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## Toward using an oxidatively damaged plasmid as an intra- and inter-laboratory standard in ancient DNA studies

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### PROLOGUE

The following paper was originally presented by Dr Thomas H. Loy at the 6th International Conference on Ancient DNA and Associated Biomolecules held in Israel, July 2002. It is included here with editorial and formatting changes with the intention of demonstrating the passion and lateral thinking that underpinned Tom's approach to the field of Molecular Archaeology. The paper represents research from three honours projects conducted during the late 1990s and early 2000s. Building a modern model for ancient DNA that could be used during routine procedures was a concept that Tom had long held as an important step forward for the burgeoning discipline. With the equipment and technology that was available at the time, the Damaged Plasmid Model concept was completely viable and worthy of detailed validation. As with all historical accounts, an understanding of more recent developments in molecular techniques and equipment will highlight the need for considerable optimisation of the model before it can be used as an inter-laboratory standard for ancient DNA.

### ABSTRACT

For some years laboratories working with ancient DNA have been optimising extraction methods using either undamaged modern DNA or authentic ancient DNA. This approach is unsatisfactory for a number of reasons, chief being the inherent variability from sample to sample. In addition, quantitative comparison of methods is generally impossible using typically small samples of ancient DNA, as well as being ethically questionable. We have now perfected a method whereby we can oxidatively damage the plasmid pUC19 using copper sulfate, ascorbic acid and hydrogen peroxide to create artificially damaged DNA that mimics the behaviour of ancient DNA. We have used this damaged plasmid to assay our extraction methods to quantitatively monitor the

yield and degree of damage induced during the extraction, purification, and storage of DNA. We suggest that the damaged plasmid can be used as a monitoring standard within the one laboratory, as we have done, and more importantly to compare yields and efficiencies between different laboratories.

## KEYWORDS

ancient DNA, plasmid, model, laboratory standard

## INTRODUCTION

The analysis of degraded, forensic and ancient (archaeological, palaeontological) DNA (aDNA) using polymerase chain reaction (PCR) amplification has demonstrated significant applications to such diverse areas as origins of domestication, palaeoepidemiology, evolution and population studies, and forensic and archaeological identification of taxa of origin from tissue, bones and blood residues. Despite these achievements, however, considerable obstacles remain to be overcome. One such obstacle is the state of degradation of aDNA samples that has prohibited the amplification of DNA fragments larger than a few hundred base pairs - making the elucidation of genetic sequences a time-consuming, and at times, impossible assignment. Damage and typically small sample sizes significantly reduce amplification yield and create a situation where extremely low-levels of copurified inhibitors and contaminants have significant detrimental effects. Evidence suggests that the major culprit in DNA degradation, especially in aDNA, is oxidative damage. This type of damage causes weaknesses in the chemical bonds that hold the nucleotides together, leading to fragmentation of DNA upon the application of heat – the first (denaturing) step in any PCR amplification (Pääbo 1989; Rogan and Salvo 1990a, 1990b).

Here, we propose that the use of deliberately oxidatively damaged modern DNA that mimics the degradation seen in ancient samples provides one avenue for increasing the reliability of conclusions drawn from aDNA studies, by presenting a standard for assessing extraction, amplification and verification of aDNA protocols and results.

## DNA DAMAGING AGENTS

To create a model system of artificially damaged DNA, an understanding of the degradative process is needed. Studying aDNA creates a problem because it is difficult to determine the amount or type of degradation exhibited by the DNA sample. A way around this issue can be through using analogies drawn from studies of modern DNA that has undergone chemical/physical alteration (i.e. damage; Rogan *et al.* 1990b). Since the issue of damaged DNA is of major concern to those studying the effects of carcinogens and mutagens, a considerable body of research exists on the types of chemical alterations to DNA and the agents that induce them.

## OXIDATION

Damage caused through oxidation is the most common form recognised. A large proportion of oxidative damage in living systems occurs through the action of hydroxyl radicals ( $\bullet\text{OH}$ ) and this also appears to be the major contributing factor in the oxidative damage of aDNA (Rogan *et al.* 1990b).  $\bullet\text{OH}$  attacks the integrity of the DNA molecule by adding to either the C5 or C6 of pyrimidines via their shared double-bond or the C4, C5 and C8 of purines (Dizdaroglu 1993). Radicals thus created are unstable and undergo further reactions in the presence of oxygen ( $\text{O}_2$ ) to form other reactive radicals. For example thymine (T), once attacked by  $\bullet\text{OH}$ , goes through a series of reactions in the presence of  $\text{O}_2$  to produce thymine peroxy radicals. The presence of radicals has the net effect of causing strand breaks in the DNA molecule (Halliwell and Gutteridge

1989). The sugar backbones of DNA can also be attacked by •OH through removal of hydrogen (H) atoms from any of the five carbons (Dizdaroglu 1993).

## HYDROLYSIS

DNA is also hydrolysed by water both contained within the DNA matrix and surrounding the molecule. Again, a large body of work exists on the effects of hydrolysis on DNA (Demple and Linn 1980; Levin and Demple 1990; Lindahl 1993; Lindahl and Andersson 1972; Lindahl and Nyberg 1972). However, it has also been shown that DNA can bind to other substrates, for example, the inorganic hydroxyapatite matrix of bone (Tuross 1994), thus reducing the effects of hydrolysis, extending the period of time in which DNA may still be recovered. It is generally believed that hydrolytic attack affects the base-sugar bonds of DNA causing the production of abasic sites (also known as apurinic/aprimidinic (AP) sites) (Doetsch and Cunningham 1990; Lindahl 1993).

## IONISING RADIATION AS AN AGENT OF •OH

A considerable number of papers discuss the effects of ionising radiation on DNA in living cells. A large proportion of the damage is thought to be caused by free radicals - particularly •OH (Dizdaroglu 1991; Steenken 1987). Because of this, the damage can be classified as oxidative (Halliwell and Aruoma 1991). Types of damage caused by radiation include alkali- and heat-labile sites (Lafleur *et al.* 1979), various base modifications, single-strand and double-strand breaks and AP sites (Breimer 1988). The alkali/heat-labile lesions are generally brought about by chemical reactions between •OH and the sugar moiety of the nucleotide (Lafleur *et al.* 1979). DNA containing such lesions, when subjected to alkaline and/or high heat environments (as in a PCR), dissociates at these positions. This phenomenon is particularly relevant and most pronounced in single stranded DNA (Lafleur *et al.* 1979). The most common forms of base modifications are guanine converted to 8'-hydroxy guanine and thymine converted to thymine glycol (Halliwell 1993; Lindahl 1993).

## CO-FACTOR DEPENDENCE

Research has also been conducted into the effects of certain chemicals upon the structure of DNA. Generally, the chemical action occurs by way of an oxidation reaction (e.g. potassium bromate [KBrO<sub>3</sub>]) (Ballmaier and Epe 1995). One substance mentioned throughout the literature is hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) - a significant source of •OH. However, research also shows that co-factors aid in the production of free radicals. Importantly, certain transition metal ions, particularly iron (Fe<sup>2+</sup>) and copper (Cu<sup>2+</sup>) (Halliwell *et al.* 1989), are necessary to catalyse these oxidative reactions (Halliwell 1993; Halliwell *et al.* 1989; Meneghini and Martins 1993). The presence of ascorbic acid in Cu<sup>2+</sup>-catalysed oxidative reactions has also been shown to increase the extent of damage.

## DAMAGE AFFECTS REPLICATION

Studies have been carried out both *in vivo* and *in vitro* to determine the effects of lesions on DNA replication. One important question centres upon whether the various types of damage cause miscoding or non-coding events during replication. That is, does damage cause the incorporation of the wrong nucleotide or does copying stop completely at such lesions? Evidence suggests, for example, that the presence of a thymine glycol causes the termination of copying *in vitro* and is lethal *in vivo*. On the other hand, the presence of 8'-hydroxy guanine residues is thought to cause miscoding but does not inhibit replication (Breimer 1988).

Under normal conditions (room temperature and pressure) damaged strands of DNA are held together because the complementary strand is often undamaged. Heat denaturation during the course of amplification causes nicked or AP portions to break into smaller single strand fragments. The result is that both template length and DNA polymerase activity are reduced. The presence of long DNA fragments extracted from ancient sources (e.g. Nielsen *et al.* 1994) cannot guarantee successful amplification of long products – a well-known phenomenon that reflects the chemical alteration of DNA, especially in regions not protected by histone or histone-like proteins within the chromatin structure (Wolffe 1995).

### **DEVELOPING A DAMAGED PLASMID MODEL**

Over the past ten years we have been using a combination of modern and ancient sources of DNA to optimise DNA extraction, purification, amplification and sequencing reactions. The advantage of using modern DNA is that usually it can be accurately quantified, allowing losses during purification and the efficiency of PCR processes to be closely monitored. The use of modern DNA, however, even in very dilute concentrations (1 fg/ $\mu$ l) does not provide a real comparison for aDNA because the modern DNA is largely intact and undamaged.

On the other hand, using authentic aDNA sources for methods development and optimisation is problematic for several reasons. First, yield is often variable and difficult to quantify – even from the same sample. Second, because of the unique relationship between taphonomic processes and sample material (soft tissue, bone and blood residue on stone tools), no two samples can be said to have undergone the same history of degradation. A general trend of increasing DNA damage can be extrapolated from our experiences with extraction, purification and amplification with ancient samples. Blood residues generally provide longer sequences of intact DNA, followed by bone, while mummified tissue often contains the most nucleic acid modification (damage). Third, archaeological and forensic samples have intrinsic value that introduces a serious ethical concern regarding their use for purposes such as optimisation and quantification experiments. Finally, issues revolving around the detection and prevention of contamination events have cast doubts on the veracity of many aDNA analyses. Current practice dictates the repetition of key experiments at independent laboratories. While admirable in intention, the requirement necessitates the production of both identical laboratory conditions and utilisation of common methodologies, requirements that are often beyond the capacity of isolated research teams.

Developing and using a modern damaged DNA model that is representative of aDNA provides a multi-locational means for testing protocols and experimental hypotheses that could not, and indeed should not, be undertaken on ancient specimens. The current paper presents results on the production and use of a Damaged Plasmid Model (DPM). Our aim was to produce a reliable mimic for authentic degraded DNA samples that could be easily substituted into current laboratory procedures.

### **METHODS**

The plasmid pUC19 was chosen as the model to better understand and characterise the damage to aDNA because its sequence is known, it can be produced in abundance at high purity, and amplification primers are easily designed.

### **DNA DAMAGE REACTION PROTOCOL**

Linearised plasmid DNA is oxidatively damaged in 20  $\mu$ l reactions. The  $\bullet$ OH that damage the DNA are generated by a combination of  $H_2O_2$ ,  $CuSO_4$  and ascorbic acid. The reaction is stopped on ice and with the addition of EDTA (Aruoma *et al.* 1991; Prutz 1990). The extent of damage can be controlled by altering the concentration of  $H_2O_2$  and the incubation time.

## PURIFICATION PROCEDURES

### 1. Ethanol precipitation

A solution containing the DNA sample to be purified, 1/10 volume 3 M sodium acetate and 2 volumes 100% EtOH was incubated at -70°C for 30 min. The solutions were centrifuged at 15,000 X g in a cool room (4°C) for 30 min. The supernatant was removed and pellets were washed in 100 µl 70% EtOH. A further centrifugation was performed for 15 min. The second supernatant was removed, pellets were air-dried and resuspended in Milli-Q water to an equivalent volume as the original DNA sample.

### 2. Sephadex G50 columns

DNA in solutions was purified using Amersham G-50 Sephadex columns, in accordance with the manufacturer's directions.

## POLYMERASE CHAIN REACTION

### 1. Enzymes and Primers

AmpliTaq DNA Polymerase, Stoffel Fragment (10 U/µl) was used for PCR amplification. Five plasmid primers were designed using both MacVector and Amplify 1.2 software. The sequences are:

pUC19A forward	5'-GGCCGAGCGCAGAAGTGGTC-3'
pUC19B reverse	5'-GCAATGGCAACAACGTTGCG-3'
pUC19C reverse	5'-ACAACATGGGGGATCATGTA-3'
pUC19D reverse	5'-CGCCGGGCAAGAGCAACTCG-3'
pUC19E forward	5'-GCAAGCAGCAGATTACGCGCAG-3'

Amplification of the following products is possible; (i) 823 bp with primers pUC E and pUC D; (ii) 420 bp with primers pUC A and pUC D; (iii) 217 bp with primers pUC A and pUC C; and (iv) 122 bp with primers pUC A and pUC B.

### 2. Amplification protocol

PCR was performed at 50 µl final volumes. To 10 µl volumes of DNA in solution (variable concentrations) 2.25 mM MgCl<sub>2</sub>, 1x PCR buffer, 0.2 mM combined dNTPs, 30 pM primers (5' and 3'), 0.03 U/µl Taq DNA polymerase and milliQ H<sub>2</sub>O were added. PCR amplification for plasmid DNA consisted of 36 cycles with an initial cycle of 95°C for 3 min, 58°C for 20 seconds and 72°C for 2 min. The remaining cycles consisted of 95°C for 15 seconds, 58°C for 10 seconds and 72°C for 2 min. A final extension period (72°C) was 4 min before holding at 4°C.

## EXTRACTION PROCEDURES:

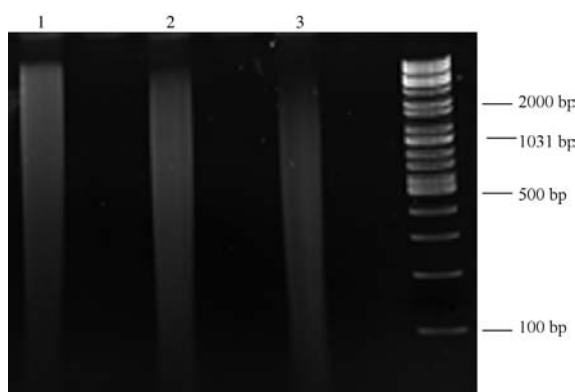
The extraction buffer III (EBIII) extraction method was developed by one of the authors and has been described previously (Matheson and Loy 2001). The sample is incubated at 57°C in a solution of EBIII (10 mM Tris-HCl, pH 8.0; 100 mM NaCl, 250 mM EDTA, pH 8.0; 1:20 10% SDS; 0.45% NP-40; 0.45% Tween-20; 0.45% Triton X-100; 100 mM DTT) and proteinase K (25 µg/ml) for 8 hours. The DNA is then purified using a SpinBind silica membrane column.

The EBIIIβ extraction method is based on the same buffer used in the above extraction. However, this buffer contains β-mercaptoethanol in addition to the other ingredients. The presence of this chemical facilitates the dissociation of DNA from proteins by breaking the di-sulfide bonds. The sample and buffer are incubated at 90°C for 45 minutes. The DNA is then purified using SpinBind silica membrane columns, as in the EBIII extraction method.

## RESULTS AND DISCUSSION

### *The Damaged Plasmid Reaction*

Using the techniques described above, the plasmid can be damaged to varying degrees. Published data on the extraction of degraded DNA consistently describe samples that contain a large proportion of low molecular weight fragments but also contain a small proportion of high molecular weight sequences (Cherry 1994; DeSalle and Grimaldi 1994; Handt *et al.* 1996; Pääbo 1989, 1990). The current Damaged Plasmid Model successfully mimics both the range and the relative proportions of DNA fragments found in ancient and forensic DNA samples (Figure 1). The fragment sizes present in the plasmid (Figure 1, lane 3) range from 2000 bp to less than 100 bp. Given that the total size of pUC19 is roughly 3000 bp, the damaged DNA size range is similar to the range of fragment sizes seen in other published aDNA extraction data (e.g. Nielsen *et al.* 1994).



**Figure 1. Effects of changing  $H_2O_2$  concentration and/or time of incubation on the extent of damage to the plasmid. The increased extent of damage seen in lanes 2 and 3 when compared with that in lane 1 reflects the effects that different reaction conditions can have on the damage reaction. Polyacrylamide 4-20% gradient gel.**

Plasmid digestion prior to the damage reaction was an important innovation that removed the possibility of accidental retention of supercoiled DNA. An accurate model required the retention of a long smear of DNA including fragments of  $\geq 1000$  bp. The presence of supercoiled DNA in the smear would have introduced an unidentifiable structural variable into the reaction, complicating the results and reducing the model's merit.

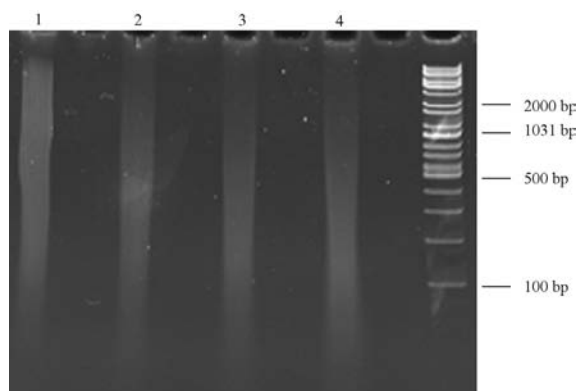
Increasing the concentration of  $H_2O_2$  in the damage reaction or lengthening the period of incubation has the effect of altering the ratio of long versus short fragments in the resultant damaged DNA (Figure 1). Altering these two variables allows the extent of damage to be controlled. Decreasing the concentration of DNA has a similar effect (data not shown).

### *The effect of heat on damaged DNA*

The first step of any PCR reaction is heating to denature the DNA. This step causes DNA with single strand breaks to fragment. Oxidatively damaged DNA (like that present in the DPM and in aDNA) contains many heat-labile sites that form single strand breaks upon heating. Therefore the first heating step of PCR causes greater fragmentation of the already damaged aDNA. Heating of the damaged plasmid results in a shift of the range of fragment sizes present (Figure 2). Many PCR reactions use an extended heating step at the beginning of the reaction. As can be seen in Figure 2, extended initial heating decreases the amount of longer DNA fragments. The damaged plasmid was also used to assess the effect of longer PCR cycles on the template DNA. The amount of high molecular weight fragments is significantly reduced following a longer PCR reaction.

### *The effect of concentration on amplification*

Dilution series PCR analysis provides a striking illustration of the amplification differences between undamaged and damaged DNA. The results indicate that while low molecular weight fragments can be amplified from degraded DNA samples - even at very low concentrations ( $0.1 \times 10^{-6} \mu\text{g}/\mu\text{l}$ ), high molecular weight sequences are only amplified from undamaged DNA. The precise reason for this phenomenon is not known but is thought to be derived from the types of oxidative damage known to occur in aDNA. Single strand nicks in the degraded, double stranded



**Figure 2. Effects of heating/PCR on damaged DNA. Heating the damage reaction to 95°C results in denaturation and strand breaks at heat-labile sites caused by oxidative damage. The unheated, damaged DNA in lane 1 has a relatively high molecular weight compared with the damaged DNA in lane 2 that has been heated to 95°C for 5 minutes or that in lanes 3 and 4 that have been subjected to the heating and cooling cycles of a short and long PCR reaction (respectively). Polyacrylamide 4-20% gradient gel.**

these results is striking and is further support for the Damaged Plasmid Model as a valid tool for understanding damaged DNA.

## USING THE DAMAGED PLASMID MODEL TO ASSESS EXTRACTION TECHNIQUES

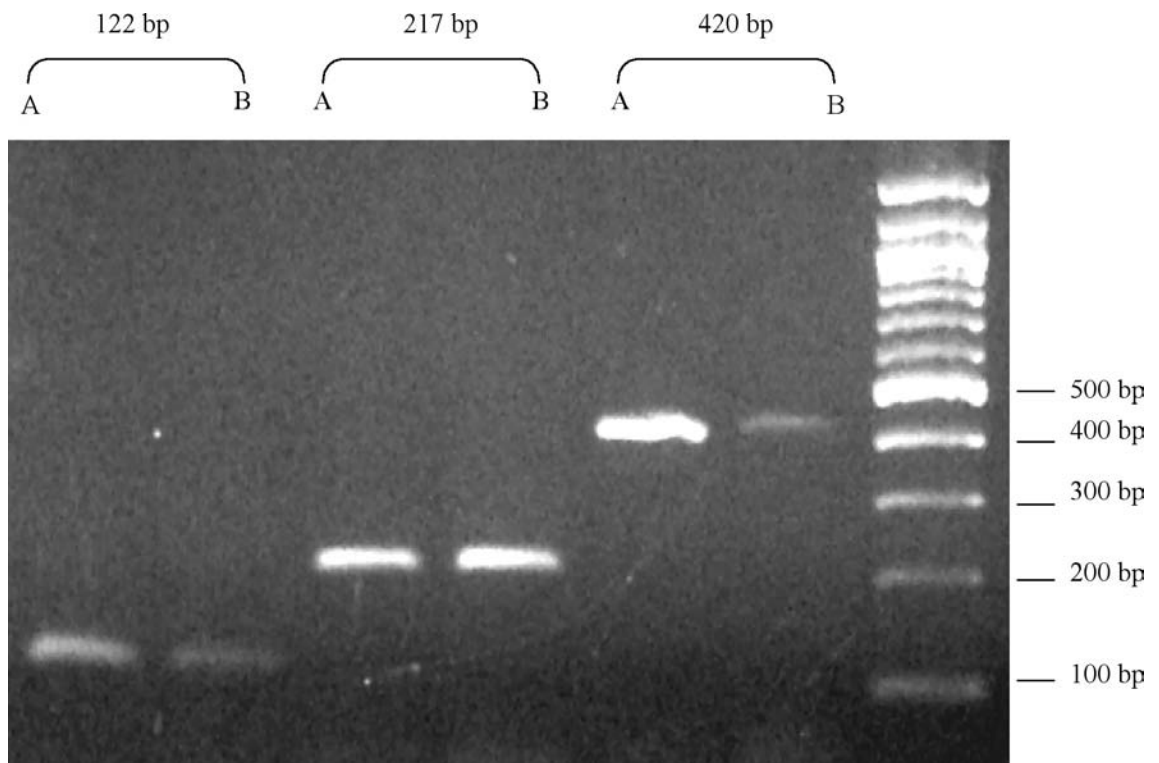
As an example of how the DPM can be used to assess the damage caused to DNA by routine laboratory procedures, a modification to a routine extraction method used in the University of Queensland Archaeological Sciences Laboratory for many years was assessed. Recently  $\beta$ -mercaptoethanol has been used as an additional ingredient in the EBIII (see methods) extraction buffer due to its ability to dissociate proteins and disulfide bonds. The modified method has produced successful results in extracting DNA from ancient samples (Loy, unpublished data).

To fully characterise the new buffer (EBIII $\beta$ ) and the effect it has on DNA, side-by-side extractions were carried out on both damaged and undamaged pUC19. Each extraction used 8 ng of substrate DNA. The extractions were assessed via PCR. While the original EBIII buffer allows amplification of up to 400 bp of damaged DNA following extraction, the EBIII $\beta$  buffer and method appear to cause more damage to the DNA (Figure 4). In comparison, undamaged DNA subjected to either extraction buffer could be amplified. The results indicate that while the addition of  $\beta$ -mercaptoethanol produces a buffer that is effective for separating aDNA from its encompassing organic matrix, care must be exercised in order to minimise further damage to the already fragile DNA.

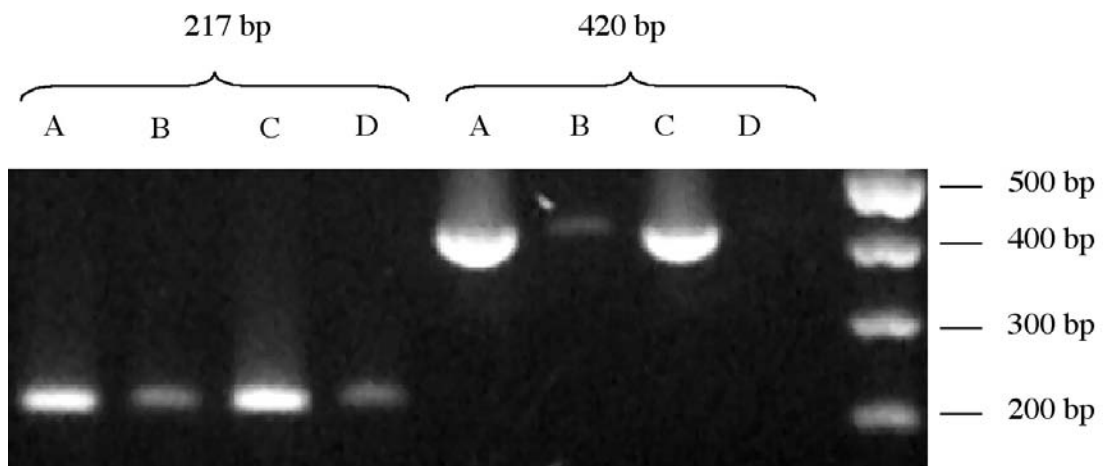
## CONCLUSIONS

Using techniques designed to cause predictable oxidative damage to modern DNA, it has been possible to create a Damaged Plasmid Model that accurately and repeatably mimics extraction and amplification results commonly obtained from aDNA. Damage present in the DPM is consistent with damage caused by hydroxyl radical attack that produces single strand nicks and AP sites in DNA.

DNA are 'invisible' after extraction since the short lengths of DNA between nicks are held together by their complementary base pairs. denaturing the DNA during PCR causes the strands to 'fall apart' at the nicked sites, leaving only short strands available for amplification (see Figure 2). Compounding the amplification problem is the ratio of long to short fragments of extracted aDNA. Since shorter pieces are in relative abundance, they generally will be preferentially amplified during any PCR, which dramatically reduces the likelihood of detecting longer DNA sequences. The compilation of amplification results for damaged and undamaged plasmid DNA at 0.02 pg/ $\mu$ l (Figure 3) can be compared directly with Pääbo's (1990) amplifications of mitochondrial DNA from 7,000 year old and modern human brains. The same pattern can be seen in his results as in both the Damaged Plasmid Model amplifications, where low molecular weight fragments can be retrieved from degraded DNA but not higher molecular weight sequences. The similarity between



**Figure 3. Amplification of the damage reaction.** The damaged plasmid can display the same characteristics of aDNA when it comes to PCR. Like most aDNA samples (e.g. Pääbo 1990) the fragment of higher molecular weight cannot be amplified from the damaged plasmid. Here 20 fg of undamaged (A) or damaged (B) DNA was amplified using primers targeting fragments of increasing size.



**Figure 4. Using the DPM to Assess Extraction Techniques.** 8 ng of either damaged or undamaged pUC19 was extracted and purified with two extraction methods (EBIII and EBIII $\beta$ ) commonly used in the UQ Archaeological Sciences Laboratory. DNA fragments of sizes up to and including 420 bp could be amplified from undamaged extracted plasmid when either EBIII (A) or EBIII $\beta$  (C) was used as the extraction method. However, only the EBIII method allowed amplification of all fragment sizes when the damaged plasmid was used as substrate (B). When EBIII $\beta$  is used to extract damaged plasmid DNA only the 217 bp fragment could be amplified (D).



Distinct advantages are conveyed by using the DPM, including (i) the original size and sequence of the plasmid are known; (ii) the types of damage are controlled; (iii) any quantity of damaged plasmid DNA can be produced and used; and (iv) the plasmid DNA has no forensic or cultural significance and is thus available for fundamental experimentation, unlike archaeological or forensic material.

We propose that the Damaged Plasmid Model presents an important step forward in providing feasible tools for inter- and intra-laboratory assessments of current damaged DNA extraction and amplification protocols. We also suggest that with further development the model could potentially be used as a verification standard against which authentic degraded DNA results can be measured.

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**terra australis 30**

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**ARCHAEOLOGICAL SCIENCE  
UNDER A MICROSCOPE**

Studies in Residue and ancient DNA Analysis  
in Honour of Thomas H. Loy

Edited by Michael Haslam, Gail Robertson,  
Alison Crowther, Sue Nugent and Luke Kirkwood





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Dr Thomas H. Loy (1942-2005)

# 1

## PREFACE

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Dr Thomas H. Loy (1942-2005) was a master storyteller, an innovative archaeologist and an inspiring teacher. He was equally at home walking a survey line in the red dust of northern Australia as he was enthusiastically lecturing on starch identification to an undergraduate audience, or sitting back with a cold beer spinning tales of escapes from helicopter crashes and bear attacks in frozen Canada. With an Apple Mac and a microscope always somewhere close by, Tom dedicated his working life to understanding the world around him by systematically examining the details that others may have overlooked, and inventing new methods of doing so if none existed. His sudden passing in October 2005 deprived us of the opportunity to present these papers to Tom personally, but both the diversity of research represented and its global coverage stand as testament to an enduring legacy, appropriate for an archaeological pioneer who demonstrated the value of examining the smallest traces for answers to the biggest questions.

The genesis of this collection was a symposium held to honour Tom's memory on 19 August 2006 at the University of Queensland, Brisbane, where he was Senior Lecturer in the School of Social Science. Papers delivered at that event have been augmented with invited contributions from colleagues not able to be present on the day. The symposium was memorable for the breadth of research presented (from residues on the tools of *Homo floresiensis* to the DNA of Henry VIII's warship) as well as for a stone knapping demonstration and discussion by Colin Saltmere of the Dugalunji Aboriginal Corporation, with whom Tom had established a strong friendship through his final field project near Camooweal in northwest Queensland. Following Tom's lead, many of the presentations recognised the responsibilities we as archaeologists bear towards both the past and present people that we deal with, a responsibility that requires vigilance in getting our stories straight. That recognition continues in the pages that follow. The central theme of this volume lies in using the detailed information recovered from microscopic and molecular archaeology to tell the most accurate stories we can about the human past, and doing so in a manner that encourages never-ending inquiry about the further avenues we may follow.

Beginning with a reproduction of the keynote address given by Richard Fullagar at the symposium, the volume is divided into two main sections. The first is titled 'Principles: synthesis, classification and experiment' and includes overviews and experimental or collection-based studies that aim to strengthen the fields of microscopic residue and ancient DNA analysis by examining the underlying principles on which these disciplines operate. In soliciting papers, the editors aimed to present an integrated if broad snapshot of the microscopic residue analysis field as it stands in the first decade of the twenty-first century. The synthesis of recent South African work provided by Lombard and Wadley provides a clear indication of the promise for residue studies to contribute to the important issues of human evolution, including the advent of hunting and the definition of modern human behaviour. From an historical perspective, Haslam's review of microscopic residue study sample sizes likewise stresses the need for thoughtful application of residue results if the field is to reach its full potential.

Specific residue types such as starches, raphides and blood proteins all played prominent roles in Tom's career as a residue analyst. This work is continued, first in the investigations of Jones and Barton into residue taphonomy over timescales from weeks to millennia, and second by Lentfer and Crowther in establishing archaeobotanical databases that will bring new rigour to discussions of past plant-use practices in Indonesia and the Pacific Islands. Watson *et al.* report on a decade-long search by Tom Loy and his students for an ancient DNA analogue to use in maintaining inter-laboratory standards (a topic continued by Hlinka *et al.*), emphasising again both respect for ancient remains and the need for procedural scrutiny to ensure reliable results.

The second section, titled 'Practice: case studies in residue and ancient DNA analysis', presents a series of studies with coverage from Europe to the Americas and Australasia. The full gamut of microscopic residue work is on display, from rockshelters (Hardy and Svoboda; Robertson) to open sites (Cooper and Nugent; Fullagar *et al.*) and private collections (Field *et al.*), and a number of stone tool types and materials receive close attention. An integrated battery of tests employed on Mesoamerican ceramic artefacts by Matheson *et al.* mirrors those used by Tom in his own early blood protein work, and reveals possible ceremonial use of the examined vessels. All these studies demonstrate the importance of detailed specialised analyses for adding social value to objects either newly or previously recovered, a theme continued in the DNA studies of museum pieces large and small by Spiers *et al.* and Hartnup *et al.*. There are few places in the modern world where you can still hunt the mighty moa, and the ancient DNA laboratory is one of them.

Reflecting on the work in this collection it is evident that one of the central strengths of residue and ancient DNA analyses lies in bringing rigorous yet innovative science to bear on otherwise intractable problems. That said, amongst the researchers reporting here there appears little room for science done for its own sake, and the anthropological and humanist underpinnings of archaeology are very much apparent. For example, the DNA case studies echo Tom's knack of identifying a specific topic of interest to modern audiences and then using that starting point to address wider issues. Microscopic residue analysis has yet to experience the same global explosion of output as ancient DNA research, but even in this selection important commonalities are emerging. One of these is the influence of rapid desiccation of residues as a significant aid to their long-term preservation, a point reiterated in various contexts by Jones and Matheson *et al.* regarding blood proteins, Barton for starches and Cooper and Nugent for a variety of residues. Identifying these common outcomes allows for future targeted research agenda, and the approaches demonstrated in these pages take important steps towards such coordinated effort.

Much was said about Tom Loy during his lifetime, and we will conclude by saying just one thing more: Tom was not always right, and he knew it. He also knew that the best way forward was to use his generosity and enthusiasm for revealing past human lives to inspire others to find the answers he did not have the time to find himself. This volume is testament to the success of that vision.

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upon the work that forms a career. For those who worked with and learned from Tom, and those who will never meet him, we hope that there remains something of his inquiring spirit within these pages.