DKT426(2); Lane 10. Positive Control.

After the PreCR™ repair treatment to the Daklah Oasis samples all three samples tested positive showing strong bands in the target region free of any smearing or unspecific product (Figure 25). The Extraction Negative which had tested positive in the unrepaired/untreated Daklah Oasis PCR also tested positive when the smaller 230bp amplicon was used in the PreCR™ treated/repaired Daklah Oasis PCR. PCR negative and the PreCR™ negative were both negative for amplified DNA confirming the reagents purity and contamination free. DKT380(1) in lane 4 and DKT426(2) in lane 9 failed to amplify in replicate but did have strong bands in one

of their two replicates. DKT388 had strong bands in both replicates in lanes 6 and 7. Positive Control had strong band in target region indicating a successful PCR amplification.

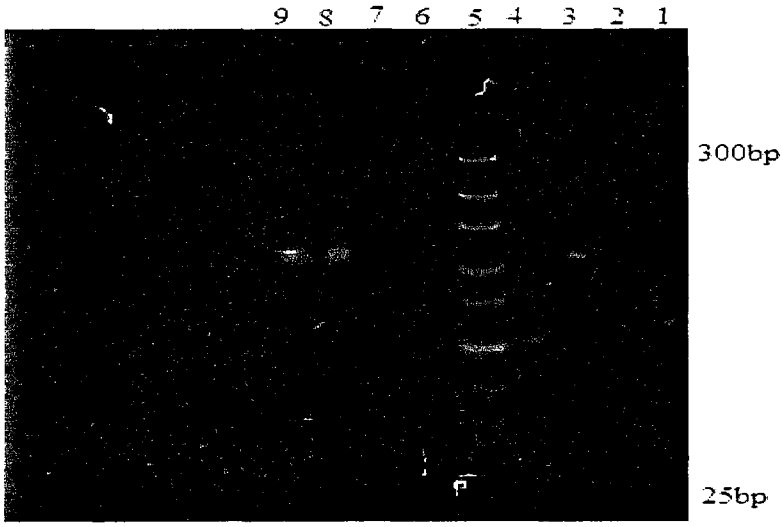


Figure 26. Repaired Çayönü Tepesi sample amplified with sexing primers

Lane 1. Phi 29 Neg PCR control; Lane 2. Phi 29 Çayönü Tepesi (1); Lane 3. Phi-29 Çayönü Tepesi (2); Lane 4. T4 DNA Polymerase-Neg PCR; Lane 5. Ultra Low Range DNA Ladder; Lane 6. T4 DNA polymerase Çayönü Tepesi (1); Lane 7. T4 DNA polymerase Çayönü Tepesi (2); Lane 8. Male positive control 0.5; Lane 9. Male positive control 2.

The repaired Çayönü Tepesi sample 9 shows a single clear band in lane 3 indicating a female result with Phi 29 in the second replicate (Figure 26). The phi 29 negative PCR reaction was negative confirming reagents purity and that the result came from the Çayönü Tepesi extract sample and not contamination. Lanes 8 and 9 were the positive male controls used for comparison at two different concentrations. PreCR™ on Çayönü Tepesi sample had negative results and the gel is not shown.

4.0 Discussion

It is through this project that current *in vitro* DNA repair methods were evaluated for overall efficiency and for their repair ability on three common types of damage encountered with preserved and degraded DNA samples. Through a combination of PCR and GCMS specific damage types and relative amounts were able to be detected and then measured (Jenner et al. 1998) against the methods relative ability to overcome the damage product present in the sample to retrieve a successful amplification. The hypothesis is that each *in vitro* method will have a varying degree of success on the repair and recovery of degraded DNA influenced by the concentration and composition of individual damage products present in the sample. Many DNA polymerases contain some degree of proofreading ability which is a correction of misincorporated bases at the time of synthesis. Newer engineered *Taq* DNA Polymerases include high fidelity and proofreading ability to help in the PCR amplification. The *Taq* DNA Polymerase used in this study lacks detectable 3'-5'exonucleolytic proofreading activity for the *in vitro* amplification of DNA and produces single-base substitution errors at a rate of 1 for each 9000 nucleotides but does possesses a non-processivity 5'-3' polymerase activity and double stranded specific 5'-3' exonuclease activity in the presence of magnesium. Allowing higher fidelity in amplification but limited in template repair ability (Tindall and Kunkel 1988). Higher fidelity *Taq* DNA Polymerase such as Platinum *Taq* DNA Polymerase with some proof reading ability could have been used but the evaluation was for the repair methods and the choice of *Taq* DNA Polymerase was to reduce the amount of influence on the results so methods could be evaluated for their repair ability. In future studies other thermo stable enzymes could be used to enhance results but that would be an optimization of method after process is understood and evaluated.

Optimization

To obtain optimal efficiency the methods evaluated had to be modified and in some cases changed completely from their originally intended role and adapted to the repair and recovery of degraded DNA. The PCR used to evaluate the efficiency of the repair protocols after treatments was optimized for the least number of cycles to reduce artificial positives from contamination and to use the least number of cycles needed to amplify undamaged template. Twenty five cycles was the threshold chosen for amplification for the undamaged template to be visualized on a 2% agarose gel under UV with EtBr. The repair method had to be able to recover the band within the same number of cycles to be considered successful. QIAquick silica bind elute columns were used to purify the experimental DNA template as well as standard ethanol precipitations with a 70% ethanol wash. It was found that the ethanol precipitation purification did not yield a pure enough product for reliable GCMS analysis but did not produce a difference in PCR analysis. The GCMS method used also was modified slightly from the original published method by actually decreasing the amount of DNA template by a final dilution in acetonitrile which is the solvent used for GCMS analysis. This dilution combined with a slightly longer run time allowed increased resolution and sensitivity. Individual peaks were to be identified corresponding to modified base products that have very similar molecular weights and which were masked by the merging of the peaks into one in the previous method because of the resolution. This is important to DNA analysis especially in aDNA and highly degraded DNA where recovered samples are extremely limited because the limiting factor for accurate analysis is not the quantity of the DNA but its purity.

Strand Breaks

In section 3.1 dealing with strand break damage which was artificially induced with DNase it was shown that the major factor for the repair of simple strand nicks where the 5' and 3' ends were unmodified from their OH and phosphate residues was T4 DNA Ligase with other factors such as pretreatments less significant when dealing only with simple nicks. The accumulation of single strand nicks can eventually accumulate in a random fashion so that they can be lined up on adjacent strands creating a double strand break which is beyond the ability of the DNA ligase to repair because of the fragmentation of the molecule which may explain why higher concentrations at of DNase caused greater damage then lower concentrations even at longer exposure times. The more enzyme present exponentially increases the potential for double strand breaks to occur up to a maximum level (Obe et al. 1992). The first amplicon used was 800bp in size and attempts to recover the amplicon after repair treatments failed in the 1U DNase exposure time interval when the amplified damaged unrepaired template was used and also in the 0.5U DNase time treatment interval. The smearing all was below the 800bp ladder indicating the accumulation of double strand breaks which caused fragmentation of the DNA template leaving no intact sections of DNA in the target region. The use of a 425bp amplicon enabled recovery up to the 0.5U DNase longest damage inducing time of 5 minutes. The 425bp failed in the 1U DNase treatment even though it was within the smearing region that showed product. The amount of unspecific product and the degree of fragmentation induced by the DSB is believed to have caused the failure due to the inability to successfully repair DSB.

Oxidative damage

Oxidative damage was evaluated through a combination of PCR and GCMS. Several methods were tested for their efficiency at repairing the damage template and to restore an

expected profile through PCR. The GCMS was used as a diagnostic tool to evaluate and quantify relatively the types of damage products generated and to what degree the methods were able to deal with each individual oxidatively modified base product. There is a direct negative correlation between the hydantoins and Fapy A and Fapy G in inhibiting PCR using *Taq* DNA Polymerase (Gasparutto et al. 2009; Hoss et al. 1996). The glycol products have also been identified as PCR blocking lesions *in vitro* but are inherently unstable and rapidly convert to other products and back again depending on environmental factors (Dizdaroglu et al. 1986; Ide et al. 1985)

Experiments with Klenow Fragment - T4 DNA Ligase

Klenow was tested first without the addition of T4 DNA Ligase step and had no success in increasing recovery time for target region DNA. This is probably the inability of the enzyme to ligate the 3' end of the newly incorporated base when it cleaves out a recognizable mismatch or modified base in its proofreading capacity. With the addition of the T4 DNA Ligase step recovery time was increased by 100% but with fairly weak bands and much smearing indicating a high degree of unspecific product (figure 10). Using the GCMS data shown in table 7 there is a reduction in the blocking lesions 5-hydroxthydantoin, uracil glycol, thymine glycol, Fapy A which would help to increase amplification product by removing these blocking lesions for the *Taq* DNA Polymerase. There is unfortunately a corresponding increase in uracil, cytosine glycol, 5-hydroxy-5-methylhydantoin. The relative percentages indicate that uracil glycol is being converted to cytosine glycol. The thymine glycol is being converted to 5-hydroxymethylhydantoin so the only real decrease would be in the percentage of 5-hydroxyhydantoin which did experience a 35% drop from treated to untreated and could account for the increase in recovery time but the decrease in thymine glycol would increase efficiency.

Several other oxidative products shown in Table 7 did have considerable percentage drops but are not considered blocking lesions but will induce transversion mutations through wrong base pairing these indicate that the Klenow treatment did improve the quality of the template fidelity through the reduction or elimination of such transversion inducing products such as 1-hydroxyguanine and 8- hydroxyguanine which were virtually eliminated in the treated versus untreated damaged DNA samples. The increase in uracil content coupled with the decrease in the oxidative damaged uracil products such as 5-hydroxymethlyuracil and 5 hydroxyuracil indicate a conversion to uracil and the probability of a disproportionate transversion of U→G in PCR amplification. The relative unchanged concentration of 5,6-dihydroxyuracil is highly suspect with the reduction of the similar uracil damage products and possibly is from the preferred conversion of 5-hydroxycytosine through the deamination on the C4′ carbon amine group caused by the heat denaturation of the Klenow enzyme or a product of the derivitization protocol (Halliwell and Dizdaroglu 1992).

Experiments with T4 DNA Polymerase - T4 DNA Ligase

The T4 DNA Polymerase enzyme (figure 10) increased recovery by100% with strong bands low smearing but only when coupled with T4 DNA Ligase. Without the T4 DNA Ligase step bands were still visible indicating some degree of ligation may have been occurring in the DNA polymerases proofreading capacity. The strong smearing indicates it is unable to deal with strand nick ligations outside of its proofreading capacity. The GCMS data listed in Table 5 shows the relative changes in modified base products after treatment with T4 DNA Polymerase - T4 DNA Ligase. The T4 DNA Polymerase method unlike the Klenow method has a significant reduction in both hydantoin blocking lesions which could explain its slightly better ability to repair damaged template versus the Klenow method. The fapy blocking lesions had a small

decrease and may have a small contribution to overall performance but are not the main factors. Thymine glycol, cytosine glycol, and uracil glycol all had significant reductions and are considered blocking lesions so would have an impact on DNA repair but the 86% increase in uracil and the less significant changes in the other uracil modified products of 5,6-dihydroxyuracil, 5-hydroxymethlyuracil and C modified product 5,6-dihydroxycytosine indicate some of the reductions are due to conversions rather than repair (Dizdaroglu et al. 1986). The high uracil content just like in the Klenow method could lead to a U→G transversion in downstream PCR. The T4 DNA Polymerase method was excellent at reducing or eliminating other non blocking transversion lesions such as 1-hydroxyguanine and 8-hydroxyguanine, 8-hydroxyadenine all major products of oxidative stress.

Experiments with Endo IV -T4 DNA Polymerase - T4 DNA Ligase

The addition of Endo IV glycosylase was added to both Klenow - T4 DNA Ligase and T4 DNA Polymerase - T4 DNA Ligase methods and increased efficiency by 300% (figure 12). Only one of the major blocking lesions in the hydantoin and fapy groups considered major barriers to downstream PCR had significant decrease over the Klenow and T4 DNA Polymerase methods. 5-hydroxy-5-methlyhydantoin was such at low ion counts to be undetectable from base line noise in GCMS (Table 10). Increases in cytosine glycol and 5,6-dihydroxyuracil can be correlated to drops in 5,6-dihydroxycytosine which can convert to 5,6-dihydroxyuracil in oxidative environment and 5-hydroxycytosine can also convert to cytosine glycol in an oxidative environment. Uracil increased 57% from damaged to treated which was smaller than previous methods without the addition of the Endo IV but still quite significant. As in the previous methods the decreases in 5-hydroxymethlyuracil could contribute to the uracil increase but contrary to the previous methods uracil glycol and 5-hydroxyuracil had virtually no change in

levels from treated from damaged indicating some other product is being converted to uracil at least in this method. The fact that thymine glycol also known as a blocking lesions seems to be at low enough levels to be undetectable in from the base line indicating complete removal or conversion to some other possible modified base such as uracil and would help explain the increase. The removal of thymine glycol would definitely contribute to the overall efficiency of recovery. As in the Klenow - T4 DNA Ligase, and T4 DNA Polymerase - T4 DNA Ligase methods 1-hydroxyguanine, 8-hydroxyguanine and 8-hydroxyadenine were all eliminated to undetectable levels from treated sample (Table 10) which would prevent transversion base mutations in downstream PCR and would increase the fidelity of the method.

Experiments using the PreCR™ Enzyme Repair Mix

PreCR™ was very effective on oxidative damage with an efficiency increase of 650%. From figure 13 it can be seen that there is very minute smearing even into the farthest damage intervals and had no virtually no unspecific product right up to the point of inhibition. The significant decreases in all four major blocking lesions, hydantoins and fapys would definitely be a major factor in PreCR™s superior performance in the recovering DNA profiles from heavily oxidatively damaged DNA . One of the constituent enzymes Fpg is probably responsible for the major decreases in Fapy G, Fapy A and reduction to undetectable levels of 5-hydroxy-cytosine and 5-hydroxyuracil because this enzyme is very effective at removing these base oxidative damages (Boiteux et al. 1992). The Endo IV Glycosylase used in both PreCR™ and the Endo IV - T4 DNA Polymerase - T4 DNA Ligase as seen table 10 was very effective on 5- hydroxy-5-methylhydantoin reducing it to undetectable levels in the Endo IV - T4 DNA Polymerase - T4 DNA Ligase method so it is probably responsible for the same drop in the PreCR™ method. Other hydantoins, cytosine glycol and 5,6-dihydroxyuracil also had an increase but in

comparison to the other methods where GCMS data was available it was much less (Table 13) and as previously discussed is probably the result of some of the other products being converted. Uracil had a 21% drop which was contrary to the other methods where GCMS data was available which all exhibited a rise in uracil content in repaired samples. One of the enzymes in the PreCR™ mix is Uracil-DNA Glycosylase which is very effective at removing uracil from DNA template (Nilsen et al. 2002; Nilsen et al. 2000) but may be inhibited due to the tradeoff in having so many enzymes together balancing competitive optimum reaction conditions. As in the other methods 8-hydroxyguanine, 1-hydroxyguanine and 1-hydroxyadenine all has significant reductions but the 1- and 8-hydroxyguanines had substantially less reduction than previous methods. Both oxidative products showed up in the same peak at the retention time 19.290 and both registered a 56% decrease but because of their similar molecular weight the resolution for discriminating the individual products on this particular GCMS run could explain the results or the PreCR™ mix could be equally efficient at reducing both types of hydroxyguanines. The 56% drop will also mean there could possibly be half as many more transversions caused by the G base damage in downstream PCRs.

Experiments with the Phi 29 Enzyme

Unfortunately GCMS data was not available at the time of writing so the oxidative repair capability for Phi 29 was evaluated strictly through PCR amplification. The price of the enzyme was cost prohibitive for this study allowing only for PCR optimization in the oxidative and hydrolytic damage categories. To obtain GCMS data often multiple runs were needed to optimize the required resolution to identify individual base modifications and it was believed that Phi 29 would be much more effective on hydrolytic damage.

Hydrolytic Damage

Hydrolytic damage was evaluated only through PCR. GCMS turned out to be an ineffective tool for identifying or quantifying hydrolytic damage. The randomness of the damage coupled with the hydrolysis procedure used to prepare the DNA for derivitization for GCMS masked any identifiable trends in damage products or between treatments. The heat/acid buffer protocol used to induce the damage which generates predominately AP sites within the DNA (Nakamura and Swenberg 1999). Even though *Taq* DNA Polymerase can transcribe over AP sites within a DNA strand (Belousova et al. 2006) in the unrepaired heat acid damaged DNA complete inhibition of the *Taq* DNA Polymerase came at the 8 hour mark which is the time interval where it is possible that the AP sites have accumulated in multiple adjacent sites preventing transcription.

In comparing the repair methods Klenow - T4 DNA Ligase, and T4 DNA Polymerase - T4 DNA Ligase effectiveness in recovering DNA profiles both methods had approximately the same effectiveness with recovery of DNA profiles over damage intervals with both methods doubling the recovery time from 8 to 16 hours or a 100% increase in efficiency. The T4 DNA Polymerase - T4 DNA Ligase method was superior with slightly brighter bands, less smearing and less unspecific product indicating a higher quality of repaired DNA. The additional added step of including Endo IV as a pretreatment to the Klenow - T4 DNA Ligase, and T4 DNA Polymerase - T4 DNA Ligase methods (Figure 18) did not extend the recovery time but did drastically reduce the smearing, unspecific product formation and brightened target bands considerably. Endo IV being a glycosylase which is effective in repairing many oxidative damage products indicates there may have been some potentially oxidative damage to the sugar moiety as a side product of the treatment process (Evans et al. 2004). Even though some

oxidative damage appears to have been generated the major factor still appears to be the hydrolytic damage based on the inability of the Endo IV treatment to increase recovery time even if it was able to improve the resolution.

The PreCR™ enzyme repair mix shown in Figure 19 was slightly better in time recovery with a visible band at the 18 hour mark but it was very weak. The absence of smearing and unspecific product in all lanes similar to the Endo IV treatment but the relative faintness of the bands over all time intervals indicates that the overall efficiency of the recovery method is approximately the same as the previous methods but its mix of glycosylases is effective at reducing the oxidative by products to increase resolution but at the cost of reducing the quantity of recovered DNA.

The Phi 29 DNA Polymerase (Figure 20) had the best results in the hydrolytic damage category with a 200% increase in efficiency with no smearing and very little unspecific product. The ability of the enzyme to replicate the DNA at room at below denaturing temperatures, excellent proofreading ability, high fidelity (Paez et al. 2004) and ability to perform translesional synthesis across multiple AP sites allowed the Phi 29 to have the greatest recovery time. It also has some ability to deal with oxidative damage products as seen in the experimentally damaged H_2O_2 results which is why the lanes had very little unspecific product and no smearing. It is unknown at this stage if Phi 29 treatment method induces transversions within the genetic code by reading over the oxidative damage products or if it actually has some glycosylase ability to remove and replace modified base products.

Comparison of Repair Methods.

The T4 DNA Ligase was found to be the determining factor for simple strand breaks and the addition of other enzymes tested had no real contributing factor and actually reduced the

repair process probably because the loss and damage of template when additional purification steps between enzyme treatments were needed. No purification method is a 100% efficient and some template loss will be realized at each stage (Krsek and Wellington 1999) which is a factor in how many steps are involved in a repair method and how many side products are formed or template loss. When all the tested repair methods are compared for recovery the Phi 29 is the most effective overall, followed by the PreCR™ repair enzyme mix both for their excellent results and their one tube one reaction protocol, Endo IV - T4 DNA Polymerase - T4 DNA Ligase, T4 DNA Polymerase - T4 DNA Ligase and finally the Klenow - T4 DNA Ligase method being the least effective. This ranking doesn't break down equally though between categories though. PreCR™ does excellent in oxidative damage repair but only slightly better than the Endo IV/T4 DNA Polymerase - T4 DNA Ligase and much less than the Phi 29 in the hydrolytic category. The Phi 29 method far surpasses all other methods in the hydrolytic category but its bands are extremely weak even though its recovery times are comparable to the other methods in the oxidative category. The Endo IV - T4 DNA Polymerase - T4 DNA Ligase gave the most consistent results for quality with very sharp bands in PCR gels and comparable recovery times in both hydrolytic and oxidative categories. Without DNA sequencing which is the next stage in this research the amount of base mutations that each repair method may induce is not known but the possibility for some transversions can be inferred from the presence of transversion inducing modified base products present in the final repaired product that could become base mutation later on in downstream PCR amplifications.

Ancient Samples

The ancient samples were chosen to assess the repair methods on degraded samples with different types of damage. Theoretically the Copan samples would have higher hydrolytic DNA

damage coming from the tropics, while Daklah Oasis had higher oxidative damage as demonstrated by the miscoding lesion study of Lamers et al (2009) and the Çayönü Tepesi sample was to assess older material. It has so far been the oldest human material genetically analyzed (Matheson and Loy 2001).

Phi 29 was able to fully restore the Copan samples to close to or maybe even slightly better than the undamaged positive controls. The T4 DNA Ligase method also worked on the Copan samples with bright bands and no smearing. These results support the hypothesis that these remains contain highly hydrolytic damaged DNA. However there could have been some oxidative damage present in these samples as well as hydrolytic damage. This clearly demonstrates the viability of the Phi 29 enzyme being capable of repairing ancient and degraded DNA. The T4 DNA Ligase method producing results suggests a degree of strand breaks consistent with the taphonomy of the samples.

Only PreCR™ worked on the Daklah Oasis samples and only with the smaller amplicon suggesting heavy fragmentation and oxidative damage. This confirms the previous research of Lamers et al. (Lamers et al. 2009). These samples have previously produced negative amplification results specifically chosen to assess the ability to repair the ancient and degraded DNA. The negative extraction showed a positive result with the smaller amplicon in both extraction before and PreCR™ treatment but the Daklah Oasis samples were negative except the Daklah DKT380 sample had some smearing but no bands before treatment. After treatment with PreCR™ all samples had excellent bands with no smearing.

The Çayönü Tepesi sample 9 had only the Phi 29 method work to create one strong band indicating a female result. This sample has only been analyzed by male researchers, therefore if there were any contamination present after this analysis it should have shown a male individual.

This was not the case so this result supports the reliability of the methods and controls used in this study. The sample is a skull sample from the "Skull building" at the site of Çayönü Tepesi. The morphological sex of this individual has been previously identified as a female but has previously failed to amplify due to the degraded and fragmented DNA within the sample (Matheson and Loy 2001). It is possible that this sample had highly hydrolytic damaged DNA with strand breaks due to its age and the fact that this was the only sample of ten originally analyzed that did not produce any results.

5.0 Conclusion

The use of enzymatic repair methods were tested on highly purified DNA template using different damaging methods to induce particular forms of DNA modification to test the effectiveness of current *in vitro* DNA repair methods. The degraded DNA was then analyzed for previously characterized modified base products and attempted to measure the relative repair ability against the individual modified base products for each method through PCR and GCMS.

This research was successful in indentifying and quantifying 16 individual oxidatively modified base products induced through an H_2O_2 damaging treatment and the *in vitro* repair methods relative ability to deal with these products. The reduction in the Fapy A and Fapy G modified base products and the hydantoins; 5-hydroxyhydantoin and 5-hydroxy-5-methlyhydantoin had direct correlations to quantity present in repaired sample and its ability to be successfully amplified in later PCR. There were still some transversions present in the samples after repair which probably won't have a great effect on recovery but will potentially cause misleading base pair coding errors in amplifying PCRs later on. The biggest increase was in uracil content which suggests that some of the other uracil oxidative products were being converted into uracil because of their relative decrease compared to the relative increase of uracil content in repaired samples.

One of the problems encountered was the measuring and interpretation for some of the glycol modified base data. Thymine glycol, uracil glycol, and cytosine glycol there were substantial reductions in thymine glycol which would help efficiency by removing a blocking lesion but there were increases in cytosine glycol and uracil glycol had varying amounts in the different samples depending on the method used. This was slightly suspect do to the unstable nature of the glycols and increases in some of their conversion products. Modifying the GCMS

derivitization method to a softer method with a lower derivitization temperature could provide a clearer picture by reducing chemical conversions induced by the high temperatures and possibly any residual oxygen.

Fragmentation was concluded to be the major factor to DNA recovery in dealing with strand breakage and that T4 DNA Ligase or Ligases in general are the most important factor in dealing with such.

Also in dealing with hydrolytic damage which is usually the formation of an AP site a fairly new enzyme which whose study for recovery of degraded DNA was scarce in the literature and no findings of its excellent ability to deal with AP sites induced through hydrolytic damage.

Evaluating the repair methods showed that each method had varying degrees of success depending on the damage type present. So by evaluating the damage present or making an educated guess by the environment conditions and age of sample it was recovered in and knowing what the major type of damage that would be caused in such environment one could target the repair method that would have the highest chance of success based on damage type present.

DNA repair using these *in vitro* repair methods was demonstrated successfully on several aDNA samples ranging in age from 1400-9600 years before present from different locations around the world. These samples had all had been previously attempted to be amplified without success and were deemed to contain no DNA or no viable template. The locations and environmental factors were used to predict what damage would be present and which method if any would be most likely to recover viable template. In the Copan samples recovered from Central America it was predicted to have mainly hydrolytic damage and the results were successful for the Phi 29 enzyme which was very effective on hydrolytic damage and the T4

DNA Polymerase methods also had success with the Copan samples. In the Egyptian Daklah Oasis samples, the predicted major damage present was oxidative damage and the PreCR™ mix was able to recover viable DNA template but due to excessive fragmentation the amplicon was smaller. The last sample was the Neolithic Çayönü Tepesi sample 9 and positive sex identification was successful with the Phi 29 identifying a female and confirming the morphological sex identification. The recovery of viable template in this sample where the major damage type was unknown but where the sample was considerably older than the others analyzed in this research confirms the success of the repair methods. The major form of DNA damage in this sample can be inferred from the results as hydrolytic damage. In the original simple ligation experiment between Pusch et al (1998) and Di Bernardo et al (2002) it is likely that the damage present in the ancient samples from the Alamannic burial site at Neresheim in Germany likely had hydrolytic damage due to its climate and taphonomy which would have made the Pol I method ineffective on that type of damage explaining their limited success. The Pompeii site on the other hand had much different taphonomic characteristics being dryer and slightly basic volcanic ash surrounding the samples. In addition the rapid burial of these individuals in hot ash that burnt the soft tissues off these individuals so quickly that a cast was formed around them from the vacant soft tissues demonstrates the rapid removal of water from the body a primary cause of post-mortem hydrolytic degradation of DNA. This would have protected the DNA from hydrolytic damage but would have made them vulnerable to oxidative damage. This likely explains why the Di Bernardo team had greater success with the Pol I method that is more effective on oxidative DNA damage.

So in conclusion we have identified in this study several key blocking lesions in oxidatively damaged DNA through a more sensitive GCMS technique that uses minute amounts

of template DNA which is of great importance in aDNA where samples are limited. We have successfully assessed current *in vitro* DNA repair techniques to deal with different types of damage and lastly we have successfully retrieved DNA from ancient samples previously thought to be unviable.

6.0 Future Considerations

In future studies the repair methodologies need to be optimized and refined allowing for greater sensitivity and the identification of more modified oxidatively damaged products. Using internal standards of many of these modified base products which are commercially available would also allow for the quantification of damage type as a percentage of sample rather than relative to unrepaired to repaired sample further allowing researchers to target their specific repair methods.

In particular the derivitization methodology needs to be further refined to exclude the possibility of artifacts of oxidative damage products induced through a softer derivitization method. In particular a longer time with lower temperatures would be most suitable.

A more in depth study of Phi 29 as a repair method for aDNA and its mechanisms could be of great value to recovery of aDNA researchers because this enzyme was very effective on hydrolytic damage which it is not in its reported abilities in the body of literature.

The repaired DNA in this study also needs to be sequenced to identify the transversion base mutations and try to link them to the specific base damage in the samples.

Additional enzyme combinations need to be tested and optimized for individual damage types which would allow targeting of damage by type more effectively. The biggest challenge to this is competition from competing enzymes and creating reaction conditions where all the enzymes are able to work efficiently.

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8.0 Appendix I

Table 22. DNA quantification of samples for standardization using Q bit quantification method

DNA Samples	First	Second	Third	Fourth	Fifth	Average ng/ul
Extractions 1 to 10	22.2	22.2	22.3	22.3	22.4	22.3
Extractions 11 to 20	17.9	18	18	18	18	18
Extractions 21 to 30	31.4	31.5	31.5	31.6	31.7	31.5
Extractions 31 to 40	27	27.1	27.1	27.1	27.2	27.1
Extractions 41 to 50	26.3	26.3	26.4	26.4	26.5	26.4
Damaged DNA template	17.2	17.2	17.2	17.3	17.3	17.2
Klenow	15.5	15.4	15.5	15.5	15.6	15.5
T4 Polymerase	15.1	15.1	15	15	15	15
Endo Klenow	13.1	13.2	13.2	13.3	13.4	13.2
Endo T4 Polymerase	15.6	15.7	15.7	15.7	15.8	15.7
PreCR™	22.2	22.2	22.3	22.3	22.4	22.3
Phi 29	23	23.1	23.2	23.2	23.1	23.1