The effect of radioactive materials on forensic DNA evidence: Procedures and interpretation

Serena F. Abbondante

A thesis submitted for the degree of Doctor of Philosophy

University of Canberra

2009

A research collaboration between
the University of Canberra, the Australian Federal Police and the Australian Nuclear Science and Technology Organisation

Supervisory Panel:
Christopher Lennard, University of Canberra
Cindy Lim, Australian Federal Police
David Hill, Australian Nuclear Science and Technology Organisation

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Acknowledgements

To my supervisors, Prof. Chris Lennard, Mr. David Hill, Dr. Cindy Lim and Prof. Bill Maher, thank you so much for your expertise, commitment and support. Each of you has provided invaluable guidance and much appreciated encouragement – thank you. Thank you also to Prof. Jennelle Kyd and Dr. Stuart Thomson for introducing me to the world of radiological forensics and starting me on my PhD journey.

I would like to sincerely thank the University of Canberra, the Australian Federal Police, the WJ Weeden Family, the Australian Nuclear Science and Technology Organisation and the Australian Institute of Nuclear Science and Engineering for providing financial support without which this research would not have been possible. A special thanks also to the management of Australian Federal Police, Forensic and Data Centres for being so generous in providing time, laboratory space and resources for my study.

This research was a true collaborative effort and would not have happened without the expertise, dedication and support of individuals from the University of Canberra, Australian Federal Police and the Australian Nuclear Science and Technology Organisation.

I would particularly like to thank Dr. Simon Walsh and everyone in the Biological Criminalistics family – thank you for your encouraging words, flexibility with your time and the instruments, and for just being a wonderful bunch of people. You have all made my PhD experience so much richer.

To Dr. George Koperski and everyone at the CBRN Data Centre thank you so much for your support and encouragement throughout the final stages of my PhD.

To Mr. Michael Colella your specialist knowledge and advice has been invaluable. A special thanks also to Mr. Robert Chisari for providing much appreciated advice and on-

going support, organising my caesium-137 experiments, and for reading through my thesis.

Thank you also to Mr. Henri Wong, for your expertise in ICP-MS analysis and microwave-assisted digestion, Dr. Rainer Siegele and Mr. Ed Stelcer for their wealth of knowledge and invaluable assistance with STAR, and Connie Banos and everyone at GATRI for irradiating the samples.

A special thank you must also go to Assoc. Prof. Paul Smith of the Trauma and Orthopaedic Research Unit and the Ms. Allison Banks of the ACT Bone Bank for coordinating bones samples for my research.

Many thanks to Dr. Paul Roffey for your constant encouragement and very welcome chats, and to Dr. Dennis McNevin for your helpfulness and much appreciated feedback.

A thank you must also go to Michelle, Ali and Ezequiel for showing me the finer arts of cell culturing and also to the HEp-2 cells for showing resilience in the face of distain and neglect.

To my wonderful family, Mum, Dad, Rosanne and Tony, Michael and Monica (and Ruby), thank you for your love and never-ending encouragement – and to my new little nephews, Sebastian and Sam, you are two of the most wonderful distractions I could have hoped for.

And finally to my Gino – you have been with me, supporting me, from day one of my research and you are (to my amazement) still here at the end. Your patience, kindness and unending belief in me kept me going – Oh, and my shoes hurt too!

Abstract

This research sought to investigate the impact that radioactive materials may have on the analysis and interpretation of forensic DNA evidence. Experiments were designed to examine the effects of ionising radiation, specifically gamma and alpha radiation, on the DNA profiles of forensically-relevant biological matrices. In addition, this work explored issues of both sample contamination and the management of radioactively-contaminated evidence in order to provide risk minimisation guidance for the forensic analyst and for the laboratory environment.

Initial experimentation investigated the effects of γ-radiation, from a cobalt-60 source, and alpha particles, from a particle accelerator, on DNA from a range of biological matrices. From the experiments, the potential impact of time-to-analysis on the qualitative and quantitative aspects of DNA analysis was examined, in addition to establishing limits of exposure for successful profiling. The purpose of the experiments was to obtain an insight into the stability of the DNA sample post-irradiation, as well as address concerns regarding sample integrity and dose thresholds for DNA degradation.

The pattern of DNA profiling results obtained for blood, saliva, bone and standard genomic male DNA following gamma-irradiation demonstrated a progressive loss of the higher molecular weight loci as the radiation dose increased (from 0 to 100,000 Gy). One of the largest target fragments, D18S51 (264-344 bps), was typically absent at both the 50,000 and 100,000 Gy doses. These observations reflect the typical pattern of degraded DNA, where the longer fragments present a greater opportunity for interaction with ionising radiation than the shorter fragments. It is proposed that degradation of the DNA molecule in these cases is likely due to fragmentation of the DNA strand, in addition to inter-strand cross-linking, deamination and dimer formation.

This trend was also evident in the findings from the alpha irradiation of blood, saliva, and a human epithelial cell line, HEp-2 (with doses ranging from 0 to 26,400,400 Gy). DNA profile degradation was observed across all matrices at doses at and above

66,000 Gy. Allelic dropout was again observed as first occurring at the higher molecular weight loci.

With regards to time-to-analysis, general trends in the data suggest a marginal reduction in DNA profiling response for the gamma-irradiated samples over time, especially between 1-day and 1-week post-irradiation. Therefore, if possible, steps should be taken to process samples within this timeframe. The data was more difficult to interpret for alpha-irradiated samples, although general trends over the three time periods suggest a reduction in response as the time-to-analysis increased.

The findings from these experiments demonstrate that gamma-irradiated biological matrices are relatively robust for DNA analysis; little degradation was observed up to an exposure of 1,000 Gy for all samples tested, and it was possible to obtain a full DNA profile at doses at least up to 10,000 Gy. Alpha-irradiated samples proved even more robust at significantly higher doses, an effect likely due to the limited penetrability of the alpha particle. Where observed, the effects of ionising radiation on DNA appear to be consistent with other degradative processes. Therefore, current standard operating procedures used for the interpretation of profiles from degraded DNA can be applied if exposure of the samples to radiation has occurred.

This research also critically examined the DNA extraction step to investigate methodologies capable of both effective decontamination of the sample and recovery of purified DNA for downstream profiling. DNA IQ™ and ChargeSwitch® solid-phase extraction systems, as well as conventional Chelex^{100} resin extraction, were investigated for their effectiveness in the removal of non-radioactive caesium-133 salt. In addition, the contaminant was characterised for its potential interference with DNA extraction efficacy. Confirmatory studies were then conducted using the corresponding radioactive caesium-137 species, with special attention given to establishing guidelines for safe working practices.

Both the DNA IQ™ and ChargeSwitch® solid-phase extraction systems proved particularly effective for the purification of DNA samples contaminated with the

representative non-radioactive caesium-133 (>99.95% removal for DNA IQ™ and 99.99% for ChargeSwitch®, compared to 98.8% for Chelex^{100}/Microcon® extraction). The findings demonstrate that contamination of the samples with caesium-133 did not result in any significant effects on the quantitation, amplification or profiling of DNA at the concentrations tested. DNA profiling results from all the contaminated samples were consistent with those from the control samples, with no significant effect of the contaminant at the targeted loci/alleles. The amount of remaining caesium-133 in the extraction eluants was extrapolated to reflect dose rates of radioactive caesium-137 (in μSv/h), which demonstrated that both the DNA IQ^{TM} and ChargeSwitch® protocols significantly reduced dose rates compared to the Chelex^{100}/Microcon® extraction. Therefore, numerous extracted samples from the DNA IQ™ or ChargeSwitch® systems could be handled before the dose rate limit of 0.5 μSv per work hour for a non-radiation worker is exceeded.

The results of the radioactive caesium-137 experimental series confirmed the extraction and decontamination efficacy of these systems, as both the DNA IQ™ and ChargeSwitch® extraction protocols proved capable of removing contamination by caesium-137 at the levels tested. In addition, the presence of caesium-137 did not affect the capability of either protocol to obtain a sample of DNA suitable for profiling. From extraction, the activity was reduced to levels approaching zero, with corresponding exposure dose rates being negligible. The efficiency data generated can be used to provide guidance when planning for the volume of samples processed and the maximum exposure time per laboratory analyst. Workplace risks may be further mitigated through the use of appropriate personal protective equipment, exposure monitoring, contamination monitoring, and waste disposal.

This innovative research has contributed to an improved understanding of the effects of ionising radiation on forensic DNA evidence. The findings have revealed the capabilities of select extraction systems in meeting the practical needs of forensic laboratories preparing standard operating procedures for investigations involving radiological incidents. The study has also provided valuable insights into associated operational procedures.

Abbreviations

A	Adenine
Å	Angstrom
AFP	Australian Federal Police
Am-241	Americium-241
AmelX	Amelogenin - X chromosome
amu	Atomic mass units
ANSTO	Australian Nuclear Science and Technology Organisation
ARPANS	Australian Radiation Protection and Nuclear Safety
BHQ1	Black Hole Quencher 1
Bq	Becquerel
C	Cytosine
CE	Capillary electrophoresis
Ci	Curie
Co-60	Cobalt-60
Cs-137	Caesium-137
CTAB	Cetyltrimethylammonium bromide (buffer)
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DVI	Disaster victim identification
EDTA	Ethylenediaminetetraacetic acid
ENSDF	Evaluated Nuclear Structure Data File
eV	Electron volts
FAM	N-(3-Fluoranthyl) maleimide (fluorescent dye)
FBI	Federal Bureau of Investigation (USA)
G	Guanine
GAO	Government Accountability Office (USA)
GATRI	Gamma Technology Research Irradiator (ANSTO)
GBq	Giga Becquerel
gDNA	Genomic DNA
Gy	Gray
h	Hour

Abbreviations (continued)

HEp-2	Human epithelial cell line
HPGe	High purity germanium (detector)
IAEA	International Atomic Energy Agency
ICP-AES	Inductively-coupled plasma - atomic emission spectrometry
ICP-MS	Inductively-coupled plasma – mass spectrometry
ICRP	International Commission of Radiological Protection
kDa	Kilo Daltons
keV	Kilo electron volts
KGB	Komitjet Gosudarstvjennoj Bjezopasnosti (Former Soviet Union's Committee for State Security)
kGy	Kilo gray
Laminar flow hood	Class II laminar flow hood
LCN	Low copy number (STR)
LD_{50}	Dose lethal to 50% of sample population
LET	Linear energy transfer
M	Molar (molarity)
MeV	Mega electron volts
mGy	Milli gray
miniSTR	Mini Short Tandem Repeats
mL	Millilitre
n	Neutron
nm	Nanometer
NNDC	National Nuclear Data Center
NRC	Nuclear Regulatory Commission
OPAL	Open Pool Australian Lightwater
PBS	Phosphate-buffered saline
PCIA	Phenol/chloroform/isoamyl alcohol
PCR	Polymerase chain reaction
PMMA	Polymethylmethacrylate
PPE	Personal protective equipment
Pu-238	Plutonium-238

Abbreviations (continued)

qPCR	Quantitative polymerase chain reaction
RDD	Radiological dispersion device
RED	Radiological emission device
RFLP	Restriction fragment length polymorphism
RFU	Relative fluorescence unit
RNA	Ribonucleic acid
SNP	Single nucloetide polymorphism
SOP	Standard operating procedures
SPE	Solid-phase extraction
Sr-90	Strontium-90
STAR	Small Tandem Accelerator for Research (ANSTO)
STR	Short tandem repeats
Sv	Sievert
T	Thymine
TE	Tris-EDTA Buffer
TFM™	Tetrafluorrmethaxil™
UNSCOM	United Nations Special Commission
UV	Ultraviolet (radiation)
VNTR	Variable number tandem repeats
YSTR	Y chromosome short tandem repeats
α	Alpha (particle) radiation
β	Beta (particle) radiation
γ	Gamma (ray) radiation

Presentations

Conferences and Workshops

Abbondante, S.F., Hill, D., Maher, W.A., Lim, C., Kyd, J., Lennard, C. (2007). The effect of radioactive materials on forensic DNA evidence: procedures and interpretation. The 15th Australian Conference on Nuclear and Complementary Techniques of Analysis, Melbourne, Australia. Oral presentation - awarded Best Presentation in Open Category.

Abbondante, S.F. (2007). Investigation of the impact of radioactive contaminants on the analysis of forensic biological evidence. Australian CBRN Data Centre Research Workshop, Australian Federal Police Forensic Services, Canberra, Australia. Oral presentation.

Abbondante, S.F. (2007). Investigation of the impact of radioactive contaminants on the analysis of forensic biological evidence. Australian Federal Police Quarterly Research and Development Workshop, Australian Federal Police Forensic Services, Canberra, Australia. Oral presentation.

Abbondante, S.F., Thomson, S. Maher, W.A., Creagh D., Kyd, J.M. (2006). Extraction of radiologically contaminated DNA evidence. Australian and New Zealand Forensic Science Society, Fremantle, Australia. Poster presentation.

Abbondante, S.F. (2006). Extraction procedures and the effect of radioactive materials on biological forensic evidence. Australian Federal Police Quarterly Research and Development Workshop University of Canberra, Australia. Oral presentation.

Abbondante, S.F. (2005). Extraction procedures and the effect of radioactive materials on biological forensic evidence. Australian Federal Police Quarterly Research and Development Workshop University of Canberra, Australia. Oral presentation.

Presentations (continued)

Abbondante, S.F. (2005). Extraction procedures and the effect of radioactive materials on biological forensic evidence. Divisional Research Institute Postgraduate Research Corroboree, University of Canberra, Australia. Oral Presentation.

Abbondante, S.F. (2004). Radiologically-contaminated evidence: Required sampling procedures and the effect of radioactive materials on traditional forensic evidence. Australian Federal Police Quarterly Research and Development Workshop, Australian Federal Police Forensic Services, Canberra, Australia. Oral presentation.

Table of Contents

List of Figures

List of Tables

Chapter 1: Literature Review

This chapter will provide relevant and important background information that outlines the basis for this research. Contextualisation of the research project will be provided with a discussion of the current radiological threat and related issues that are faced by the forensic science community, specifically within the field of forensic deoxyribonucleic acid (DNA) analysis.

The literature review in this study will focus on techniques involved in the analysis of forensic DNA evidence, particularly with regard to the impact that radioactive materials may have on these procedures and on the interpretation of results. A focused discussion will be provided on biological matrices, to include the types of specimens commonly encountered in the forensic environment, and factors influencing evidentiary processing.

DNA extraction techniques will be discussed for their application to radioactive contaminant removal, including Chelex 100® resin with Microcon® filtration, organic extraction, and solid-phase extraction. In addition, safety considerations for the analyst working with radiologically-contaminated evidence and in a radiologically-contaminated work environment will be reviewed.

The information presented in this chapter will provide the justification for investigating the impact that radioactive materials will have on DNA evidence and on the analysts performing each step of the DNA profiling process.

1.1 Project design

1.1.1 The purpose of this research

The events of 11 September 2001 set the scene for a global change, not only in day-to-day living but also in perceptions of risk and threat. Five years after these tragic events devastated the United States; al Qaeda again changed the face of modern day terrorism. In 2006, the leader for al Qaeda in Iraq, Abu Hamza al-Muhajir, also known as Abu Ayyub al-Masri, called for “experts in the fields of chemistry, physics, electronics,

media and all other sciences -- especially nuclear scientists and explosives experts" to join the group's holy war with the promise that "the field of Jihad can satisfy your scientific ambitions and the large American bases [in Iraq] are good places to test your unconventional weapons, whether biological or dirty, as they call them" (Rising 2006).

As disturbing as these statements were, they also prompted the forensic science community to address the need for a greater understanding of the impact that these unconventional weapons might have on forensic evidence and criminal investigations, as well as the implementation of personal protection strategies in the forensic science arena.

Of particular interest for this research is the impact that an unconventional weapon incorporating radioactive material, such as a Radiological Dispersion Device (RDD), may have on analytical processes and the interpretation of forensic DNA evidence.

1.1.2 The structure of the research

This research begins with an investigation of the impact of ionising radiation on the DNA profiles of forensically-relevant biological matrices. The effects of electromagnetic gamma radiation is explored and characterised with regard to blood, saliva and bone specimens, in addition to a genomic DNA control (Chapter 2). Furthermore, the effects of alpha particle radiation are also investigated concerning blood, saliva and a human epithelial cell line, HEp-2 (Chapter 3). Chapters 2 and 3 address concerns regarding sample integrity and dose thresholds, and also provide insight into stability of the DNA sample post-irradiation.

Chapters 4 and 5 address issues of sample contamination and the impact that radioactive material will have on the forensic analyst in a working laboratory. Chapter 4 presents an examination of traditional and novel DNA extraction methodologies for their effectiveness in removing a contaminating caesium-133 salt, a representative non-radioactive material, via analysis with inductively-coupled plasma mass-spectrometry

(ICP–MS) and extrapolation of dose rates. Furthermore, the novel extraction methods were investigated for the non-radioactive caesium's potential interference with extraction efficacy. Confirmatory studies were then conducted using the corresponding radioactive material with the novel extraction techniques, and sample extraction and decontamination efficacy was determined (Chapter 5).

1.2 Radioactive Dispersion Devices (RDD)

Since the break-up of the Soviet Union in 1991, there has been a rise in international concerns that information, nuclear weapons and radioactive materials have been put at risk of theft or clandestine sale (Moody *et al.* 2005). Most recently, these concerns were voiced by the Director General of the International Atomic Energy Agency, Dr. Mohamed ElBaradei, at the UN General Assembly on 28 October 2008, who delivered a serious and sobering warning of the threat of nuclear terror (ElBaradei 2008). His address highlighted that "the possibility of terrorists obtaining nuclear or other radioactive material remains a grave threat…" and stressed that "…the number of incidents over the past year demonstrated a persistent problem with the trafficking, theft, losses and other unauthorised activities involving nuclear or radioactive materials" (ElBaradei 2008).

These materials have the potential to be used for terrorist or malicious/criminal purposes. Of particular concern is the Radiological Dispersion Device (RDD), a tool with which radioactive materials can be disseminated over an area by clandestine or overt means, and one proposed to be a weapon of interest amongst terrorist organisations (Ferguson *et al.* 2004; Zimmerman and Loeb 2004; Colella *et al.* 2005; Thomson *et al.* 2006; Coughlin 2008).

The dispersion mechanism of an RDD is typically described as passive or explosive (Ferguson *et al.* 2004; Colella *et al.* 2005). Passive dispersion of a radioactive material provides several advantages for implementation in terrorist actions, such as secrecy of deployment, ease of spread, and possible long term exposure. An example of passive

dispersion may include the distribution of radioactive materials in a city's water supplies, throughout a building via the air conditioning system, or as a dissolved substance in a spray (from an aeroplane or via a hand-held device). However, it does not have the psychological and social impact of an explosive RDD.

Explosive dispersion, a method that has traditionally received more international and national attention, can be in the form of a dirty bomb. The principle type of dirty bomb combines a conventional explosive (e.g. trinitrotoluene or dynamite) with radioactive material in the form of powder or pellets. Its primary purpose is to create an initial state of terror as well as long-term social, commercial and medical services disruption. It will create immediate damage to the surrounding area via the conventional explosive and the spread of radioactive material over a larger area, without creating a nuclear explosion (Ferguson *et al.* 2004). Through the RDD's dual nature as a weapon, it creates immediate and long-lasting effects.

Recent articles in the media have demonstrated the interest of radical Islamist terrorists to develop a "dirty bomb" for use against Western targets (Coughlin 2008). Terrorist interest has also been demonstrated in various high profile criminal proceedings. In 2002, U.S. citizen José Padilla (aka Abdullah Al-Mujahir) was arrested by the U.S. government for allegedly attending an al Qaeda training camp in Pakistan where he received instruction on the mechanics of dirty bombs and explosives. While the documentation and research conducted by Padilla supported his intention to build a dirty bomb, he was arrested and convicted of conspiring to kill, fund and support overseas terrorism (Sherman 2005; Williams 2008). Similarly, in 2006, U.K. citizen Dhiren Barot was convicted of conspiracy to commit murder after documents planning the use of radiological materials, explosives and other chemicals were discovered in his possession (Zagorin and Shannon 2004). Furthermore, information supporting al Qaeda's interest in developing an RDD was presented by British officials to the British Broadcasting Corporation in January 2003, who allegedly uncovered evidence that al Qaeda had been trying to build an RDD in Afghanistan and had assembled sufficient radioactive material (Gardner 2003).

To date there has not been a documented case of an RDD being utilised. There has, however, been reported interest by various government and non-governmental groups in employing the weapon. In 1995, the Iraqi Government produced documentation describing their experimentation in 1987 with RDDs for application in war (UNSCOM 1995; UNSCOM 1996). Three devices, containing 74 GBq (2 Ci) of zirconium-95 and hafnium-181, were constructed and tested for their ability to cause injury or to deny access to an area through dispersal of radioactive material. Based on reported outcomes, these 1.4 tonne devices caused minimal contamination, and were viewed as unlikely to elevate radiation levels above normal background exposure levels (UNSCOM 1995; UNSCOM 1996). In addition, non-government groups, such as the Chechen resistance group, have demonstrated their ability to procure radioactive material for use in an RDD. In 1995, the group deposited a quantity of caesium-137 in Izmailovskiy Park, Moscow (Figure 1.1a).

(a) Images have been removed due to copyright restrictions (b)

Figure 1.1: Potential dirty bombs uncovered in (a) Russia 1995 [image of bag of radioactive material with detector] and (b) Chechnya 1998 [image of device next to railroad tracks] (Krock and Deusser 2004)

Bale (2004) reports that the leader of the Chechen resistance group, Shamil Basayev, informed a Russian television network where to find a container of caesium-137, allegedly sourced from a medical therapy unit. In addition, the same group was suspected to have been involved in a second incident involving radioactive material in 1998. An explosive mine attached to a container filled with an undisclosed amount and

type of radioactive material was reportedly discovered hidden near a railway line in the town of Argun, 10 miles from Groznyy in Chechnya (Figure 1.1b) (Bale 2004; Krock and Deusser 2004; Moody *et al.* 2005).

Other reports have demonstrated the theft and clandestine sale of radioactive material across the world. Some incidents of note were presented by Karam (2005), including the theft and ransom of five industrial indium sources in Ecuador in 2002, the theft of a caesium-137 source from well-logging equipment in Nigeria which was later recovered in 2002 in Germany, and the interception in Georgia of radioactive material being smuggled possibly into Turkey or Iran in 2003.

Perhaps more relevant for the Australian context are reports detailed in Karam (2005) and Colella *et al.* (2005) disclosing the interception of a quantity of caesium-137 in Thailand in 2003 (Ferguson and Andreoni 2003; Colella *et al.* 2005; Karam 2005). While reports vary as to the quantity of caesium-137 confiscated and no direct terrorist links were reported, the trafficking of this material in such close proximity to Australia is of serious concern.

Other mechanisms that may utilise radioactive materials for malicious purposes include the radiological emission device (RED), whereby a radioactive source is left in a public place to cause injury or death through external exposure, the assassination or poisoning of an individual or small group of individuals, or a targeted attack on a nuclear/radiological installation, such as the Open Pool Australian Lightwater (OPAL) nuclear reactor at the Australian Nuclear Science and Technology Organisation (ANSTO), or an industrial sterilisation plant. For example, in 2002, a Chinese nuclear scientist illegally purchased an industrial device containing iridium-192 metal pellets and placed these pellets in the ceiling of the office of a work rival. The intended victim experienced radiation exposure symptoms from the RED, as did 74 other staff members at the institution (Anonymous 2003). In addition, perhaps the most well publicised assassination with a radioactive material is that of the ex-KGB agent Alexander

Litvinenko (Harrison *et al.* 2007). Mr Litvinenko died after being poisoned with a small quantity of the alpha-emitter polonium-210.

While there have been numerous reports of attacks on nuclear/radiological installations by criminal or terrorist groups, none have been known to be successful to-date (Mohtadi and Murshid 2006; Hosken 2007). However, the seriousness of this threat is clearly demonstrated by criticality accidents, such as the one that occurred in Chernobyl, former Ukrainian Soviet Socialist Republic in 1986 and most recently by the armed attack on the Pelindaba nuclear facility in South Africa (IAEA 2001; IAEA 2006a; Hosken 2007).

The emergence of a radiological threat has created a need within the forensic science community to address the implications for traditional evidentiary processing and methodologies. These implications range from specialised training and the implementation of radiation detection equipment for crime scene personnel to the establishment of the new area of nuclear forensic science to identify the source or origin of illicitly-trafficked radioactive materials or those that are used for malevolent purposes.

A number of research papers have recently been published in the area of source determination, in addition to the International Atomic Energy Agency's (IAEA) Nuclear Forensic Support document, and are proof of the development and applicability of the nuclear forensics discipline (Colella *et al.* 2005; Mayer *et al.* 2005; IAEA 2006b; Thomson *et al.* 2006; Wallenius *et al.* 2006). For example, Wallenius *et al.* (2006) recently published two case studies examining analytical techniques for the determination of signatures to identify the origin of uranium pellets and uranium powder.

While great strides are being made in the nuclear forensic science discipline, certain traditional forensic disciplines are yet to address implications. This includes the discipline of forensic biology.

1.3 Forensic DNA analysis and its use in human identification

Forensic Science is the application of scientific and technical knowledge to the examination of evidence for use in a court of law. Of particular relevance to this project are applications in the field of forensic biology, specifically DNA analysis.

Prior to 1985, serological genetic markers were relied upon for forensic identity testing (Butler 2005; Carracedo and Sánchez-Diz 2005). Protein markers, such as ABO blood groups and human leukocyte antigen, as well as other polymorphic proteins and enzymes, were used for a number of purposes, including criminal investigations, paternity testing and individual identification. However, serological genetic markers showed significant limitations due to limited variation between individuals; protein expression was restricted to particular tissues or biological fluids, they exhibited rapid degradation when exposed to the elements or subject to destructive processes such as incineration, and a large amount of the biological sample was required (limiting its usefulness in forensic investigations) (Nasidze 1995; Butler 2005). In addition, this method of testing was useful for excluding an individual from an investigation but not of significant evidential value when an inclusion was made (Butler 2005).

In 1985, Alec Jeffreys introduced a breakthrough method in genetic identity testing that dramatically changed the field of forensic biology (Jeffreys *et al.* 1985a; Jeffreys *et al.* 1985b; Jeffreys *et al.* 1985c). Jeffreys *et al.* (1985b) described the presence of hypervariable regions in the genome which, when analysed, addressed the limitations of protein typing, as nuclear DNA is found in almost all human cells (except red blood cells), is unique to each individual (except identical twins), and is much more stable over time than proteins (Jeffreys *et al.* 1985a; Jeffreys *et al.* 1985b; Jeffreys *et al.* 1985c).

Genetic typing of DNA is a convenient and reliable method for differentiating and identifying individuals, or groups of individuals, in a closely related population of organisms (Jeffreys *et al.* 1985c). The concept of genetic typing of DNA, termed DNA

profiling, is based on the ability to detect small, abundant, and highly polymorphic genetic markers within the genome, and identify the alleles present.

DNA profiling has made it possible to easily address questions of genetic linkage such as the determination of paternity and familial relationships (Jeffreys *et al.* 1991; Pastore *et al.* 1996), evolutionary taxonomy (Bowcock *et al.* 1991; Meyer *et al.* 1995), human genetic mapping (Nakamura *et al.* 1987), population diversity (Edwards *et al.* 1992), individual identification (Jeffreys *et al.* 1985a; Sasaki *et al.* 1997), and the verification of animal bloodlines (Bowling 2001). Of particular relevance is the use of DNA profiling in criminal cases where biological evidence can identify the source of human genetic material found at a crime scene.

Biological evidence has been associated with many types of forensic investigations, such as murder (Hagelberg *et al.* 1991; Staiti *et al.* 2004), sexual assault (Monaghan and Newhall 1996; Petricevic *et al.* 2006), threatening letters (Barbaro *et al.* 2004a), and familial relationships (Jeffreys *et al.* 1992; Pastore *et al.* 1996). DNA analysis has also played a role in counter terrorism to connect and identify persons involved in planning, preparing and carrying out terrorist acts. In the event of an incident involving radioactive material, it is important to establish the identity of the person or persons of interest for intelligence purposes and to identify their associates.

Hagelberg *et al.* (1991) first demonstrated the capacity of DNA analysis for identification with its application to the case of a murder victim. This capacity was further supported by the work of Jeffreys *et al.* (1992) with the identification of Josef Mengele, the Auschwitz "Angel of Death", and Gill *et al.* (1994), with the analysis of the remains of the Romanov family. There were, however, limitations to the Variable Number Tandem Repeats (VNTR) approach as this type of analysis requires large quantities of undegraded DNA not typically associated with the remains resulting from mass fatalities (Clayton *et al.* 1995b; Whitaker *et al.* 1995; Olaisen *et al.* 1997). Significant progress was made when Short Tandem Repeat (STR) analysis was

introduced, as one can use smaller target fragments and exponentially as well as accurately amplify the target fragments (Clayton *et al.* 1995a).

DNA analysis also plays a major role in disaster victim identification (DVI). Mass disasters and catastrophic events can lead to severe body fragmentation and the process of DVI can be further complicated by the large number of remains that can be recovered and the extent to which the remains have deteriorated. Traditionally, fingerprints, odontological analysis and other forensic pathology are the preferred methods of confirmative identification (Corach *et al.* 1995). However, within incidents where there is high fragmentation of the decedents, it is not always possible to apply these identification methods.

DNA analysis has proven to be an effective tool for identification of victims from traumatic events such as plane crashes, fires, and wars where the remains are no longer viable for other methods due to the fragmentary conditions of the remains, or for confirming uncertain identifications made with other methods (Clayton *et al.* 1995a; Clayton *et al.* 1995b; Corach *et al.* 1995; Whitaker *et al.* 1995; Olaisen *et al.* 1997; Alonso *et al.* 2001; Hoff-Olsen *et al.* 2003; Leclair *et al.* 2004a; Piccinini *et al.* 2004). DNA typing enables identity information to be derived from any type of tissue and, as such, DNA analysis has been used successfully in a number of large accidents to associate body parts and for the purposes of identification, by comparing victim's DNA profiles to ante-mortem samples from personal items or with those of relatives (Hagelberg *et al.* 1991; Jeffreys *et al.* 1992; Gill *et al.* 1994; Olaisen *et al.* 1997; Leclair *et al.* 2004a).

Specific examples of where DNA analysis has contributed to mass victim identifications include the 1993 siege in Waco, Texas. Most of the remains recovered from the scene were fragmented by the collapsing building and munitions explosions, charred and partially incinerated by the fire, and generally in an advanced state of decomposition (Clayton *et al.* 1995a; Clayton *et al.* 1995b). Types of biological materials used in such analysis include bone, soft tissues such as deep muscle, skin, and internal organs.

1.3.1 Biological matrices in forensic DNA analysis

As stated above, common biological matrices collected from mass incidents are bone, soft tissue, skin and internal organs. However, there are a number of other typical biological matrices submitted to forensic laboratories for DNA analysis depending on legislative requirements (e.g. reference samples from suspects) and availability (e.g. sample collected from crime scenes). As such, biological samples are numerous and include whole blood and bloodstains, semen and semen stains, buccal cells, saliva on cigarette butts, teeth, hair roots, fingerprint deposits, debris under fingernails, faeces, urine, dandruff and trace biological material (Wiegand *et al.* 1993; Cattaneo *et al.* 1995; Lorente *et al.* 1998; Vigilant 1999; Graw *et al.* 2000; Hoff-Olsen *et al.* 2001; Balogh *et al.* 2003; Cerri *et al.* 2003; Bright and Petricevic 2004; Roeper *et al.* 2007). In addition, the biological material can be processed from any number of substrates, such as swabs, clothing, shoes, firearms, knives, cigarette butts, plastic drink bottles, postage stamps, envelope sealing flaps and personal items including toothbrushes, chewing gum, mobile phones as well as many others (Allen *et al.* 1994; Komonski *et al.* 2004; Polley *et al.* 2006).

In addition, the nature of crime often results in potential evidence being exposed to harsh environments and less than pristine conditions. The following section discusses some of the factors that influence the integrity of biological matrices and can impact on the successful analysis of DNA evidence.

1.3.2 Biological matrix quality, degradation and contamination

The persistence of biological evidence at a crime scene will depend on the quantity of biological material originally deposited and the conditions to which it is exposed (Graw *et al.* 2000; Prinz *et al.* 2002; Balogh *et al.* 2003; Arismendi *et al.* 2004; Meissner *et al.* 2007). For example, samples collected from crime scenes and mass disasters can be challenging as exposure to certain environmental conditions such as sunlight, fire, heat,

soil and water can accelerate the process of degradation (Corach *et al.* 1995; Höss *et al.* 1996; Graw *et al.* 2000; Bender *et al.* 2004; von Wurmb-Schwark *et al.* 2004).

Under normal conditions, DNA becomes degraded by endogenous nucleases, catabolic enzymes that degrade DNA by fragmentation through oxidation and hydrolysis of the DNA molecule (Lindahl 1993; Hofreiter *et al.* 2001). However, exposure to direct sunlight (UV radiation) can result in the formation of thymine dimers, where adjacent thymine bases on the DNA molecule become cross-linked (Lindahl 1993; Durbeej and Eriksson 2003). Exposure to fire, heat, soil and moist environments results in oxidation and/or hydrolysis of the DNA molecule. This is in addition to the bacterial activity, which also facilitates DNA fragmentation by nucleases through hydrolysis and oxidation (Iwamura *et al.* 2004). A review of DNA degradation is presented by Iwamura *et al.* (2004) which discusses contributing factors such as rapid tissue degradation caused by bacterial colonisation, temperature, humidity, and many organic compounds.

The action of oxidation and hydrolysis of the DNA molecule is described in Lindahl (1993) and is further supported by the work of many authors including Höss *et al.* (1996), Yang (1997) and Hofreiter *et al.* (2001). Hydrolytic processes destabilise and break the DNA molecule by deamination or depurination of the phosphodiester bond between the nucleotide bases and the sugar residues (Lindahl 1993; Höss *et al.* 1996; Yang 1997; Hofreiter *et al.* 2001). Oxidation will modify the nitrous bases and the sugar-phosphate backbone of the DNA molecule. Oxidative damage causes strand breaks, chemically alters nucleotide bases and creates DNA-DNA cross-links (Lindahl 1993; Höss *et al.* 1996; Yang 1997; Hofreiter *et al.* 2001). The combined action of these processes can result in inter- and intra-strand crosslinks, single and double stranded breaks, and exposed sugar, phosphate and hydroxyl groups.

The origin and age of the biological specimen will also influence the amount of DNA able to be analysed; for example, aged bone, teeth or telogen phase hair typically have only small amounts of DNA present and have been exposed to catabolic enzymes that degrade DNA by fragmentation (Roeper *et al.* 2007). In addition, sample quantity,

particularly with trace DNA evidence such as saliva stains on cigarette butts or trace DNA transfer onto objects, will also affect the recoverable amount.

Bär *et al.* (1988) and Ludes *et al.* (1993) evaluated the post-mortem stability of different tissues utilising VNTR analysis. Overall, there was good post-mortem DNA stability in these tissues, however, there was a detectable level of DNA degradation in blood and kidney after a period of one week. In addition, Hoff-Olsen *et al.* (2001) investigated brain, hair, cartilages, liver, thyroid, and blood by STR analysis, but the most suitable tissue for STR analysis could not be established.

Analysis can also be compromised when contaminants interfere with the analytical process and inhibit the recovery of a full DNA profile. Heme (hematin) in blood, melanin in tissues and hair, polysaccharides and bile in faeces, humic compounds in soil, urea in urine, and indigo dyes in denim have all been demonstrated to interfere with the DNA analysis process. It is proposed that these contaminants bind in the active site of the DNA polymerase and prevent its proper functioning (Hoff-Olsen *et al.* 2001; Butler 2005).

All of these factors play a role in the destruction of the DNA molecule and influence the persistence of biological material for forensic analysis. The implications of DNA degradation on DNA profiling and interpretation will be discussed later in Section 1.8.6.

1.4 Radioactive materials

1.4.1 Ionising radiation – Background

There are constant exposures to natural sources of ionising radiation in daily living, such as cosmic (carbon-14, tritium), internal (potassium-40), terrestrial (natural decay series uranium, thorium and actinium), and radiogenic gases (radon and thoron) (Martin and Harbison 1996d). Radioactive materials are usually described in terms of radioactive decay or radioactivity. Radioactive decay is the process by which an unstable element is

transformed into a more stable element through the emission of a charged particle or a photon (Martin and Harbison 1996d). Substances that undergo this transformation are said to be radioactive. There are five main types of ionising radiation emitted during radioactive decay: alpha, beta (electron and positron), gamma, X-ray and neutron. Beta (positron), neutron and X-ray emissions are beyond the scope of this project and will not be discussed further.

1.4.1.1 Alpha radiation

Alpha radiation is a particle emission that consists of a group of two protons and two neutrons that are bound tightly together (Martin and Harbison 1996d). The structure is therefore identical to a helium nucleus. As a result, the alpha particle is relatively large, heavy, and has two units of positive charge. These particles are typically emitted by a larger unstable nucleus, such as americium, plutonium and uranium, and a common example is the reduction of uranium-238 to thorium-234 by the expulsion of an alpha particle:

$$^{238}_{90}U \rightarrow ^{234}_{90}Th + ^{4}_{2}\alpha \qquad \text{Equation 1.1}$$

with two alpha particle energies of 4.20 and 4.15 MeV (NNDC 2009).

Alpha particles are released with energies that usually fall within the range 3.5 to 10 MeV, and more commonly within 4 to 6 MeV. All alpha decays result in a transmutation of the element to one of a lower atomic number.

1.4.1.2 Beta radiation (electron emission)

Beta radiation consists of high speed electrons that originate in an unstable nucleus. Beta radiation occurs when there is an excess of neutrons in the nucleus and the neutron transforms into a proton and an electron. The electron is emitted from the nucleus. An example of beta decay is the transformation of hydrogen-3 to helium-3:

$$^{3}_{1}H \rightarrow ^{3}_{2}He + \beta^{-}$$ Equation 1.2

with a beta particle energy of 18.59 keV (NNDC 2009).

A beta particle is small compared to an alpha particle, with a mass of 5.488×10^{-4} amu (compared to 4 amu for alpha particles), and has a single negative charge. All beta decays result in a transmutation of the element to one of a higher atomic number.

1.4.1.3 Gamma radiation

In most cases, after the emission of alpha or beta radiation, the electrons in the nucleus undergo rearrangement and emit energy in the form of gamma radiation (Martin and Harbison 1996d). Gamma radiation is a class of electromagnetic radiation whereby the emission consists of a photon rather than a charged particle. Gamma rays therefore do not have a mass or a charge. An example of gamma decay is presented in Equation 1.1, where the alpha particle decay of uranium-238 to thorium-234 also results in two low abundance gamma rays of 49.55 and 103.0 keV (NNDC 2009).

1.4.2 Quantification of radiation – activity and dose

There are several specialised units utilised in the measurement and quantification of radioactivity (Table 1.1). Activity of a source is described in terms of the Becquerel (Bq), which gives the number of atoms disintegrating per second. The energy of the radiation is measured by the electron-volt (eV). Absorbed dose is the amount of energy absorbed per kilogram by the material and is described by the gray (Gy). Equivalent dose is measured by the Sievert (Sv) and describes the relative amount of damage caused by different radiations to living tissue (Martin and Harbison 1996c).

Table 1.1 Specialised units used in the measurement and quantification of radioactive materials.

Measurement	**SI Unit**
Activity	Becquerel (Bq)
Energy	Electron volt (eV)
Absorbed dose	Gray
Equivalent dose	Sievert

1.4.3 Interaction of radiation with matter

The interaction of radiation with matter is commonly described in terms of linear energy transfer (LET), which is a measure of the average energy deposited along the track of a particle per unit length or mean energy released (keV) per micrometer (μm) of the tissue traversed (keV/μm) (Martin and Harbison 1996c). The LET is affected by the velocity and charge of an emission. In terms of biological damage, the higher the LET value the more the damage.

In order to affect matter, either living or non-living, radiation must interact with it and cause excitation or ionisation of the atoms of the material (Coggle 1971). Ionisation of an absorber atom occurs when an outer electron of the atom receives sufficient energy to leave the atom, resulting in the formation of a positive and negative ion pair. Excitation occurs when an inner electron receives enough energy to move it to a higher energy level but not enough to leave the atom.

As alpha particles have a relatively large mass and the particles carry a +2 charge, they travel at a slower velocity and readily interact with their surroundings (Coggle 1971; Martin and Harbison 1996a). As velocity is slow, there is an increased opportunity for the particle to interact with and exert its electric field on atoms in the surrounding area. As the alpha particle penetrates deeper into media, more and more interactions occur,

and, as the speed reduces, the chance of further ionisations is also increased. Alpha particles therefore have a high LET value.

The beta particle has a higher velocity, smaller mass and smaller electric charge (-1), than the alpha particle (Coggle 1971; Martin and Harbison 1996a). Their smaller size allows them to travel further and therefore they are less likely to cause ionisations than alpha particles. When the beta particle interacts with an orbital electron of an atom, ionisation occurs when the electron is ejected from its orbit. An atom that has lost one of its orbital electrons is now an ion. As the energy of the electron falls, so does its velocity, and there is a resultant increase in the probability of interactions between it and the atoms of the medium.

Gamma radiation is in the form of photons which have no mass or charge, and photons lose energy by interacting with the electrons of an atom (Coggle 1971; Martin and Harbison 1996a). Gamma radiation travels very large distances and therefore produces very few interactions per unit length and resulting in a low LET value. Gamma radiation usually accompanies ejection of an alpha or beta particle to bring the nucleus of the emitting atom down to a ground energy level.

Table 1.2 describes the properties and ranges of alpha, beta and gamma radiation, including an approximate range in air and tissue, as well as appropriate shielding material to block penetration of the radiation.

Table 1.2 Properties of alpha, beta and gamma nuclear radiation

Radiation	Mass (amu)	Charge	Range in air	Range in tissue	Shielding material
Alpha	4	+2	0.03 m	0.04 mm[a]	Paper
Beta	1/1840	-1	3 m	5 mm[a]	Aluminium or Perspex
Gamma	0	0	Very large	Though body	Lead or Concrete

[a] These values are examples only, as the range is influenced by the energy of the particle.

1.5 Radioactive materials for an RDD

From the many known radioactive isotopes that exist, there are only a few materials that stand out as being suitable for use in a radiological device, such as an RDD. These radioactive isotopes include caesium-137 (Cs-137), cobalt-60 (Co-60), americium-241 (Am-241), strontium-90 (Sr-90), iridium-192 (Ir-192), and plutonium-238 (Pu-238) (Ferguson *et al.* 2004; Colella *et al.* 2005; Karam 2005). A list of their radioactive properties is provided in Table 1.3.

There are a number of factors that influence a radioactive material's suitability for such a device; including availability of a particular isotope, its half-life, the type and strength of radiation emitted and the ability to access the material.

As shown in Table 1.3, the isotopes of interest demonstrate relatively long half-lives, from over 400 years for americium-241 to less than 74 days for iridium-192. The half-life of an isotope will determine its persistence in the area of dispersal. A longer half-life will ensure area denial, and could cripple an economy if dispersed in a city centre. The half-life of an isotope is the time required for the activity of the material to reduce by half and can vary from less than a millionth of a second to more than a billion years. However, there is an inverse relationship between the strength of the source and half-life and typically, as the half-life increases the strength of the source decreases.

While isotopes such as cobalt-60 and iridium-192 have relatively shorter half-lives as compared to other isotopes of interest, other attributes contribute to their suitability for a radiological weapon, such as activity and availability of the sources.

Table 1.3 Radioactive properties of selected radioisotopes

Isotope	Half-life	Decay mode	Energy[a] (MeV)
Caesium-137	30.2 years	Beta Gamma	0.514, 1.18 0.662
Cobalt-60	5.27 years	Beta Gamma	0.318 1.17, 1.33
Strontium-90 (Yttrium-90)	28.79 years (2.67 days)	Beta	0.546 (2.28)
Iridium-192	73.83 days	Beta Gamma	0.535, 0.672 0.316, 0.468
Americium-241	432.6 years	Alpha Gamma	5.486, 5.449 0.059
Plutonium-238	87.7 years	Alpha	5.499, 5.456

[a]Energies represent the most abundant emission intensities. Stated beta particle energies represent the maximum energy. Elemental properties were derived from the National Nuclear Data Center's Evaluated Nuclear Structure Data File (ENSDF) Database (NNDC 2009).

Availability of a particular isotope is generally governed by its ubiquity of use. Radioactive materials are used heavily in industry, medicine and research (IAEA 2005). Examples of industrial uses include well-logging gauges (cobalt-60; caesium-137; americium-241/beryllium), for geological surveys of oil and gas deposits and in industrial radiography cameras for weld or materials inspection (iridium-192; cobalt-60; caesium-137). Medical uses include radiation treatment with teletherapy units (caesium-137) and brachytherapy sources (iridium-192; cobalt-60; caesium-137), and research applications include animal or equipment sterilisation in irradiators (cobalt-60; caesium-137). Examples of the various types of radiation equipment and activities associated with the isotopes of interest can be found in Table 1.4.

Table 1.4 Radiation equipment and activities of high risk radioactive sources

Type of radiation equipment	Radioactive Components	Chemical Composition	Typical Radioactivity (TBq)
Radioisotope thermoelectric generators	Plutonium-238 Strontium-90	Plutonium metal Strontium carbonate pellet	7.4×10^{2} 1.0×10^{1}
Sterilisation and food preservation irradiators	Cobalt-60 Caesium-137	Cobalt metal Caesium chloride powder	1.5×10^{5} 1.1×10^{5}
Teletherapy	Cobalt-60 Caesium-137	Cobalt metal Caesium chloride powder	1.5×10^{2} 1.9×10^{2}
Industrial radiography	Cobalt-60 Iridium-192	Cobalt metal Iridium metal	2.2 3.7
High/medium dose rate brachytherapy sources	Cobalt-60 Caesium-137 Iridium-192	Cobalt metal Caesium chloride powder; Iridium metal	3.7×10^{-1} 1.1×10^{-1} 2.2×10^{-1}
Well-logging gauges	Caesium-137 Americium-241/beryllium	Caesium ceramic Americium oxide pellet / beryllium metal	7.4×10^{-2} 7.4×10^{-1}
Low dose rate brachytherapy sources	Caesium-137 Iridium-192	Caesium ceramic Iridium metal	1.9×10^{-2} 1.9×10^{-2}
Thickness/fill level gauges	Strontium-90 Caesium-137 Americium-241	Strontium carbonate pellet Caesium ceramic Americium oxide pellet	3.7×10^{-3} 2.2×10^{-3} 2.2×10^{-2}
Portable gauges (e.g. moisture, density)	Caesium-137 Americium-241/beryllium	Caesium ceramic Americium oxide pellet / beryllium metal	1.9×10^{-3} 3.7×10^{-4}

Table derived from IAEA's Safety Standards Series Categorization of Radioactive Material (IAEA 2005).

As a consequence of their wide use, radioactive materials can be purchased legally, purchased illegally, or stolen. A recent study from the US Government Accountability Office (GAO) demonstrated a possible avenue for individuals to illegally purchase radioactive materials. The GAO was able to set up a fake construction company and receive a licence from the Nuclear Regulatory Commission to purchase radioactive materials (Kutz *et al.* 2007). The operation revealed that the NRC provided a licence to purchase a moisture density gauge without requiring proof of an office, website, or employees. In addition, the GAO modified the licence to be able to purchase an unlimited quantity of radioactive material and successfully obtained quotes with an intent to provide the gauges from two suppliers. A moisture density gauge is used to read the density of soil and pavement when constructing roads, and contains caesium-137 and americium-241 / beryllium. If combined with explosives, these materials can be used to create a dirty bomb (Ferguson *et al.* 2004; Kutz *et al.* 2007). In addition, portable devices such as industrial equipment from sites of mining operations are at risk of theft and, in some instances, may be left behind after operations are complete (Ferguson *et al.* 2003; Streeper *et al.* 2007). Items such as the caesium-137 source from medical teletherapy units may prove more difficult to procure, due to the size of the equipment and the restricted access surrounding its use.

The activity of the source describes the amount of radiation released over time. Higher activity sources would result in a greater release of radiation and would result in a greater overall impact. For example, approximately six cobalt-60 brachytherapy sources (370 GBq) would be required to make up a single cobalt-60 source from an industrial radiography camera (2220 GBq).

In addition, certain characteristics, such as chemical form, will also make a material more suited to explosive dispersion. For example, higher activity sources of caesium-137 are in the form of a caesium chloride powder that is soluble and dispersible (IAEA 1988). For other types of radiation weapons, such as a RED a highly penetrating gamma emitter would be suitable; and conversely, high LET alpha emitters would be more suitable for assassinations or poisoning events.

It should be noted that in some cases, regardless of the radioactive material employed, the psychological impact from the public fear of radiation may prove more damaging than the device itself; hence dirty bombs are often called weapons of mass disruption (Ferguson *et al.* 2004). To this end, material with low levels of activity such as uranium oxide, or yellowcake, would be as effective in satisfying the radiation component as the radioactive materials listed in Tables 1.3 and 1.4.

Of the radioactive materials presented, the beta/gamma emitters cobalt-60 and caesium-137 and alpha emitter americium-241 were chosen for further investigation due to their potential for use in an RDD. This includes their ubiquity of use in industry, medicine, and research, the potential for dispersion and the diversity in decay mode. These radioactive materials are described in detail.

1.5.1 Caesium-137

Caesium-137 is an artificially produced radioactive isotope. During radioactive decay, it emits a monoenergetic gamma ray at 0.662 MeV and beta particles with energies in the range of 0.51 MeV to 1.17 MeV (NNDC 2009). As stated in Section 1.4, beta radiation travels short distances in air and can penetrate a short distance into body tissues, while gamma radiation can travel much further and penetrate the entire body. Therefore, the radiation emission from caesium-137 can be harmful to the body via external or internal exposure.

Toxicological studies have shown that beta and gamma radiation can penetrate through the body and induce tissue damage and disruption to cellular function. While the gamma radiation can penetrate the entire body, beta is normally not a serious concern unless in contact with the skin, as this allows the particles to penetrate deeper into the tissue, or taken internally. Once taken into the body, soluble caesium salt is readily absorbed into the system, by replacing potassium, and is cleared from the body within approximately 73 to 84 days (Miller 1964).

Caesium-137 is used in moisture density gauges, levelling gauges, thickness gauges, well-logging devices, teletherapy units, brachytherapy sources, industrial irradiators, calibration standards, and blood irradiators (IAEA 2005). For higher activity sources, caesium-137 is found in a powder form that resembles table salt and, when collected in large amounts, may glow (This was demonstrated in the well-known case study of caesium-137 contamination that occurred in Goiania, Brazil, where a 50.6 TBq caesium-137 chloride source was accidentally dispersed around the village after scrap metal thieves removed the source from an abandoned teletherapy unit (IAEA 1988)). Powder is typically sealed in a double encapsulated and welded stainless steel container, which does not shield the gamma radiation (IAEA 2007). Based on IAEA documents, caesium sources contained within moisture density gauges are in ceramic form. Caesium salts are soluble in water and form a monovalent cation (positive). It can therefore form electrovalent bonds with nearly all the inorganic and organic anions.

1.5.2 Cobalt-60

Like caesium-137, cobalt-60 is an artificially produced radioactive isotope that is found in metal form, typically as a pellet (1 mm × 1 mm) or slug (2.54 cm × 0.635 cm) (IAEA 2007). It decays by emission of beta particles with a maximum energy of 0.318 MeV and gamma rays at 1.17 and 1.33 MeV (NNDC 2009).

The beta and gamma emissions produce the same effects as discussed for caesium-137 (Section 1.5.1), where the emission of beta particles and gamma radiation results in an internal and external radiation hazard. However, as cobalt-60 is usually produced as a metal, internal contamination as a result of inhalation or ingestion is not considered a significant issue.

Cobalt-60 is used for a number of industrial, commercial and medical applications including batch sterilisation of medical and food products, weld inspections of pipes, external radiation therapy utilising such instruments as a teletherapy unit and internal radiation therapy utilising brachytherapy sources for implantation (IAEA 2005). Cobalt-

60 sources commonly consist of a double encapsulated stainless steel container with several thousand pellets or long 'pencils' of zircaloy tubes that contain around 16 slugs (IAEA 2007).

1.5.3 Americium-241

Americium-241 is an artificially made radioisotope that can be in the form of a ductile, malleable, silver-white metal or americium oxide powder. It decays by emission of alpha particles at two energies of 5.48 and 5.45 MeV, as well as three low-level gamma rays at 59, 26 and 14 keV (NNDC 2009). As discussed in Section 1.4, alpha particles travel only short distances in air and even shorter distances through denser material; however, if taken into the body, it presents significant danger to internal tissues. Americium-241 therefore represents a serious internal radiation hazard. While americium-241 has a half-life of over 400 years, the body is usually able to clear powder forms within 11 days; however, americium is understood to concentrate in the bone and remain there for up to 920 days (Newton *et al.* 1983).

Americium is typically used in thickness gauges and smoke detectors, and, when combined with beryllium, releases neutrons for use in well-logging and moisture/density gauges (IAEA 2005). In industrial, medical or commercial devices, americium is typically in the form of metal or plastic discs (IAEA 2007). Americium-241 oxide/beryllium mixtures are usually in pressed powder pellet and metal forms, and are typically sealed in a double-encapsulated welded stainless steel container.

Americium-241/beryllium mixtures within moisture/density gauges pose an interesting problem. The material not only produces alpha radiation as the americium-241 decays, but also produces neutrons from the alpha particle interaction with beryllium. The transmutation reaction of an alpha particle (He) hitting beryllium is:

$$ {}^{4}_{2}He + {}^{9}_{4}Be \rightarrow {}^{12}_{6}C + {}^{1}_{0}n \qquad \text{Equation 1.3} $$

However, if the two materials are separated (e.g. by dispersion), the alpha particle interaction with beryllium reduces dramatically and neutron production virtually stops.

1.6 Ionising radiation and its effects on DNA

1.6.1 DNA structure

DNA is found in almost all cells (except red blood cells), and in humans is found in the B-form of a right-handed double helix (Fowler *et al.* 1988). DNA is also observed in A- and Z-structural conformations. It is a complex molecule comprised of a long chain polymer of nucleotides. Nucleotides consist of a nitrogenous base [adenine, thymine, guanine, or cytosine (A, G, T or C)], a deoxyribose sugar, and a phosphoryl group. DNA exists as a two chain helical structure formed by hydrogen bonding between complementary base pairing (Watson and Crick 1953b; Watson and Crick 1953a; Fowler *et al.* 1988).

Nucleic acids are polyanions and therefore require counter ions in order to neutralise the negatively charged phosphate groups and stabilise the double helix within a biological system (Denisov and Halle 2000). Cellular genomic DNA undergoes local conformational changes, such as supercoiling of transcription sites, chromosomal formation, which may be factors that mediate the effect and extent of radiation damage (Michalik *et al.* 1995). For example, the DNA molecule is condensed into the chromosome structure by associating with proteins called histones. Approximately 140 base pairs coil around each histone core to form a nucleosome with 20 to 50 base pairs of DNA linking each nucleosome (Hartl and Jones 2001). The study by Michalik *et al.* (1995) demonstrated an increase in DNA damage during mitosis (cell division) where the DNA is no longer coiled in a chromosome. This may be of particular importance when comparing and evaluating the effects of radiation damage on raw genomic DNA molecules compared to cellular DNA molecules.

1.6.2 Radiation damage and the biological consequences of exposure

Ionising radiation can induce a large variety of molecular damage in the DNA molecule. Diversity in cellular responses, such as inactivation, chromosomal rearrangement, and mutations may lead to cancers or hereditary diseases (Upton 1986; Kramer and Kraft 1994; Georgakilas *et al.* 2000; de Oliveira *et al.* 2001). The efficiency of producing biological damage is dependent on the quality of radiation, and varies with absorbed dose, type of radiation and its energy (nature of the radiation tracks) (Coggle 1971).

Biological consequences depend on how the damage is subsequently processed by the cell (e.g. if they are repaired, fidelity of repair, etc.). Mettler and Moseley (1985) noted that single strand breaks in DNA may be produced at acute doses up to 100 mGy. In a living cell, these single strand breaks would be repaired quickly. At higher doses, such as those above 0.5 – 5 Gy, double-stranded DNA breaks occur and are typically irreparable in a living cell.

1.6.3 Mechanisms and types of DNA damage

Ionising radiations differ from most other physical or chemical agents in that they act indiscriminately on all molecules of an exposed sample (Hutchinson 1985). The damage caused to the DNA molecule by interaction with ionising radiation is typically described by two distinct mechanisms: direct and indirect (Hutchinson 1985; Cullis and Symons 1986; Baverstock and Will 1989; Jones *et al.* 1994; Symons 1994; Michalik *et al.* 1995; Kuipers *et al.* 2004).

Experimental models using dried or frozen DNA have been utilised to demonstrate the effects of *direct* damage on the DNA molecule (Cullis and Symons 1986; Baverstock and Will 1989; Jones *et al.* 1994; Michalik *et al.* 1995). These studies have shown that direct DNA damage is caused when energy particles traverse the DNA molecule and lead to ionisation and/or excitation of DNA constituents at the site of interaction. Cullis and Symons (1986) reported that the direct-damage mechanism causes electron-gain or

electron-loss centres to become localised within the DNA. Direct damage may be particularly significant in that the cationic and anionic centres (G^+ and T^-), if formed close enough together (within approximately 16 base pairs), are thought to lead ultimately to double stranded breaks.

The model system for analysis of *indirect* DNA damage is DNA in a dilute aqueous solution (Jones *et al.* 1994; Kuipers *et al.* 2004). These systems have demonstrated that indirect damage is caused when the energy particles traverse the DNA environment and produce highly reactive free radicals by radiolysis of the water and proteins surrounding the DNA. These free radicals are only able to diffuse a few nanometres due to the highly reactive nature of the cellular environment. Of the great number of free radicals produced during exposure to ionising radiation, the hydroxyl radicals are of major importance, with attack on the sugar or base units frequently leading to strand breaks (Hutchinson 1985; Cullis and Symons 1986). The hydroxyl radicals are thought to chiefly react with organic molecules either by adding to a double bond or by extracting a hydrogen atom from a carbon-hydrogen bond to form H_2O and a carbon radical (Hutchinson 1985). Attack by hydroxyl radicals in dilute solutions has been shown to be essentially random; producing strand breaks at all possible sites with almost equal probability.

Nevertheless, both the direct and indirect mechanisms are capable of producing hundreds of ionising radiation-induced products from interactions with the DNA molecule (Hutchinson 1985). Key examples include the following:

- Strand breaks: These are caused when the particle or electromagnetic radiation traverses the DNA molecule causing ionisation and either fragmentation of the strand (double-strand breaks) or creating nicks on one side of the phosphodiester backbone (single strand breaks).
- Alkali-labile sites: These are specific sites on the DNA molecule made highly reactive by excitation or ionisation.
- Chemical alteration to nucleotide structure: This may involve covalent binding of nucleotide bases such as the production of thymine dimers under UV irradiation

(Snopov *et al.* 1995; Durbeej and Eriksson 2003), or the formation of DNA adducts on both nucleotide bases and sugars. For example, the compound 5-hydroxy-5-methylhydantoin is one of the major degradation products of thymine gamma-irradiated DNA and its presence in ancient DNA samples, has been shown to inhibit DNA sequencing efforts (Hofreiter *et al.* 2001).

There are a number of effects that only occur in replicating cells such as point mutations, where there is loss of a nucleotide, substitution of one nucleotide for another, or insertion of a nucleotide or chromosomal translocations (Hlatky *et al.* 2002; Watanabe and Nikjoo 2002; Tavera *et al.* 2003). These occurrences are, however, only specific to a replicating cell and are not expected within a forensic sample as specimens are no longer living. All the above induced effects have negative implications for PCR amplification. These implications will be further discussed in Section 1.8.6.1.

1.6.4 Implications of radioactive materials in forensic science

Within the forensic science arena, there have been relatively few investigations into the impacts and effects of radioactivity (mostly electromagnetic radiation) and chemicals on DNA profiling. Most of these studies have dealt with the impact of techniques from one field of forensic examination on another.

For example, Anderson and Bramble (1997) examined the effect of forensic light sources, utilised for the examination of fingermarks, on DNA profiling of whole blood. Forensic light sources, including an argon-ion laser (514 nm), the Polilight® 530 (513–555 nm), the Polilight® UV (315–385 nm), the Superlite® (320–400 nm), and a shortwave UV lamp (255 nm), were tested for their effects on the DNA profiling of whole blood. Anderson and Bramble (1997) showed that a blood smear exposed to shortwave UV light (255 nm) resulted in a reduction in peak height of the DNA profile proportional to the UV exposure time, with exposure over 30 seconds resulting in complete degradation of the profile. Castle *et al.* (2003) explored the effects of longwave UV irradiation (with an output wavelength over 315 nm; UV-A) on whole

blood. Findings suggested that the whole blood exposed to longwave UV light sources did not exhibit any degradative effect in subsequent DNA profiling (Castle *et al.* 2003). Shortwave UV-C (200–300 nm) light is typically used for germicidal treatment.

Grubwieser *et al.* (2003) conducted an extensive study on the effects of chemical and optical fingermark and blood / saliva enhancers on DNA profiling from blood and saliva stains. Their examinations showed that specific chemical enhancers did reduce the quality and completeness of the DNA profiles (Grubwieser *et al.* 2003).

Similarly, Withrow *et al.* (2003) investigated the use of electron beam irradiation for the destruction of anthrax and other biological agents sent through the postal service, and its effect on nuclear and mitochondrial DNA in saliva. Irradiations were conducted at 29.3 and 51.6 kGy doses with DNA extraction and analysis at 40 and 56 days after irradiation (Withrow *et al.* 2003). Results from these experiments demonstrated a general reduction in the total genomic DNA present when the irradiation dose is increased and at the longer extraction time point. There are, however, various experimental variations with this publication. Control samples from 56 day extraction show an overall decrease in total genomic DNA present compared to the 40 day extracted samples. The authors suggest that this observation may have been the result of omission of dithiothreitol from the extraction buffer in the 40 day extraction, allowing degradation in the sample over time (especially as it is also occurring in the control samples), or a generally higher amount of DNA in the 40 day extraction samples over the 56 day extraction samples. The last two points seem unlikely and the variations in the extraction procedures prevent a direct comparison of the two time points as the extraction procedure may have altered the results.

Forensic Biology is not the only field that has needed to address the impacts of radioactivity. A similar experiment to that reported by Withrow *et al.* (2003) has been investigated for the effect of electron beam irradiation on latent fingerprint recovery. Ramotowski and Regen (2005) used several donors, porous and non-porous substrates, and numerous visualisation reagents for investigation and the results demonstrated that

for the majority of substrates there are detrimental effects with respect to the successful visualisation of latent fingermarks using certain reagents. Specifically, there is a diminished quality and quantity of ridge detail after irradiation. Recent work by Colella *et al.* (2009) has supported these observations through experiments involving the cobalt-60 irradiation of latent fingermarks.

1.7 Occupational health and safety risks

1.7.1 Radiation safety considerations

Given the potential hazards associated with radioactive materials, several issues must be addressed in the development and/or modification of analytical strategies for DNA analysis. This focuses predominantly on reducing the exposure of the analyst and avoiding contamination of the working environment.

The International Commission of Radiological Protection (ICRP) provides recommendations for dose limits in planned exposure situations. The occupational dose limit for a radiation worker is 100 mSv over 5 consecutive years (averaged 20 mSv per year), while members of the public may receive up to 1 mSv per year (ICRP 1991; ICRP 2007). In addition, there are deterministic dose limits for the extremities for radiation worker including a 500mSv equivalent dose for the hands and feet.

Depending on the type of radiation emitted by the source (i.e. alpha, beta or gamma), it is possible to reduce the radiation dose to an acceptable limit by utilising shielding. While alpha radiation is not usually considered an external hazard, beta radiation can be shielded by Perspex or aluminium, and gamma radiation by lead, iron or concrete (Martin and Harbison 1996b). The effectiveness of the shield can be determined through calculations of the half value layer, which is the thickness for a particular shielding material required to reduce the intensity of the radiation by half its value. Other shielding such as glove boxes, hot cells, fume hoods, splash trays, and various barriers

may also be used to contain contamination and, in some instances, to protect from radiation emissions.

In addition, distance to the source and time of exposure can be manipulated to reduce the dose received. Distance from the source will also provide some protection according to the inverse square law; whereby doubling the distance from the source will decrease the dose rate to ¼ of its original value (Martin and Harbison 1996b). The overall dose an individual receives can be managed by limiting the time spent with the radioactive materials, as integrated dose is directly proportional to the time spent in the presence of the materials (dose rate × time = integrated dose) (Martin and Harbison 1996b). The dose can be determined by measurement of the dose rate of the particular material.

Lastly, the use of personal protective equipment (PPE) can also enhance the other safety parameters to produce the safest possible working environment. The use of personal dosimeters, hand-held detectors, and encapsulated suits can be used to protect from internal contamination, as well as gloves, lab coats, overshoes, and respiratory protection.

A combination of the above will provide the most effective protection from radioactive sources and need to be considered when addressing policy and operational procedures for dealing with radioactive materials for biological processing. These strategies – and more – will be further discussed in Chapters 2 to 6, examining the effects of alpha and gamma radiation on DNA profiling, and investigating contaminant removal.

1.8 Forensic DNA analysis

Within the forensic biology laboratory, a number of standard operating procedures guide analyses. These processes are: (1) screening of the exhibit or item (including presumptive and confirmatory testing of suspected biological materials and sample collection); (2) extraction of DNA from the biological material; (3) quantitation of the DNA to determine an appropriate quantity for amplification; (4) DNA amplification;

(5) electrophoretic separation of amplified DNA fragments; and, (6) subsequent profile interpretation and comparison. Of particular relevance to this research are the extraction and profile interpretation steps.

1.8.1 Screening of exhibits – presumptive and confirmatory testing, and sample collection

Forensic evidence from a crime scene may be in many different forms, such as a ski mask from a burglary or a knife from a murder. Therefore, different screening protocols are required depending on the item that is seized.

Presumptive and confirmatory tests are performed either before or after collection of biological evidence. Presumptive testing provides an indication of the presence of a particular type of biological material (e.g. blood, semen or saliva) and is limited through either specificity or sensitivity.

Techniques for searching include presumptive screening techniques such as ortho-tolidine and luminol for blood, acid phosphatase or prostate specific antigen (PSA) tests for semen, Phadebas test or the starch iodine radial diffusion test to detect the amylase enzyme in saliva, and all of the above with a forensic light source such as the Polilight (Bulter 2005; AFP 2007). Confirmatory tests are conducted if a positive result is obtained from a presumptive screen. These tests include the Ouchterlony and Haemochromogen tests for blood, the Christmas Tree Stain and Laurell tests for spermatozoa, and the Ouchterlony test for species identification (Bulter 2005; AFP 2007).

For the collection of samples for DNA analysis, procedures depends upon the type of biological material (such as saliva, blood, semen, hair, bone or other tissues), and the substrate that the material is found on.

Principal types of sampling procedures used by forensic biologists include (AFP 2007):

- **Hand picking** – usually relating to physical evidence such as hairs (which may or may not be suitable for DNA analysis).
- **Cutting** – relating to sectioning of a stain on clothing (or postage stamps or envelope flaps) for direct extraction.
- **Tape lifts** – usually used for collection of trace DNA such as on clothing, fabrics or paper. Clear adhesive tape is generally used to collect surface material.
- **Swabbing** – used for collection of trace DNA from smooth, non-absorbent surfaces (combination of a wet [70% ethanol or sterile water] and a dry swab).
- **Scraping** – usually relating to fingernail scrapings (samples from each hand collected separately).

Samples pertaining to DVI are usually sent by the pathologist at the scene to the forensic biology laboratory. Samples received are usually in the form of muscle tissue, bone and teeth. Depending on the sample, a section is usually taken from within the cleaned sample, such as bone marrow or flesh, with any charred sections removed. In the case of DVI, the biological evidence is provided by the coroner and the majority of time is therefore spent cleaning, documenting and sampling for DNA analysis.

1.8.2 DNA extraction

Ling (2001) described the criteria for an effective DNA isolation method as maintaining purity, integrity and yield of the DNA. In addition, it was stated that three conditions must be satisfied to achieve this:

- Cell lysis. DNA is released from the cellular and nuclear membrane. Cell lysis can be achieved utilising protein digestion enzymes such as Proteinase K (Walsh *et al.* 1991), chaotropic reagents such as guanidinium chloride or guanidinium isothiocyanate to digest enzymes, dithiothreitol (DTT) to reduce disulphide bonds and unwind the DNA molecule, and detergents such as sodium dodecyl sulphate (SDS) to disrupt the cell membrane.

- Nuclease inactivation. Deactivation of degradative enzymes is achieved by utilising SDS (denature enzymes), ethylenediaminetetracetic acid (EDTA) (ion scavenging), Proteinase K (digest enzymes), and chaotropic reagents (digest enzymes)
- Purification of DNA (isolation from non-DNA components). This involves purification of the DNA by separation from contaminants such as protein and non-protein material that may interfere with downstream processing. This may be achieved through filtration, binding the DNA to an absorbent, or by liquid separation (Ling 2001).

Consideration must also be given to the time required for the procedure, the level of labour involved, cost and resource efficiency, and analyst safety.

Traditionally, there are two principal DNA extraction techniques utilised in the DNA analysis of forensic biological material: Chelex® 100 extraction and organic extraction. These DNA extraction techniques will be discussed in the following section, in addition to the novel application of solid-phase extraction (SPE) in forensic biology.

1.8.2.1 Chelex® 100 extraction

Chelex® 100 resin (Bio-Rad Laboratories, Hercules, USA) is a chelating resin composed of styrene divinylbenzene co-polymers containing paired iminodiacetate ions (Walsh *et al.* 1991). The iminodiacetate ions act as chelating groups that have a high affinity for divalent metal ions such as magnesium (Walsh *et al.* 1991). These divalent metal ions are responsible for activating nucleases that destabilise and degrade the DNA molecule, and therefore their removal is paramount to obtaining a stable DNA extract. The Chelex® 100 resin extraction procedure results in a stable single-stranded DNA extract with metal ions bound to the resin (Walsh *et al.* 1991). The DNA is typically extracted in a 5% suspension of Chelex® 100 resin, in a stable solution of TE Buffer (10mM Tris-HCl 0.1mM EDTA pH 8.0). It is a simple procedure that can be performed within a relatively short time frame (usually 2 hours for 24 samples), with minimal equipment (Class II laminar flow hood, heating block, microcentrifuge, and pipettes)

and low cost, non-hazardous reagents (e.g. deionised H_2O, Chelex® 100 resin, TE Buffer). Phipps *et al.* (2006) have demonstrated automation of the Chelex® 100 resin procedure for reference samples on FTA paper, although this application has proven difficult to implement (Phipps *et al.* 2006).

The Chelex® 100 resin extraction procedure does, however, present certain limitations regarding its suitability for application to forensic samples contaminated with radioactive materials. The Chelex® 100 resin specifically binds only divalent ions, and therefore may be limited to radionuclide salts that include americium oxide and strontium salts such as strontium chloride, strontium fluoride, and strontium titanate, but not to monovalent ion salts such as caesium chloride. While Chelex® 100 does have the capacity to bind some radioactive materials, the presence of both radioactive contaminants and cellular metal ions may saturate the resin and require the sample to undergo further purification to remove all contaminants from the extract. A typical protocol, such as dialysis and/or concentration with Microcon® YM-100 (Millipore, Billerica, USA), can be time consuming and requires extensive handling and manipulations of the sample, which not only increases the risk of error or contamination but also places the analyst at higher risk of exposure to radioactive materials.

Microcon® filtration units (Millipore, Billerica, USA) are designed to desalt, purify and concentrate DNA samples (Millipore 1998). The filtration units contain a low binding anisotropic hydrophilic regenerated cellulose membrane that allows molecules smaller than the designated size to pass through. The exclusion size of the membranous filter can range from 3kDa, 10kDa, 30kDa, 50kDa, and 100kDa, allowing molecules of a particular size to pass through while capturing the DNA on the membranous filter. The smaller sized filters allow for degraded or minute traces of DNA to be captured in the filter for purification (Millipore 1998). The cellulose membrane requires rinsing prior to use to equilibrate the membrane and to assess the flow of liquid through the filters. The DNA binds to the membrane and the washing of the DNA strands allows for the removal of radioactive material. Micro-concentration, however, is again time consuming and extremely labour intensive, with high risk for sample and analyst contamination.

Alternatively, an increase in the concentration of the Chelex® 100 resin in the extraction suspension may accommodate the increase in metal ion concentration due to the presence of radioactive ions, and also improve the likelihood of scavenging ions in the sample (Sweet *et al.* 1996; Kalamár *et al.* 2000). However, the concentration of Chelex® 100 may only be increased by up to 20%, after which using the suspension becomes too viscous to pipette.

In addition, contaminants such as haemoglobin, cellular debris and external contaminants, such as soil and indigo dyes from denim, are not removed as the procedure does not allow for isolation of the DNA from these contaminants (which are known to interfere with downstream analysis).

1.8.2.2 Organic extraction (with ultrafiltration concentration)

Organic extraction is used for the purpose of extraction and purification of the DNA from other cellular components such as lipids, polysaccharides and proteins (Cattaneo *et al.* 1995; Alonso *et al.* 2001; AFP 2005). As a preliminary step, a digest buffer containing Proteinase K and DTT is added to the sample to denature proteins and reduce disulphide bonds, respectively, and EDTA is added to act as a metal ion chelator to decrease nuclease activity. The digest buffer acts to lyse cells, break down cell membranes and degrade proteins and is followed by addition of phenol/chloroform/isoamyl alcohol (PCIA). PCIA also contains the antioxidant compound 8-hydroxyquinoline, a weak metal chelator to inhibit nuclease activity.

After centrifugation, lipids and other non-polar molecules separate into the organic phase, while the double-stranded DNA partitions into the aqueous phase. The aqueous phase then undergoes purification and concentration using Microcon® YM-100 filtration units (Millipore, Billerica, USA), after which the supernatant is directly added to PCR reagents. This type of extraction procedure is suitable for tissue and bone, and is very effective at obtaining high concentrations of high molecular weight DNA.

The organic extraction procedure, however, does present several limitations with respect to its potential use as a method to remove radiological contaminants. The process can be time consuming (up to 2–3 days depending on in-house laboratory validations) and complex, and involves the use of hazardous materials such as phenol, which causes severe burns on contact, and chloroform, which is highly volatile and toxic. These materials pose a significant risk to the analyst. In addition, inorganic radionuclides will partition into the aqueous layer with the DNA, and will therefore require subsequent purification and concentration.

Alternative measures may include the addition of another organic layer with a density greater or less than that of water. For example, Alonso *et al.* (2001) use n-butanol, which is sparingly soluble in water and less dense than water (density = 0.809 to 0.813 g/cm^3), to further purify samples. However, this adds an additional step and includes another toxic chemical that may simply increase the level of risk to the analyst without distinct or demonstrable advantages.

It is also possible that the radioactive salt will form a separate layer under centrifugation (e.g. caesium chloride in viral protein extractions forms a layer below the aqueous phase), and it may be possible to remove the supernatant containing the DNA. However, the sample will still require micro-concentration or ethanol precipitation to reduce the volume and increase the DNA concentration.

There are many alternative, previously published DNA extraction methods, including incubation in various extraction buffers to release DNA from the cellular matrix followed by separation of the DNA from the cellular matrix. The DNA can be extracted using liquid-liquid extraction protocols, such as the organic or phenol-chloroform extraction followed by concentration with ethanol and isopropanol precipitation or using micro-concentrators, separation with glass-milk or silica suspension, or silica pellet (Prado *et al.* 1997; Hoff-Olsen *et al.* 1999; Vigilant 1999; Prado *et al.* 2002; AFP 2005)

The extraction of DNA from a sample may be conducted using a variety of methods, which in themselves have variations specific to the conducting laboratory performing the analyses or to the items extracted. For example, for the extraction of DNA from bone and tooth, proteinase K, DTT and SDS are used for enzymatic digestion, typically in association with concentrated solutions of EDTA to remove calcium salts that have leeched out of the bone.

1.8.2.3 New directions in forensic DNA extraction techniques: Solid-phase extraction

Neither the Chelex® 100 resin nor the organic (PCIA) method are suitable for both tissue/bone and trace extraction. Chelex® 100 resin extraction cannot process the cellular debris and other contaminants present in tissue or bone samples, and the organic extraction of trace DNA and saliva is laborious, time consuming, less effective and potentially hazardous to the analyst.

Furthermore, experiments comparing the phenol-chloroform and Chelex® 100 techniques concluded that, although the Chelex® 100 method was simple and fast, inhibitory substances are not eliminated in most of the cases (Kalamár *et al.* 2000).

An alternative is the use of solid-phase extraction (SPE) systems. Solid-phase extraction techniques have been available for many years; however, it is only recently that the method has been applied in the field of forensic biology. These techniques can be used in a wide variety of fields ranging from toxicology to immunology, and can be applied in the isolation of numerous chemicals, proteins, ribonucleic acid (RNA), and DNA molecules. Silica-based extraction methods have also been employed in microchip-based cell lysis and DNA extraction from sperm cells, as well as more traditional laboratory bench extraction of mitochondrial DNA (Prado *et al.* 1997; Bienvenue *et al.* 2006).

The silica particle based technology was first demonstrated by Boom *et al.* (1990) as a potential method for the simultaneous extraction and purification of DNA. Prado *et al.* (1997) and Vogelstein and Gillespie (1979) have successfully demonstrated the application of silica particles to the purification of DNA after extraction. The authors advocated the use of silica particles as a means of removing potential contaminants in aged bone to produce useful quantities of nuclear and mitochondrial DNA for analysis.

Newer methods utilise paramagnetic material to isolate DNA from surrounding contaminants (Smith and York 2000; Baker 2002). Atoms that have a single unpaired electron are paramagnetic. The electrons in such material are weakly attracted to magnetic poles (e.g. hydrogen, lithium, iron).

In addition, these solid-phase extraction systems have significant overall benefits including: the combined purification and extraction within a single procedure; amenability to automation; quick processing time; and, increased analyst safety due to the avoidance of harsh chemicals.

DNA IQ™ system

The DNA IQ™ system (Promega Corp, Madison USA) is a relatively new solid-phase extraction and purification technique that utilises paramagnetic silica beads as the DNA binding surface. The DNA binds to the paramagnetic silica beads under high concentrations of chaotropic salts such as guanidine isothiocyanate (a potential health hazard), guanidine hydrochloride, sodium iodide and/or sodium perchlorate (Vogelstein and Gillespie 1979; Boom *et al.* 1990; Prado *et al.* 1997; Smith and York 2000; Smith and York 2002; Tereba *et al.* 2004). It is theorised that chaotropic salts of high ionic strength encourage denaturation of proteins and nucleic acids by disrupting hydrogen-bonding networks (i.e. denature proteins and lyse cells) (Melzak *et al.* 1996; Tian *et al.* 2000; Tereba *et al.* 2004). This results in the proteins and nucleic acids being more thermodynamically stable in their denatured form. At higher ionic strength, the negative charge around the silica is reduced, decreasing the electrostatic repulsion with the DNA

molecule, and driving the two together. Alternatively, it has also been proposed that under a high salt concentration, a positively-charged ion forms a salt-bridge between the negatively-charged silica and the negatively-charge phosphate in the DNA backbone to assist in binding. In either case, when the chaotropic salts are removed and a low salt buffer is added, the DNA is released from the silica beads.

The DNA IQ™ procedure purifies the DNA extract by utilising the paramagnetic nature of the beads. While the beads do not have a magnetic field of their own, when one is applied the beads form a dipole and are able to be drawn out of solution with or without the DNA bound. The beads are composed of an absorptive surface of hydrous siliceous-oxide that is known to bind DNA with greater than 95% efficiency (Smith and York 2000; Smith and York 2002). Nucleic acids bind well to the silica surface of the beads but proteins such as exonucleases and other deleterious material do not bind.

The greatest advantage that the DNA IQ™ system provides is the ability to simultaneously extract and purify the DNA in a single step. Essentially, the system provides a solid support for the DNA to be adsorbed, while removing chemical interferents, cellular debris and other contaminating materials. In addition, this process can be applied to samples such as trace DNA residues, blood, bone, and tissue, as well as a large range of substrates, such as chewing gum and denim (Promega 2002b; Komonski *et al.* 2004; Lazzarino *et al.* 2008). The process can be completed in a relatively short time frame (1–2 hours) and is amenable to automation after initial sample preparation.

Potential disadvantages include manual processing, as the system provides for a maximum of twelve samples at a time. However, if dealing with a radioactive contamination and depending on the radioactive contaminant and the dose emitted by each sample, the number of samples that may be simultaneously extracted may be deliberately kept small. In addition, as the extraction system utilises salts to encourage DNA binding to the silica, the presence of radioactive metal salts in certain concentrations may alter the efficacy of the DNA binding and extraction.

Despite the potential limitations, the DNA IQ™ system has been successfully applied to the extraction of biological samples contaminated with chemical warfare agents (Wilkinson *et al.* 2007). The applications and efficacy of the DNA IQ™ system is further explored in Chapters 2, 4 and 5.

ChargeSwitch® system

ChargeSwitch® technology utilises a similar selective capture and release of DNA from a solid phase paramagnetic bead as the DNA IQ™ system. However, in the ChargeSwitch® model, the paramagnetic polypropylene beads are covalently coupled to polyamines, polyhistidine, or any compound containing an imidazole moiety that is able to reversibly bind DNA at variable pH. Figure 1.2 is provided to illustrate this procedure.

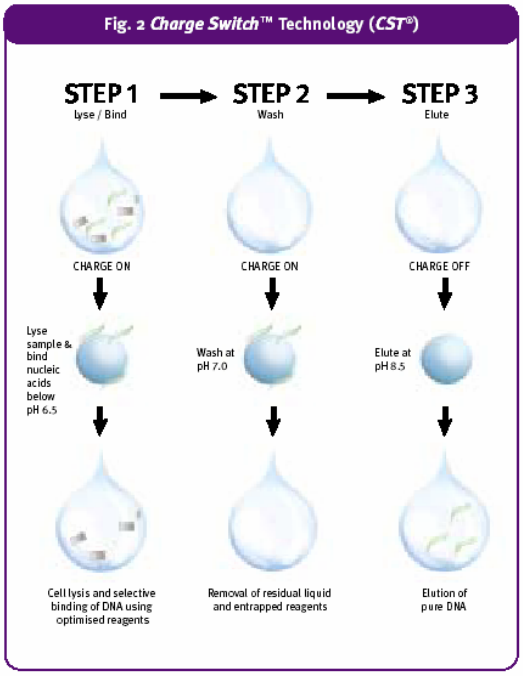


Figure 1.2: ChargeSwitch® Technology (Source: Invitrogen 2005)

At low pH (pH 4–6), the imidazole moiety has a positive charge, so negatively charged nucleic acids are able to bind to the beads in preference to any other contaminants in the biological material. Proteins and other contaminants are not bound and are washed away using the wash buffer. The DNA is then released at a higher pH (around pH 8), above the pKa of the polyhistidine, to reverse or neutralise the positive charge on the moiety (Baker 2002; Invitrogen 2005).

As previously stated, the benefits of such technology include amenability to automation, time efficiency, analyst safety (i.e. ChargeSwitch® does not use hazardous reagents such as: phenol, chloroform, ethanol, isopropanol, and ionic chaotropes such as guanidinium isothiocyanate), and a single purification and extraction step. However, unlike the DNA IQ™ system, the procedure does not provide for the extraction of bone/tissue samples.

1.8.3 DNA quantitation

In order to successfully amplify the DNA template using the polymerase chain reaction (PCR), a certain quantity of DNA must be added to the reaction. DNA quantitation techniques are utilised to provide an estimate of the quantity of the DNA in a sample, in addition to ensuring the sample contains DNA from human/primate origin. There are a number of techniques available for this process, with examples including the slot-blot procedure, fluorescence-based micro-titre plate assays and real-time/quantitative PCR (Nicklas and Buel 2003). All systems provide an estimate of the DNA quantity in the sample. The technique utilised in this research is real-time PCR and will be discussed further.

Quantitation using real-time PCR (fluorescence reporter probe method) involves the fluorescence detection of a specific site in the human genome as it is amplified by PCR (AFP 2006). As described further in Section 1.8.4, PCR involves the exponential amplification of a specific segment of DNA between two fluorescently tagged primers. In real-time PCR, the primers are not tagged, instead a DNA probe with a reporter and

quencher tag are utilised (AFP 2006). The probe binds the segment of DNA between the primers. When the quencher dye is in close proximity to the reporter dye, the reporter dye's fluorescence is not detected. However, as the segment is amplified, the reporter dye is released from the probe due to the action of the polymerase and the fluorescence can be detected. The real-time PCR process not only satisfies quantitation and human/primate specificity but also provides an indication of the likely DNA profiling success as a PCR amplification may fail due to insufficient DNA, highly degraded DNA and/or the presence of inhibitors (Section 1.3.2). The limit of detection for real-time PCR is between 1 and 20 pg DNA. However, for optimal measurement of DNA quantity, samples should be analysed in triplicate and an average calculated. For a review of DNA quantitation techniques used for forensic samples see Nicklas and Buel (2003).

1.8.4 DNA amplification

Within eukaryotic genomes, there are regions of DNA in which a single base or short sequence of bases are tandemly repeated (Jeffreys *et al.* 1985c). These repeat sequences are principally found in non-coding regions of the DNA and are therefore usually variable between individuals. The two types of human genomic DNA sequences that are used for DNA profiling are minisatellite DNA (Jeffreys *et al.* 1985c) and microsatellite DNA (Carracedo and Sánchez-Diz 2005), and are differentiated by the number of bases in the repeat unit. Microsatellite DNA regions contain repeat units of between 1 to 10 base pairs, whereas minisatellite DNA regions contain between 10 and 100 base pairs (Butler 2005).

It is now generally accepted in the scientific community that genetic typing of microsatellite DNA represents the most promising approach for DNA profiling. Microsatellites, or Short Tandem Repeat (STR) sequences, have short core repeat units, whose allelic variants are repeated up to 50 times at any given locus (Fowler *et al.* 1988). Therefore, at any given STR locus, the degree of polymorphism is high and can vary greatly between individuals. The purpose of STR fragment analysis is not for

individualisation (Fowler *et al.* 1988). As a result, DNA analysis with STR sequences is termed DNA profiling rather than DNA fingerprinting, and relies on statistical data of allelic frequency within a reference population.

STR loci occur approximately every 6 to 10kb and are typically evenly dispersed throughout the human genome, which makes them ideal genetic markers (Carracedo and Sánchez-Diz 2005). Each STR is a single locus with multiple allele sizes, and the genetic variation between individuals is based on the number of tandemly repeated core units.

STR loci are classified based on the composition of repeat units and are categorised as simple, compound and complex (Krenke *et al.* 2002; Carracedo and Sánchez-Diz 2005). The 'simple repeat' STR loci contain units of identical length and sequence, 'compound repeat' STR loci contain two or more units of simple repeats, and 'complex repeat' STR loci contain units of variable length with variably interspersed sequences (Weber and May 1989; Edwards *et al.* 1991). Alleles are assigned designations based upon the number of repeat units at a particular locus; for example, the D5S818 allele 9 contains nine repeat units of AGAT. Alternatively, microvariant alleles contain incomplete repeat units. An example of a microvariant is allele 39.2 of the D18S51 locus, which contains 39 repeat units of AGAA, in addition to one incomplete unit of two nucleotides (AA).

An advantage of utilising STR loci for DNA analysis is that is also allows for very small amounts of DNA to be analysed via PCR. PCR is utilised to exponentially amplify the fragment of DNA to be tested by an amplification factor of up to 2^{10}–2^{20} (Butler 2005). PCR is an *in vitro* method in which a specific segment of DNA is rapidly replicated between two labelled synthetic oligonucleotide primers, producing large quantities of that segment of DNA. Since the bases flanking the STR repeats are highly conserved, STR-specific primers can be readily designed. The alleles of a microsatellite locus are differentiated through variations in the number of tandem repeats, so the ability to recognize these alleles is based on the differences in molecular weight due to the number of tandem repeats present at the locus. Alleles are separated and identified by

the highly sensitive process of capillary electrophoresis, which separates the alleles, with up to single base pair distinctions, based on the fragments' molecular weight (Buel *et al.* 1998).

Use of these small polymorphic sequences, combined with sensitive PCR–based amplification of the STR locus, produces amplified products between 50 and 500 base pairs in length (Weber and May 1989; Edwards *et al.* 1991). The analysis of STR loci is therefore more tolerant of degraded DNA templates, as the smaller size allows for partially degraded and minute quantities of genomic DNA to be analysed, without compromising the allelic diversity. In addition, PCR amplification is highly suited for samples containing minute quantities of degraded DNA because relatively few target sequences need to be intact. Thus, STR sequences, combined with PCR-based sequence amplification provide a means for accurate genetic typing, with allelic variants separated and visualised through capillary electrophoresis (Buel *et al.* 1998).

1.8.4.1 Genetic typing using multiplex systems

Over the past decade, the use of multiplex systems for genetic typing utilising STR loci has come to the fore (Chakraborty *et al.* 1999). This has been demonstrated through the implementation of these systems within the forensic science community in countries around the globe.

The ability to differentiate between individuals relies on the analysis of several loci. Multiplex DNA typing systems involve the use of multiple sets of PCR primers to target STR sites throughout the genome (Clayton *et al.* 1995a). By labelling the primer for each locus with a particular fluorescent dye, it is possible to specifically identify each locus fragment and assign the correct designation. A large number of these multiplex STR systems are available as commercial kits for routine use in forensic laboratories.

Table 1.5 Descriptions of the Amp*Fl*STR® Profiler Plus® Amplification Kit loci (Applied Biosystems, USA)

STR Locus	Chromosomal Location	GenBank® Locus and Locus Definition	Repeat Sequence	Size Range (bp)	Reference
Amelogenin	Xp22.1-22.3 and Yp11.2	HUMAMEL, Human Y chromosome gene for Amelogenin-like protein	NA	106, 112	(Sullivan *et al.* 1993)
FGA	4q28	HUMFIBRA, Human Fibrogen Alpha Chain gene	TTTC Complex	322-444	(Mills *et al.* 1992)
vWA	12p12-pter	HUMVWFA31, Human von Willebrand Factor gene	TCTA Complex	123-171	(Kimpton *et al.* 1992)
D3S1358	3p	NA	TCTA Complex	115-147	(Li *et al.* 1993)
D5S818	5q23.3-32	NA	AGAT	119-155	(Lins *et al.* 1998)
D7S820	7q11.21-22	NA	GATA	215-247	(Lins *et al.* 1998)
D8S1179	8q	NA	TCTA Complex	203-247	(Barber and Parkin 1996)
D13S317	13q2-q31	NA	TATC	169-201	(Lins *et al.* 1998)
D18S51	18q21.3	HUMUT574	AGAA (21)	290-366	(Staub *et al.* 1993; Barber and Parkin 1996)
D21S11	21q11-21q21	HUMD21LOC	TCTA Complex	203-259	(Sharma and Litt 1992)

Table derived from Butler (2005)

The desire to gain more information from a single DNA sample, and the need to restrict the consumption of a DNA sample where its availability may be limited, has lead to systems in which there is co-amplification and typing of at least nine STR loci in a single reaction, as well as analysis of the amelogenin locus to permit sex determination. An example is the Ampli*Fl*STR Profiler Plus® system (Applied Biosystems, USA),

which is used routinely by government forensic science laboratories across Australia (Table 1.5).

Other multiplex typing kits are also available, such as the PowerPlex® 16 System (Promega, USA) and Identifiler (Applied Biosystems, USA), which contain 15 STR loci to increase discriminatory power. The inclusion of additional loci makes it possible to increase the significance of a DNA profile and thus bolster the evidential value.

Furthermore, new multiplex systems are currently being developed to recover information from smaller regions of DNA, which are more likely to be intact following DNA damage (Butler *et al.* 2003; Coble and Butler 2005; Hill *et al.* 2008; Mulero *et al.* 2008).

Table 1.6 Comparison of miniSTR allelic information for loci analysed with the Profiler Plus® Amplification Kit

STR Locus	Allele Range	Allele Spread (bps)	PP® Product Size (bps)	MiniSTR Product Size (bps)	Size Reduction (bps)
Amelogenin	X, Y	6	106, 112	N/A	N/A
D3S1358	8-20	48	97-145	72-120	25
D5S818	7-16	36	134-170	81-117	53
D7S820	5-15	40	253-293	136-176	117
D8S1179	7-19	48	123-171	86-134	37
D13S317	5-16	44	193-237	88-132	105
D18S51	7-27	80	264-344	113-193	151
D21S11	24-38.2	58	186-244	153-211	33
FGA	12.2-51.2	156	196-352	125-281	71
vWA	10-25	60	152-212	88-148	64

PP® = Profiler Plus ® amplification kit
N.B: Table adapted from Butler *et al.* (2003). Further miniSTR information, including primer sequences, can be obtained from Butler *et al.* (2003).

These new systems, called miniSTRs, utilise PCR primers closer to the STR repeat region producing shorter amplicons. Table 1.6 provides a selection of currently available miniSTRs that correspond to loci analysed by the Profiler Plus® amplification kit (Applied Biosystems, USA). MiniSTRs have been developed by private and commercial groups, with commercial kits, such as MiniFiler® (Applied Biosystems, USA) already available.

In addition, the development of new DNA analysis techniques such as single nucleotide polymorphisms (SNPs), where variations in single base pairs are used to differentiate between individuals, or whole genome amplification are also being investigated to determine the possibility of enhancing PCR amplifiable material from limited or damaged DNA templates.

1.8.5 DNA fragment analysis with capillary electrophoresis (CE)

Amplification of STR alleles in a multiplex reaction produces a mixture of DNA fragments that require separation and detection for analysis. Within forensic biology, fragment separation and detection can be conducted with CE (Buel *et al.* 1998). In this method, DNA fragments are separated through a liquid polymer in a capillary, which acts as a molecular sieve and separates DNA fragments by size (molecular weight) (Buel *et al.* 1998). CE utilises the negatively-charged phosphate groups on the DNA backbone to mobilise the DNA, and when placed in an electric field, DNA fragments migrate towards the anode. Samples then move through the capillary with smaller fragments travelling further as there is less resistance from the polymer.

Once the DNA fragments have separated, they are then detected by excitation of the fluorescent labels incorporated during amplification (Buel *et al.* 1998). As each sample will exhibit slightly different electrophoretic properties, the same alleles will not always travel the same distance along the capillary, for the same analysis time. To standardise across samples, an internal size standard is added to each sample as a reference marker and is used to estimate the fragment size in base pairs. In addition, an allelic ladder is

also utilised and provides fragments of almost all possible alleles for each locus analysed. It is used to compare the unknown alleles present in the sample with the known alleles of the ladder, and determine its allelic designation (representing the number of repeat units). Once the samples are run, specialised software is utilised to process the STR data, calculating the size of each fragment and applying an allelic designation to each peak. An example of a processed DNA profile is illustrated in Figure 1.3, and represents a typical DNA profile produced with the Profiler Plus® amplification kit.

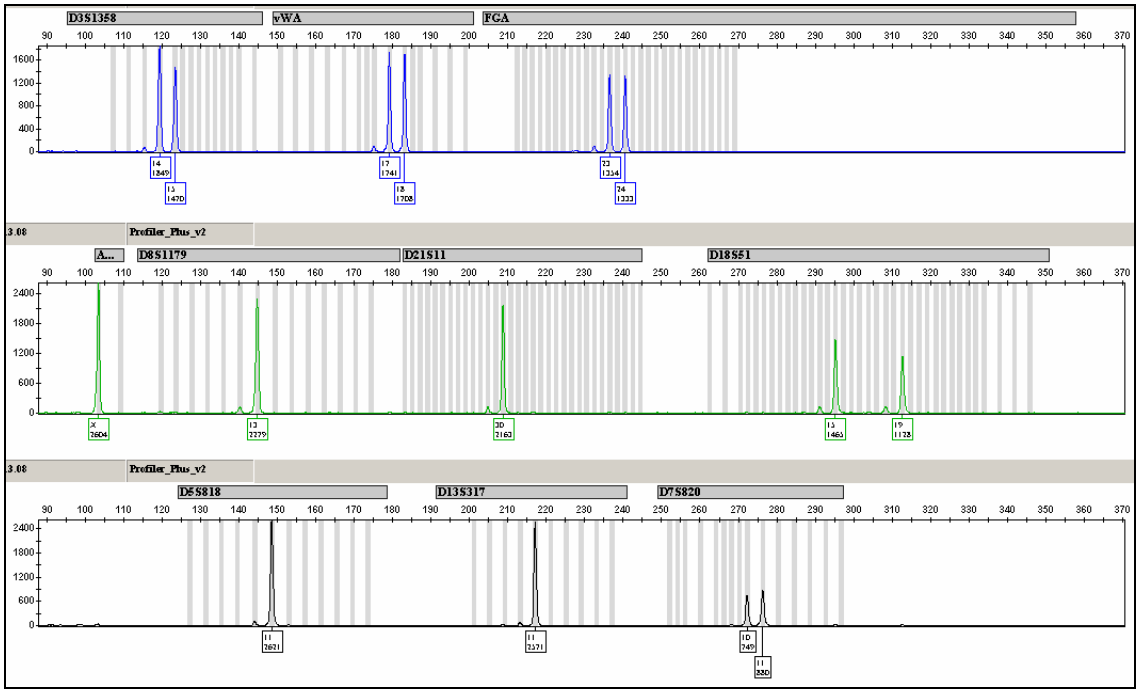


Figure 1.3: DNA profile generated from the analysis of 9947A genomic standard control with Profiler Plus®

Three fluorescent dyes are utilised for the analysis of 9 STR loci in addition to the amelogenin marker. Each locus is represented by one (homozygote) or two (heterozygote) peaks measured in relative fluorescent units (RFU).

1.8.6 DNA profiling and interpretation

The process of interpreting DNA profiles produced from the PCR and capillary electrophoresis systems, involves careful consideration of a number of characteristic features or artefacts that may be generated. These include stutter products, heterozygote imbalance, allelic dropout, and non-specific artefact peaks, as well as N and N+1 peaks "pull-up" and null-alleles.

Stutter products are a common feature in DNA profiles. When STR loci are amplified by PCR, a small PCR product, four bases shorter than the corresponding main allelic position, is generated (Leclair *et al.* 2004b; AFP 2008a). Stutter products are readily identified in a DNA profile, as they have a reduced peak intensity compared to the true allele. Stutter percentages (i.e. the percentage of the stutter peak relative to the true allele) are provided with each multiplex kit for specific loci (Applied Biosystems 1997; Promega 2002c). Stutter products are thought to result from strand-slippage of the *Taq* polymerase during the copying phase of the PCR process.

Heterozygote imbalance describes the condition where the peak heights or area of two peaks within a heterozygote locus are significantly different (Gill and Buckleton 2005; AFP 2008a). Heterozygote imbalance typically occurs where the starting template is low in concentration or is degraded, and there is variation in the sampled templates. This difference can be identified through calculations, with detailed information provided by Gill and Buckleton (2005).

Allelic dropout defines the phenomenon whereby one allele of a heterozygote cannot be visualised/detected (Gill and Buckleton 2005; AFP 2008a). Allelic dropout occurs when peak heights are very low, as a result of low starting template of DNA, DNA degradation, or the presence of inhibitors during amplification. Locus dropout is an extension of this condition where both alleles of a heterozygote, or the single allele of a homozygote cannot be visualised.

Non-specific artefact peaks are typically displayed when analysing degraded DNA samples, however they may also be visualised in unaffected DNA samples due to instrumentation (AFP 2008a). They are identified by poor peak morphology (shape), generally as narrow spikes or wide blobs. Under normal conditions, non-specific artefacts are caused by an electrical spike during analysis, air bubbles in the liquid polymer of the capillary, degraded polymer, or dust in the detection instruments. In addition, degraded or contaminated DNA samples may contain interfering fluorescent materials (i.e. substances that fluoresce in the visible region of the spectrum, around 500 – 600 nm) such as textile dyes and chlorophyll from plant extracts (Butler 2005). Bone or tooth samples with incorporated tetracycline-group antibiotics can also cause non-specific artefact peaks and interfere with DNA typing (Butler 2005).

There are also a number of characteristics that result from the addition of excessive DNA in the PCR amplification reaction (Gill and Buckleton 2005; AFP 2008a). N and N+1 peaks typically occur when the amplification reaction contains too much DNA template. During PCR amplification, the DNA polymerase catalyses the addition of an adenine nucleotide to the 3' end of the PCR product; however, when excessive DNA template is present, there is insufficient adenine nucleotide reagent in the reaction to complete this process. As a result, the allele of interest will be represented by either a broad peak with poor morphology or as two peaks separated by a single base pair (split-peaks).

"Pull-up" in a DNA profile is also caused by excessive DNA template in the amplification reaction (Gill and Buckleton 2005; AFP 2008a). Pull-up peaks occur when the fluorescence from a peak is so intense that it "bleeds though" to the fluorescence of a different colour i.e. the spectral matrix of the detection system cannot fully correct for the overlap in the fluorescent dyes. Pull-up peaks can be identified by excessively high RFU values and when a minor peak with one fluorescent dye corresponds to the same position as major peak with another fluorescent dye. One consequence of having pull-up peaks is that they may obscure peaks in other dye groups.

Null-alleles, or non-amplifiable alleles, arise when there are mutations in the primer-binding regions of the STR locus (Gill and Buckleton 2005; AFP 2008a). These mutations do not allow the primers to bind effectively and therefore prevent PCR amplification of the allele. As a consequence, loci that would normally contain two peaks, representing two alleles, would only present a single peak i.e. a false homozygote. However, null-alleles typically only become an issue when comparing two DNA profiles that were analysed with different primer sets or when establishing allele frequencies within a population.

In addition to the above phenomena, there may also be triallelic patterns, which are generated by the presence of three distinct alleles or peaks within a locus (Gill and Buckleton 2005; AFP 2008a). Triallelic patterns are typically the result of genetic abnormalities where there are extrachromosomal fragments present in the sample (e.g. trisomy 18), when chromosomal translocation has occurred (i.e. where there is a rearrangement of segments between chromosomes of different pairs), or where primers anneal to additional areas on the genome as a result of locus duplication.

Finally, important factors to consider when interpreting an electropherogram include the baseline quality in relation to peak height and the overall quality of the profile and the capillary run itself.

Following the correct assignment of alleles, the statistical weighting of the DNA profile for a relevant population must be ascertained. To this end, allele and genotype frequency data from relevant populations are utilised to determine the frequency of the profile in the population and, thus, determine the evidentiary value of the profile (Aitken 1991; Chakraborty *et al.* 1999; Holt *et al.* 2000).

1.8.6.1 Implications of degradation on DNA profiling

Many DNA samples encountered in a forensic context are insufficient, mildly degraded or severely degraded. Damage to the DNA molecule occurs either through chemical

changes to the physical structure, such as cross-linkage of DNA base pairs which blocks the polymerase action during polymerase chain reaction (PCR) amplification, or through fragmentation of the DNA molecule, which prevents strand elongation during DNA amplification (Clayton *et al.* 1995b; Chung *et al.* 2004). Similarly, the presence of contaminants, such as indigo dyes from denim or heme from blood, can inhibit the enzymes involved in PCR and prevent amplification from occurring. The impact of DNA degradation or inhibition due to the presence of a contaminant is typically reflected in the DNA profile.

Poor amplification of the loci of interest results in peak height reduction or allelic drop-out, producing a typical "decay curve" or "ski slope" effect where peak height is inversely proportional to the amplicon length (Chung *et al.* 2004). Large loci can fall below the detection threshold on a capillary electrophoresis instrument, resulting in a partial genetic profile (incomplete DNA profile). Where the DNA is degraded or an inhibitor is present, higher molecular weight loci (300–500 bps) in standard multiplex typing kits are commonly affected.

Allelic dropout is an expected occurrence in profiles generated from degraded DNA. This may be affected by the total amount of DNA and/or the presence of intact DNA template (stochastic effects) (Prinz *et al.* 2002). If high molecular weight DNA is present, then allelic dropout will only occur if the total amount of DNA present is low, and has little to do with whether or not the DNA is degraded. Alternatively, if there is significant DNA template present but it is highly degraded, i.e. there is only low molecular weight DNA present, then allelic dropout will also occur.

1.9 Contribution of this research

The presence of both traditional forensic evidence, such as biological material, and radiological material is a probable scenario in investigations related to the illegal handling and/or malevolent use of nuclear and radioactive materials. From the discussion presented in this chapter, it can be seen that very little information exists in

the current literature pool that addresses the issues surrounding radiologically-contaminated DNA evidence. Specifically, information is lacking regarding the characterisation of the effects of ionising radiation on forensic biological samples. Furthermore, there are no previously reported analytical strategies that address the extraction of DNA in the presence of radioactive contaminants or the decontamination of critical biological matrices, such as blood, bone and saliva.

This research addresses these gaps through the investigation of radiation effects and novel extraction methodologies. Therefore, the principal aims of this research were to:

- Investigate the effects of gamma (Chapter 2) and alpha (Chapter 3) radiation on forensically-relevant biological samples to identify dose thresholds and the potential impact of delayed analysis on DNA profiling;
- Examine conventional and novel extraction systems for their ability to remove a representative non-radioactive contaminant and explore the impact of the contaminant on DNA extraction efficiency (Chapter 4); and,
- Conduct extraction studies utilising radioactive material to determine efficiency of contaminant removal and DNA recovery (Chapter 5).

The following chapters present the overall findings and provide unique guidance for institutions or agencies planning to implement strategies for handling and processing radiologically-contaminated DNA evidence.

Chapter 2: Effects of Gamma Radiation on DNA Profiles Obtained from Forensically-Relevant Biological Matrices

2.1 Introduction

The production of a DNA profile from biological material found at a crime scene or mass disaster can be instrumental in the identification of deceased/human remains (e.g. disaster victim identification) and the prosecution of offenders. The identification of a perpetrator may not only be useful for criminal proceedings but also for intelligence gathering, revealing links to other members of a terrorist group or possible collaborators/suppliers, for example, which may include the illicit trafficking of radioactive materials.

However, the successful production of a DNA profile can be hampered by exposure of the DNA to extreme conditions such as high temperatures, moisture, soil, fire, acidic or alkaline pH, UV light, or contamination with bacteria resulting in degradation of the molecule (McNally *et al.* 1989; Graw *et al.* 2000; Hoff-Olsen *et al.* 2001). In addition, successful production of a DNA profile can be inhibited by the presence of contaminants in the sample such as coloured dyes, activated proteinases, or high levels of haemoglobin, which can result in degradation of the sample or may compromise PCR amplification during analysis.

2.1.1 Ionising radiation

Ionising radiation presents a further challenge as it has the potential to cause physical changes to the DNA structure through the formation of single- and double-stranded breaks, DNA adducts, and/or alkali-labile sites. There are two primary mechanisms of action for ionising radiation on DNA: direct and indirect effects (Hutchinson 1985; Cullis and Symons 1986). While the nature of forensic samples suggest that direct effects are the most likely mode of action, where changes to the DNA strand are caused by direct interaction of ionising radiation with the DNA, the indirect effects of hydroxyl radical formation and attack on sugar or base units cannot be discounted.

To date, there has been limited published baseline data investigating the effects of gamma radiation on DNA from biological matrices relevant to forensic investigations. In recent years, most research has focussed on the sterilisation of mail flowing through the US Postal Service as a protective measure against terrorism with biological agents. Withrow *et al.* (2003) investigated the effects of electron beam irradiation on nuclear and mitochondrial DNA for application to decontamination of envelopes containing anthrax spores. Results from this study indicate that, at the two doses examined (29.3 kGy and 51.6 kGy), there was a decrease in quantity of DNA obtained after irradiation; however, a full profile was obtained in all cases. The study also explored the effects of exposure after a delay of 40 and 56 days before extraction and demonstrated a decrease in DNA yield after 56 days. However, this conclusion should be taken with caution as different extraction protocols were used to conduct the investigation at the two time periods (Withrow *et al.* 2003). Castle *et al.* (2003) also conducted studies using electron beam radiation for sterilisation of mail entering the US Postal Service. Buccal cells suspended in liquid were exposed to 70-97 kGy radiation and investigated for genomic yield, DNA integrity and PCR amplification success at three single tandem repeat loci (Castle *et al.* 2003). The authors found that electron beam irradiation at 70-97 kGy resulted in reduced DNA yield and DNA integrity, and concluded that assays targeting regions of DNA longer than 989 bps may be compromised.

In contrast, a recent publication by Shaw *et al.* (2008) investigated four sterilisation techniques for application to the decontamination of extraneous DNA from items used in the laboratory and at crime scenes. The study examined the effectiveness of irradiation with gamma, beta (electron beam) and ultraviolet radiation, and treatment with ethylene dioxide, for the destruction of DNA from saliva. For the purpose of the study, researchers found the most effective decontamination method to be ethylene dioxide. However, their investigation also demonstrated that, of the radiation techniques evaluated, gamma irradiation at 56 kGy was the most effective at destroying the contaminating DNA (Shaw *et al.* 2008). The authors indicated that, in all cases, a profile was obtained, with a full profile obtained in 40% of cases, a partial profile in 30% of

cases (allele designation at four or more loci), and a poor partial in 30% of cases (allele designation at less than four loci and more than one).

From these investigations, it has demonstrated that at high dose (>50 kGy) of ionising radiation can cause damage to the DNA molecule and effect the subsequent DNA profile produced. In a similar manner to degradation caused by other means, ionising radiation results in a gradual loss of alleles from the larger to smaller molecular weight ranges.

2.1.2 Biological matrices

Within a forensic context, typical biological matrices are dictated by legislation for the collection of reference samples or by what is present at the crime scene. This research sought to characterise the effects of gamma radiation on a variety of biological matrices that are relevant to forensic investigations at the crime scene. These matrices include blood, saliva and bone samples, in addition to a genomic DNA standard that is used as a benchmark for comparisons.

As discussed in Chapter 1, blood and saliva are common biological matrices found at crime scenes. These can be in the form of saliva on cigarette butts, blood on knives, as well as many others. In addition, a genomic DNA standard was examined to provide a baseline for comparing the potential impact that the presence or absence of cellular material may have on the overall effects of ionising radiation.

Bone is also a biological matrix of interest due to its use for DVI. In the event of a radiological incident where there are mass casualties, either as a result of radiation exposure or more likely collateral elements such as explosive charges or other weapon trauma, DVI procedures will be implemented. While DNA analysis is not usually the preferred mode of identification for this process (preferential modes include dental, fingerprints, and other pathological comparisons), it has featured prominently in many cases where there is a high degree of body fragmentation. For example, in the wake of

the 2001 terrorist attack on the World Trade Centre Twin Towers, US agencies attempted to identify all biological material. Due to the devastating nature of the event, fragmentation resulted from explosions and building/structural collapse, as well as charring and partial or complete incineration by intense fire. In many instances, there were very few remains left and many of the remains had commingled between individuals. DNA analysis therefore played an important role in ensuring that as many individuals were identified as possible (Prinz *et al.* 2002).

Typical biological materials available for analysis in DVI are bone, muscle tissue and teeth. In this research, the femoral head was used as a representative biological material for DVI analysis as bone is a reliable source of genetic material. The femoral head is composed of cancellous (spongy) bone which has numerous internal cavities and is highly vascularised. The fresh bone has a smooth surface and is coated in cartilage (except for the fovea capitis femoris which is the position of attachment for the ligament) (Purves *et al.* 1997).

While blood, saliva, and the genomic DNA standard are relatively straightforward matrices to investigate, bone presents a variety of experimental issues, particularly with regard to extraction efficiency.

Extraction of DNA from calcified tissues, such as bone and teeth, is traditionally difficult. Post-mortem, DNA undergoes binding interactions with the mineral component of bone, the hydroxyapatite matrix (Ye *et al.* 2004). This chemically stabilises the DNA and aids its survival over time, however, this makes extraction of the DNA more difficult. In fresh human bone, the amount of DNA bound to the hydroxyapatite matrix ranges from 1.5 – 3.0 μg/g (Tuross 1994). In addition, the bone matrix contains PCR inhibitors, such as calcium, that must be removed before amplification. Therefore, an essential part of the extraction process is to release DNA from this binding and remove inhibitors.

In this study, three methods for extracting DNA from bone were assessed to identify an effective protocol for application to the remainder of the study. The protocols assessed include two phenol/chloroform organic extraction methods (one from the AFP Biological Criminalistics laboratory's standard operating procedure, and the other identified from Alonso *et al.* (2001)) as well as one solid phase extraction protocol utilising the DNA IQ™ System, with emphasis on the influence of the initial extraction buffer on efficiency and an investigation of solid-phase versus liquid-liquid extraction.

2.1.3 Time-to-analysis

Processing a crime scene for forensic evidence has the potential to take prolonged periods of time, especially for larger incidents or scenes. In addition, the Waco incident of 1993 demonstrated the limitations placed on investigators when timely recovery of remains was hampered by fire at the compound, triggering explosions by munitions and preventing the access of emergency services and access to the crime scene (Clayton *et al.* 1995b). This issue of time-to-analysis has previously been presented in Section 2.1.1 as having potential impact on the quantity and quality of DNA obtained after exposure to ionising radiation (Withrow *et al.* 2003).

In the event of a radiological incident, time-to-analysis would also impact on the extent of radiation exposure of the evidence. Even at low dose rates, a higher integrated dose would result in extensive exposure to the evidence. This therefore warrants investigation of higher doses. In addition, establishing a threshold dose where degradation starts to occur, as well as where complete degradation of the sample occurs, can be instrumental in the planning of evidence collection and the interpretation of results.

Since the integrity of the DNA molecule underlies the ability to obtain the full information available within the DNA molecule, it is important to establish the "post-mortem" stability of DNA over various time periods (Bär *et al.* 1988). Bär *et al.* (1988) examined the post-mortem stability of DNA in various tissues specimens over 21 time periods from 0.2 to 19 days. Using RFLP analysis, a technique requiring high molecular

weight DNA (up to 23 kb), this study showed that the amount of degraded DNA correlated directly with the duration of the post-mortem period (i.e. there were increasing amounts of low molecular weight DNA observed with increasing post-mortem period). This is consistent with apoptotic events (programmed cell death) that are triggered following cell death.

2.1.4 Aims

The effects of gamma radiation on biological materials, particularly those often encountered in forensic investigations, merit further investigation. Therefore, the objectives of this experimental series were to:

1) Identify a reliable and robust technique for the extraction of DNA from bone (femoral head);
2) Investigate the effects of γ-radiation from a cobalt-60 source on DNA from a range of biological matrices – blood, saliva, bone and standard genomic male DNA;
3) Investigate the potential impact of the time-to-analysis on the qualitative and quantitative aspects of DNA profiling; and,
4) Establish limits of exposure for the analysis of gamma-irradiated (cobalt-60) biological samples.

2.2 Experimental

2.2.1 Biological matrices and standards

Blood, saliva, bone and a genomic DNA standard were used as DNA sources for this study. Donated human male blood was provided from the Australian Federal Police standards collection and stored at -20 ^{o}C until required. Donated human male saliva was collected by expectoration and stored at -20 ^{o}C until use. Bone samples were kindly provided from the ACT Bone Bank's research collection. Ethics approval for use of

human bone was obtained from the University of Canberra Committee for Ethics in Human Research (Project No. 06-95). The femoral heads were stored at -80 ^{o}C until required for experimentation. In addition, a male single source genomic DNA standard (9947A Promega, USA) was utilised as an additional sample matrix.

The DNA extraction protocols utilised: Chelex® 100 resin purchased from Bio-Rad Laboratories (USA); the DNA IQ™ System and associated reagents from Promega (USA); and phenol/chloroform/isoamyl alcohol and associated reagents for organic extractions from Sigma-Aldrich (USA). All reagents were of analytical grade.

Real-time PCR analysis was utilised for the quantitation of DNA extracts. Primers and probes were purchased from Sigma-Aldrich (USA), with additional reagents from Invitrogen (USA).

DNA profiling analysis was conducted using Amp*Fl*STR® Profiler Plus® PCR amplification kits (Profiler Plus®) from Applied Biosystems (USA). All reagents associated with the DNA profiling analysis were purchased from Applied Biosystems (USA). The DNA profiles produced for each of the sample matrices using Profiler Plus® are shown in Table 2.1.

Table 2.1: Profiler Plus® DNA profiles for the selected matrices of blood, saliva, bone, and genomic male DNA standard

Locus	Blood	Saliva	Bone	gDNA
Amelogenin	X,Y	X,Y	X,Y	X,Y
D3S1358	16,16	14,15	16,18	15,17
vWA	16,16	17,17	18,18	17,17
FGA	21,22	21,21	22,25	24,26
D8S1179	12,13	12,13	12,13	12,13
D21S11	30,31	30,31.2	30.2,31.2	29,30
D18S51	16,17	16,17	15,18	15,18
D5S818	12,13	11,13	10,12	11,13
D13S317	8,12	8,13	11,12	11,11
D7S820	7,11	9,11	10,12	11,11

2.2.2 Laboratory preparation

Ultraviolet irradiation and treatment with 10% bleach were used to limit possible DNA contamination on laboratory work areas, equipment and consumables, in addition to the analyst's use of disposable gloves and clean laboratory coats. Safety gloves and protective eyewear were worn at all times when handling liquid nitrogen and when chipping bone fragments.

2.2.3 Preparation for the analysis of bone

2.2.3.1 Special considerations

The use of a femoral head from a single individual was proposed for the following reasons:

- DNA densities vary from individual to individual; comparing the same bone type from the same individual will create less background variation in the results.

- Each individual will have different DNA profiles; the same DNA pattern is needed for comparisons across methods to detect experimental effects on subsequent DNA analysis (i.e. allelic dropout).
- Male samples were selected to permit monitoring of amelogenin alleles from both X- and Y-chromosomes.
- Each of the bones tested should be of the same time-since-death to ensure that any variation observed is attributable to the experimental variables rather than degradation due to differential ages.

In addition, bone chip samples were powdered before irradiation due to the number of samples required for analysis, the desire to homogenise bone chips across all conditions, and to limit cross-contamination of samples from different experimental conditions.

2.2.3.2 Bone sample preparation

Remnant soft tissue was excised from the femoral head using a sterile scalpel and tweezers. The exterior surfaces of the bone were cleaned with 10% bleach to remove excess blood and potential microbial contamination from the surface. The femoral head was rinsed thoroughly with warm distilled water and air dried at room temperature. All grinding components for the freezer mill were washed in bleach and 70% ethanol, and then autoclaved before use. A wet swab was taken of the autoclaved grinding components as a negative equipment control.

2.2.3.3 Bone crushing

The femoral head was chipped into small fragments (approximately 3 × 3 × 3 mm) using a chisel and hammer on a clean plastic block. To homogenise the fragments between samples, all bone fragments were placed into a clean beaker and evenly mixed. The bone fragments were then placed into the freezer mill (6750 Freezer/Mill SPEX Certiprep Inc., USA) and pulverised in the presence of liquid nitrogen. The grinding cycle followed a 7.5 minute pre-chill of the sample; 2 × 30 second cycles at 10 impacts

per second, and 2 × 2.5 minute cool down between each cycle. The pulverised bone was quickly weighed into 2 g sample batches, placed into 10 mL tubes, and stored at -80 ^{o}C prior to experimental treatment.

2.2.4 Preparation of blood, saliva and genomic DNA standard

Sterile glass slides were wiped with 10% bleach and 70% ethanol. Following exposure of the surface to UV light for 45 minutes, three 5 µL spots of either 1:2 diluted human male blood (in TE buffer), whole saliva from a male individual, or three 2.5 µL spots of male genomic single-source standard (9947A) were placed on a glass slide. Samples were placed in a laminar flow cabinet until dry, packaged in plastic glass slide mailer, and then stored in a clean box at room temperature prior to experimental treatment.

2.3 Experimental procedures

2.3.1 Irradiation of samples

Cobalt-60 was chosen as a representative radioactive source material for the following reasons:

- Co-60 is often listed as a potential material of interest for use in radiological dispersion devices or as a possible target in an attack on sterilisation plants (e.g. food irradiator facilities);
- Co-60 is a commonly used source within Australia, in industry and medicine, for both high and low radiation activities and in portable and fixed devices; and,
- Access was available to a source with a sufficiently high activity to produce desired doses and a facility that provides this service.

Prepared blood, saliva, genomic DNA and bone samples were irradiated at the Australian Nuclear Science and Technology Organisation's Gamma Technology Research Irradiator (GATRI) facility at Lucas Heights NSW and the Chemistry Centre

in the WA Department of Industry and Resources. Samples were irradiated at the GATRI facility up to a dose of 500,000 Gy in a Gammacell 220 Excel (MDS Nordion International, Canada) research irradiator, containing a cobalt-60 source with a dose rate of 3980 Gy/hour. Delivered doses were measured with Fricke (Ferrous ammonium sulphate) and/or Ceric-Cerous dosimeters. In addition, prepared blood, saliva, genomic DNA and bone samples were irradiated at 1,000,000 Gy at the Chemistry Centre WA with a Gammacell 220 Excel (MDS Nordion International, Canada) research irradiator containing a cobalt-60 source with a dose rate of 12.6 kGy/h. Doses were measured and extrapolated using Harwell Perspex polymethylmethacrylate (PMMA) and Ceric-cerous dosimeters. In total, samples were irradiated at 0, 25, 50, 100, 500, 1000, 5000, 10000, 50000, 100000, 500000 and 1000000 Gy.

2.3.2 DNA extraction from blood, saliva and genomic samples

2.3.2.1 Sample collection of dried biological fluids

Dried biological fluids on solid surfaces such as glass were collected with a cotton swab moistened with distilled water for blood samples and 70% ethanol for all other dried biological fluids.

2.3.2.2 Chelex® 100 extraction of DNA from blood, saliva and genomic standard

Within the AFP Biological Criminalistics laboratory, DNA extraction from reference samples is conducted with Chelex® 100 Resin (Bio-Rad Laboratories, USA). Chelex® 100 is a chelating resin composed of styrene divinylbenzene co-polymers containing paired iminodiacetate ions (Bio-Rad 2008). The iminodiacetate ions act as chelating groups that have a high affinity for divalent metal ions such as calcium, manganese and magnesium (Bio-Rad 2008). These metal ions are responsible for activating nucleases and destabilising and degrading the DNA molecule. The Chelex® 100 extraction procedure results in a stable single-stranded DNA extract (Walsh *et al.* 1991).

The entire cotton tip of each swab was removed and placed in a sterile 1.5 mL microcentrifuge tube. 180 µL of 5% Chelex® 100 resin suspension solution (in TE Buffer) and 10 µL of Proteinase K (10 mg/µL) was then added to the samples and incubated in a water bath at 56 ^{o}C for 1 h to optimise the chelating process. A negative control containing Chelex® 100 resin was also prepared to detect any DNA contamination of the reagents employed. Samples were mixed by vortex and placed in a heating block at 100 ^{o}C for 8 min to lyse the cells and release the DNA. Samples were centrifuged at 12000 rpm for 3 min and the supernatant removed and placed in a clean, labelled sterile tube. All samples were stored at -20 ^{o}C.

2.3.3 DNA extraction from bone – Pilot study

Three DNA extraction procedures were investigated for effectiveness in extracting DNA from bone samples. These included the AFP in-house organic DNA extraction procedure (AFP 2005), a modified DNA IQ™ bone extraction protocol (Promega 2002a), and protocol A from Alonso *et al.* (2001). It should be noted that mechanical reduction of each bone sample was conducted with a freezer mill (Section 2.2.3) in order to increase the surface area in contact with the extraction buffers and enzymes (Arismendi *et al.* 2004).

2.3.3.1 Bone extraction method 1: AFP organic DNA extraction procedure

The procedure was conducted as per the AFP's protocol for DNA extraction using the phenol-chloroform organic extraction technique (AFP 2005). DNA extraction buffer was prepared by adding 300 µL Proteinase K (20 mg/mL) and 60 mg solid dithiothreitol (DTT) to 10 mL TENS Buffer (10 mM Tris-HCl pH 8.0; 10 mM EDTA; 100 mM NaCl; 2% [w/v] SDS) and inverting several times to dissolve the solid. The DTT and Proteinase K were added immediately before adding the buffer to the samples.

3 mL of the DNA extraction buffer was added to immerse 2 g of pulverised bone and 350 µL was added to the equipment control swab and mixed thoroughly by vortexing. The samples were then incubated at 56 ^{o}C for 18 h, briefly vortexing every 4-10 hours. After incubation, the equipment control swabs were transferred to a spin basket with the digest solution and spun at 14000 rpm for 5 min (the spin basket and swab were then discarded). The bone samples were spun at 5000 rpm for 5 min until a tight pellet was formed. The supernatant was separated into six 500 µL aliquots for phenol/chloroform/isoamyl alcohol extraction.

500 µL of phenol/chloroform/isoamyl alcohol (25:24:1) were added to each tube and mixed thoroughly by inversion. Tubes were then centrifuged at 13000 rpm for 15 min to separate the organic and aqueous phases. The aqueous top phase was removed into a clean microcentrifuge tube, with care not to carry over denatured organic material.

40 µL of sodium acetate (3 M, pH 5.2) and 1100 µL of cold absolute ethanol were added to each sample to precipitate the DNA. Samples were then mixed by inversion and incubated at -80 ^{o}C for a minimum of 40 min. The samples were then spun at 13000 rpm for 40 min at 4 ^{o}C. The supernatant was then removed, ensuring that the DNA pellet was not disturbed. The DNA pellet was air-dried at room temperature and 50 µL of TE Buffer was then added to each sample and the DNA pellet resuspended by gentle pipetting. The DNA extract was stored at -20 °C.

2.3.3.2 Bone extraction method 2: Modified DNA IQ™ bone extraction protocol

The procedure was conducted as per the modified DNA IQ^{TM} bone extraction protocol (Promega 2002a). A Proteinase K digest solution and prepared lysis solution were made for each sample. The Proteinase K digest solution was freshly prepared by gently mixing 2832 µL of bone incubation buffer (Promega, USA) and 168 µL of Proteinase K (18 mg/mL) to give a total of 3 mL for each sample. Each sample required 6100 µL of

prepared lysis solution and was made using a ratio of 1 µL dithiothreitol to 100 µL lysis buffer.

For the extraction, 3 mL of Proteinase K digest solution was added to 2 g pulverised bone (or the equipment control swab) in a 15 mL tube. The samples were thoroughly mixed and then incubated at 56 ^{o}C for 24 hours, mixing occasionally. The samples were centrifuged at 5000 rpm for 5 min and the supernatant solution transferred to a new 15 mL tube. Two volumes (approximately 6 mL) of prepared lysis buffer were added to the solution. The DNA IQ™ resin was resuspended by vortex for 10 s, and 15 µL of the resin suspension was added to the sample. The sample/lysis buffer/resin mixture was resuspended by vortex for 5 s at high speed and incubated at room temperature for 10 min, mixing three times by inverting the tube.
The sample/lysis buffer/resin mixture was then mixed by vortex for 5 s at high speed and placed immediately on a MagneSil® magnetic separation unit (Promega, USA). The solution was carefully removed and discarded. 100 µL of the prepared lysis buffer was added to each sample, the tube removed from the stand, and mixed by vortex for 2 s at high speed. The mixture was then carefully transferred to a 1.5 mL microcentrifuge tube, ensuring that all the resin was captured in the transfer. The microcentrifuge tube containing the mixture was then mixed by vortex for 2 s at high speed and the tube returned to the magnetic stand. The remaining lysis buffer was removed and discarded. 100 µL of the prepared 1 × wash buffer was added, the tube removed from the magnetic stand and mixed by vortex for 2 s.

The tubes were returned to the magnetic stand for separation. The wash step was repeated a further two times (three times in total), ensuring that all the solution had been removed after the last wash. The resin was then air-dried for 5 min while still on the magnetic stand. After air-drying, 100 µL of the elution buffer was added to each sample, mixed by vortex, and placed in a 65 °C heating block for 5 min. The tube was then removed from the heating block and mixed for 2 s at high speed on a vortex. The sample was immediately placed on the magnetic stand and the eluate transferred into a clean 1.5 mL microcentrifuge tube. The DNA extract was stored at -20 °C.

2.3.3.3 Bone extraction method 3: Protocol A (Alonso *et al.* 2001)

DNA was extracted from the pulverised femoral head bone using a modified version of protocol A from Alonso *et al.* (2001). 3 mL of extraction buffer (10 µmol/L Tris pH 8.0; 100 µmol/L NaCl; 50 µmol/L EDTA pH 8.0; 0.5% SDS and 20 mg/mL Proteinase K) was added to 2 g of pulverised bone (or equipment control swab) in a 10 mL tube. After incubation at 56 ^{o}C for 1 h, the samples were mixed thoroughly and incubated at 56 ^{o}C overnight.

A further 2 mL of extraction buffer with Proteinase K was added to each sample and incubated at 56 ^{o}C for 5 h. Following the incubation, the sample was thoroughly mixed and each sample was separated into aliquots of 500 µL. An equal amount of phenol/chloroform/isoamyl alcohol (25:24:1) was added to each sample and mixed thoroughly by inversion. The samples were centrifuged at 13000 rpm for 15 min until layers had formed. The upper aqueous phase was transferred to new 1.5 mL microcentrifuge tubes and the procedure was repeated ensuring that the upper aqueous phase was completely clear of denatured organic material.

To concentrate the DNA, 500 µL of n-butanol was then added to the aqueous phase and thoroughly mixed by inversion. The samples were then centrifuged for 2 min at 8500 rpm. The lower aqueous phase was then transferred in aliquots of 200 µL, ensuring all n-butanol was removed from the pipette tip during transfer. Ethanol precipitation was then carried out as in Section 2.3.3.1. Samples were stored at -20 ^{o}C for quantitation.

The bone extraction method 3 (modified Alonso *et al.* (2001) protocol A) was selected for further extraction studies. Ethanol precipitation was not carried out, as sufficient DNA was extracted for DNA processing.

2.3.4 DNA quantitation

For the PCR reaction, Profiler Plus® requires a DNA concentration within the range 0.5 – 2.5 ng for optimum results. Excessive amounts of DNA within the amplification reaction will result in off-scale and artefact peaks, whereas too little DNA will result in allele or locus dropout. The Rotor-Gene™ 3000 real-time PCR (Corbett Research, Australia) was used for quantitation of the DNA samples (AFP 2006).

Real-Time PCR is a method for detecting and quantifying the amount of DNA in an extract by utilising the 5' to 3' exonuclease activity of *Taq* polymerase (AFP 2006). The PCR process amplifies a specific segment of DNA within the human amelogenin gene located on the X chromosome (AmelX). The quantity of DNA present is detected through a fluorescent oligonucleotide probe specific for the AmelX gene that consists of a 5' reporter dye FAM and a 3' quencher BHQ1 (Black Hole Quencher 1). During PCR extension, the *Taq* polymerase cleaves the reporter dye FAM from the quencher dye. This results in an increase in the fluorescence signal in proportion to the amount of DNA present. The amount of amplified DNA present is detected after each cycle by measuring the fluorescence of FAM. FAM is excited at 470 nm and the fluorescence emission detected at 510 nm (AFP 2006).

Reference standards were prepared as 10-fold serial dilutions using G147A Control DNA (variable concentration in ng/μL) (Promega, USA). An example of the DNA standards and concentrations is provided in Table 2.2.

Table 2.2: DNA standards and concentrations for Rotor-Gene™ real-time PCR quantitation (example only)

DNA Standard	Concentration (ng/µL)
1 (G147A Control DNA)	260
2	26.0
3	2.60
4	0.260
5	0.0260

The Real-Time PCR master mix was prepared in a microcentrifuge tube using the AmelX Forward Primer [5'-ACT CCT GAT TCT AAG ATA GTC ACA CTC-3'] (9µM), AmelX Reverse Primer [5'-GAG TCT CTC CTA TAC CAC TTA GTC AC-3'] (9µM), and FAM-BHQ1 Probe [5'-FAM-TCA GCA GAG GCA AGC AAG AGA CAC ACA-BHQ1-3'] (2.5 µM). In addition, 2 x Platinum® qPCR Super Mix (Invitrogen, USA), magnesium chloride (50 mM) and Bovine Serum Albumin (10 mg/mL) were added in the quantities listed in Table 2.3.

Table 2.3: Reagent list and volumes for Rotor-Gene™ real-time PCR quantitation master mix

Reagent	Volume per sample (µL)
2 x Platinum® qPCR SuperMix	12.5
Magnesium chloride (50 mM)	2.0
AmelX Forward Primer (9 µM)	2.5
AmelX Reverse Primer (9 µM)	2.5
FAM-BHQ1 Probe (2.5 µM)	2.5
Bovine Serum Albumin (10 mg/mL)	0.5
Total	22.5

To each PCR tube, 22.5 µL of the master mix and 2.5 µL of each DNA sample were added, in addition to a negative control containing 2.5 µL of deionised H_2O, a positive

control containing 2.5 µL of Amp*Fl*STR™ Control DNA 9947A (approximately 0.10 ng/µL), and a baseline blank containing 25 µL TE buffer (to test the integrity of each quantitation batch). The final reaction volume in each PCR tube was 25 µL. The samples were then transferred to the Rotor-Gene™ 3000 (Corbett Research, Australia). The Rotor-Gene™ 3000 run conditions used for real-time PCR quantitation are listed in Table 2.4.

Table 2.4: PCR conditions for Rotor-Gene™ real-time PCR quantitation

PCR Steps	**Temperature (^{o}C)**	**Time**
Hold	50	2 min
Denature	95	6 min
45 Cycles		
Denaturation	95	15 s
Annealing/Extension	60	60 s

The concentration of DNA in each sample was determined using Rotor-Gene™ 6.1.81 software (Corbett Research, Australia). The software is used to generate a standard curve to maximise the coefficient of variance (r^2) to ≥ 0.99. The concentration of the sample is then determined based on the standard curve. The appropriate dilution was then ascertained to provide 1 ng of DNA in each PCR amplification reaction volume.

2.3.5 Amplification

The production of a DNA profile for the identification of an individual requires amplification of specific non-coding regions of DNA within the human genome. The Profiler Plus® PCR amplification kit (Applied Biosystems, Foster City, USA) is a commercially available PCR amplification kit that specifically amplifies 9 non-coding regions of DNA (loci) and amelogenin. Amplification was conducted under guidelines set out by Applied Biosystems for the Profiler Plus® system (Applied Biosystems 1997).

50 µL Reaction

Deionised water was added to each labelled 0.2 mL thin-walled PCR reaction tube to allow for the appropriate dilution of each DNA sample to contain approximately 1 ng DNA. The PCR Master Mix was then prepared in sufficient quantity for the number of samples to be analysed using Amp*Fl*STR® PCR Reaction Mix, Amp*Fl*STR® Profiler Plus® Primer Set and Amp*Fl*STR® Gold DNA Polymerase (5u/µL) according to the Amp*Fl*STR® Profiler Plus® instruction manual (Applied Biosystems 1997) (Table 2.5). 30 µL of the PCR master mix was added to each of the PCR tubes. The appropriate amount of DNA was placed in each tube to allow for each reaction to contain approximately 1 ng of DNA. In addition, a negative control and a positive control were used to test the integrity of each amplification batch. The negative amplification control contained 20 µL of deionised water, while the positive amplification control contained 20 µL of Amp*Fl*STR® Control DNA 9947A (0.10 ng/µL). The final reaction volume in each PCR tube was 50 µL.

25 µL Reaction (Chapter 5 only)

A 25 µL reaction volume for amplification with Profiler Plus® was also used in this study. This revised volume has been validated by the AFP Biological Criminalistics laboratory and is routinely used. Preparation was as for the 50 µL reaction in the volumes listed in Table 2.5, with 15 µL of the PCR master mix added to each of the PCR tubes. Reagent volumes for the 25 and 50 µL PCR reaction are provided in Table 2.5. 15 or 30 µL of the master mix was then added to 10 or 20 µL of DNA/distilled water for a 25 µL or 50 µL reaction volume, respectively.

Table 2.5: Reagent volumes for 25 µL and 50 µL reactions for DNA amplification with the Amp*Fl*STR® Profiler Plus® Amplification Kit

Reaction Component	25 µL Reaction	50 µL Reaction
Amp*Fl*STR® PCR Reaction Mix	10.5 µL	21.0 µL
Amp*Fl*STR® Profiler Plus® Primer Set	5.5 µL	11.0 µL
Amp*Fl*STR® Gold DNA Polymerase (5u/µl)	1.0 µL	1.0 µL
Total Master Mix	17 µL	33 µL

The samples were then transferred to the GeneAmp® PCR System 9700 (Applied Biosystems, USA). The GeneAmp® PCR System 9700 thermal cycler run conditions used for amplification with the Profiler Plus® kit are listed in Table 2.6.

Table 2.6: PCR conditions for DNA amplification with the Amp*Fl*STR® Profiler Plus® Amplification Kit

PCR Steps	Temperature (^{o}C)	Time (minutes)
Initial Soak	95	11
28 Cycles		
Denaturation	94	1
Annealing	59	1
Extension	72	1
Final Extension	60	45
Store	4	∞

Amplified samples were then stored at -20 ^{o}C prior to fragment separation and detection by capillary electrophoresis.

2.3.6 DNA profiling by capillary electrophoresis

Capillary electrophoresis (CE) is a method for the separation and detection of fluorescent dye-labelled DNA fragments generated during PCR amplification. Separation of the mixture of DNA fragments is done according to fragment lengths (or molecular weight). Identification of the alleles present provides a profile of the amplified DNA sample and the CE-based "genetic analyser" detects the fragments present in each sample using a computer analysis program to interpret the information obtained and identify each separated DNA fragment.

GeneScan™ 500 ROX™ Size Standard (ROX-500) and HiDi™ Formamide Master Mix was prepared using 15.5 µL of ROX-500 to 0.6 µL HiDi™ Formamide per sample. ROX-500 is an internal lane standard that contains labelled DNA fragments of specific sizes to enable standardisation of fragment migration across capillaries. 15 µL of the ROX-500/HiDi™ Master Mix was transferred into a 96-well plate for each amplified sample and ladder sample.

The 96-well plate containing the ROX-500/HiDi™ Master Mix was centrifuged for 1 min at 1000 rpm. 1.5 µL of each sample or AmpFlSTR® Profiler Plus® Allelic Ladder Mix was added to the appropriate wells. The 96-well plate was sealed with a septum and denatured at 95 ^{o}C for 3 min in a GeneAmp® PCR System 9700 (Applied Biosystems, USA). The plate was placed in a centrifuge for 1 min at 1000 rpm to remove bubbles and then snap frozen at -15 ^{o}C for 3 min. The plate was then placed in an ABI PRISM® 3100 Genetic Analyzer and the sample analysis run initiated. The samples were injected into a 36 cm capillary filled with fresh 3100 Performance Optimised Polymer 4™ (POP-4™) for DNA fragment separation. The run parameters included injection of the samples for 10 s at 5 kV, and separation along the capillary for 30 min at 15 kV. The temperature remained constant at 60 ± 1 ^{o}C for the duration of the run. These parameters are utilised by the AFP Biological Criminalistics laboratory.

It is important to note that prior to conducting a run, the instrument was calibrated for spectral and spatial accuracy. Spectral calibration is conducted to correct for the spectral overlap between the fluorescent dyes used to tag the DNA fragments, and spatial calibration is conducted to establish the position of each capillary and ensure all sixteen capillaries of the ABI PRISM® 3100 Genetic Analyzer are visible to the detector.

2.3.7 DNA fragment analysis

DNA fragment analysis was conducted using an ABI PRISM® 3100 Genetic Analyzer in conjunction with associated computer software programs. DNA fragment analysis occurs in three stages: raw data collection, length approximation, and allelic assignment.

In the first stage, information is collected from the instrument using ABI PRISM® 3100 Data Collection Software V1.2. Raw data is obtained regarding estimated fragment length and dye colour. Fragment length information is collected as a ratio of time to distance travelled, which involves the time taken for each fragment to travel from the injection point to the detector. The ratio is then defined for each fragment.

The second stage involves GeneScan® Analysis Software Version 3.7 (Applied Biosystems, Foster City, USA) which provides an approximate base pair length of the DNA fragments. The GeneScan® Analysis Software compares the raw data obtained by the Data Collection Software to the internal size standard, ROX-500, using a Local Southern Algorithm. The algorithm compares the distance between each fragment (peak) detected and two peaks above and one peak below, and two peaks below and one peak above the internal size standard to give an average size in base pairs. This information is then utilised by a genotyping program, Genotyper® Version 2.0 (Applied Biosystems, USA) in conjunction with the Kazam® Macros (Applied Biosystems, USA) for Profiler Plus®. This is to assign allelic designations to the various fragments by comparison to allelic ladders provided with the kit. Genotyper® V2.0 uses a binning approach whereby an allelic designation is assigned to an unknown peak that falls

within ±0.5 bps of an allelic ladder fragment. A DNA profile (electropherogram) of the separation for each sample is then generated.

2.3.8 Typing of DNA profiles

Typing of DNA profiles involves correctly assigning allele designations to reflect the number of repeat units present in the allele. Correct typing of a DNA profile also requires consideration of various phenomena including stutter peaks, N and N+1 peaks, pull-up peaks as well as non-specific artefacts, heterozygote imbalance, and changes in peak morphology (as described in Section 1.9.6). In addition, variant alleles, (i.e. those that are not present in the standard allelic ladder and associated analysis software) require manual typing and allele designation is calculated based on the deviation from known alleles present in the profile and the allelic ladder associated with sample.

Peaks are assigned allele designations if they meet the minimum threshold for reportable peaks. All peaks above 50 relative fluorescent units (RFU) are given an allele designation by the Genotyper® V2.0 genetic typing program. While within a forensic context various other restrictions apply to peak heights for allele designation and reporting, these genotyping guidelines were not applied in this research.

2.4 Results and discussion

2.4.1 DNA Extraction from bone – method identification for further research

Three methods were examined to identify an efficient bone extraction methodology for the subsequent investigation of the effects of gamma radiation on DNA from bone. The methods examined included the AFP organic extraction protocol, a modified DNA IQ™ protocol for bone, and an organic extraction protocol from a publication by Alonso *et al.* (2001). The three extraction methods differ in their mode of extraction (i.e. organic liquid-liquid versus solid-phase) and the composition of the extraction buffer employed.

Table 2.7 and Figure 2.1 compare extraction efficiencies for the three DNA extraction procedures under investigation. The data demonstrate that a significantly higher concentration of DNA is present in the AFP (200.5 ng/µL) and Alonso *et al.* (2001) (235.8 ng/µL) organic extraction procedure extracts compared to the extract produced using the modified DNA IQ™ extraction protocol (4.3 ng/µL). Variation within each procedure's sample set demonstrates that the Alonso *et al.* (2001) protocol was the most consistent across replicates with a variation of 2.5% within samples, followed by the AFP extraction protocol at 25.1% and the modified DNA IQ™ protocol at 51.6%.

Table 2.7: DNA quantitation results from human bone

Extraction Protocols	**Replicate 1 ng/µL**	**Replicate 2 ng/µL**	**Average ng/µL**	**St. Dev.**	**% CV**
AFP Organic	164.9	236.0	200.5	50.3	25.1
Modified DNA IQ™	5.8	2.7	4.3	2.2	51.6
Alonso *et al.* (2001) Organic	240.0	231.5	235.8	6.0	2.5

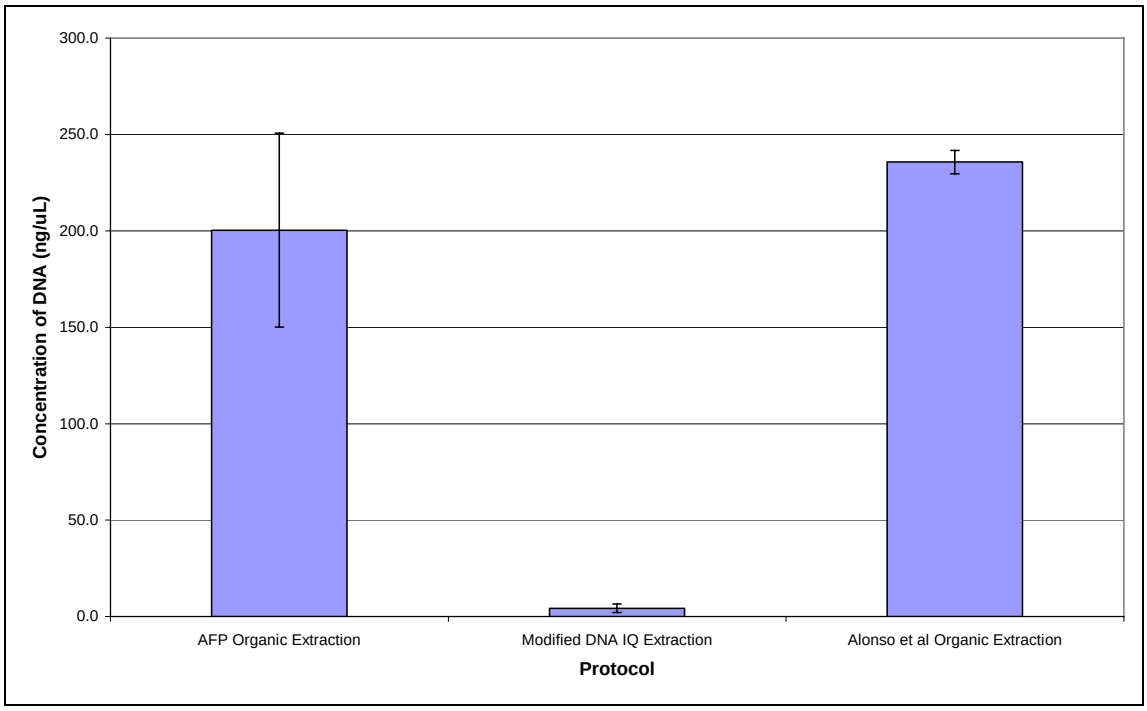


Figure 2.1: Quantitative comparison of DNA extraction protocols for human bone (ng/µL)

The modified DNA IQ™ bone extraction protocol produced a significantly lower DNA yield (average total yield 430 ng) compared to the AFP (average total yield 10,000 ng) and Alonso *et al.* (2001) (average total yield 11,800 ng) organic protocols. This outcome was not unexpected as the DNA IQ™ protocol is a solid-phase extraction procedure utilising paramagnetic silica beads with a finite DNA binding capacity. Therefore, regardless of the amount of DNA present in the initial extraction buffer, there would be a maximum amount able to bind to the beads. Based on the estimated binding capacity of the beads, given that 7 µL will hold a maximum of 100 ng DNA (Promega 2002b), it was expected that the 15 µL of the resin suspension added for the bone extraction would bind approximately 230 ng DNA. A larger yield of DNA may have resulted from variation in the number of beads added or a more complete saturation of the beads.

Based on patent information, the ability of the beads to bind the DNA is far stronger than their ability to release the DNA, and further optimisation may be required to increase the final DNA yield. Hoff-Olsen *et al.* (1999) demonstrated the benefits of a

silica-based extraction method over other methods for degraded DNA samples. The authors examined five different DNA extraction methods, specifically phenol-chloroform, silica-based, the InstaGene Matrix™ (BioTest), glass-fibre filter, and the Chelex® 100, on decomposed human liver tissue samples at differing levels of decomposition (post-mortem time varied 2-90 days) (Hoff-Olsen *et al.* 1999). The investigation showed that the silica-based method gave a full STR profile in 90% of the cases (Hoff-Olsen *et al.* 1999).

In addition, the modified DNA IQ™ method is a significantly less time consuming and laborious protocol. The shorter time for extraction and the ability to automate the DNA IQ™ system would allow for a higher throughput of samples in a shorter time and reduce the labour burden on the analyst. The lower DNA concentration may be improved by conducting more than one extraction on a single supernatant or by increasing the number of beads, allowing for a greater number of available binding sites for the DNA molecules. This issue, however, will be discussed further in Chapter 4.

While the Alonso *et al.* (2001) organic bone extraction protocol is an extremely laborious and time consuming process, it was felt that its high extraction efficiency and minimal variation across replicates supported its use in subsequent studies aimed at investigating the effect of gamma radiation on DNA in human bone (Section 2.4.2.4). The selection of this protocol, however, had implications on the ability to process the samples within the required timeframe, for example, within 1-day of exposure. On average, it was possible to process each data set (i.e. 1-day, 1-week and 4-weeks post-exposure) within 2.5 days.

2.4.2 The effects of gamma radiation on biological samples

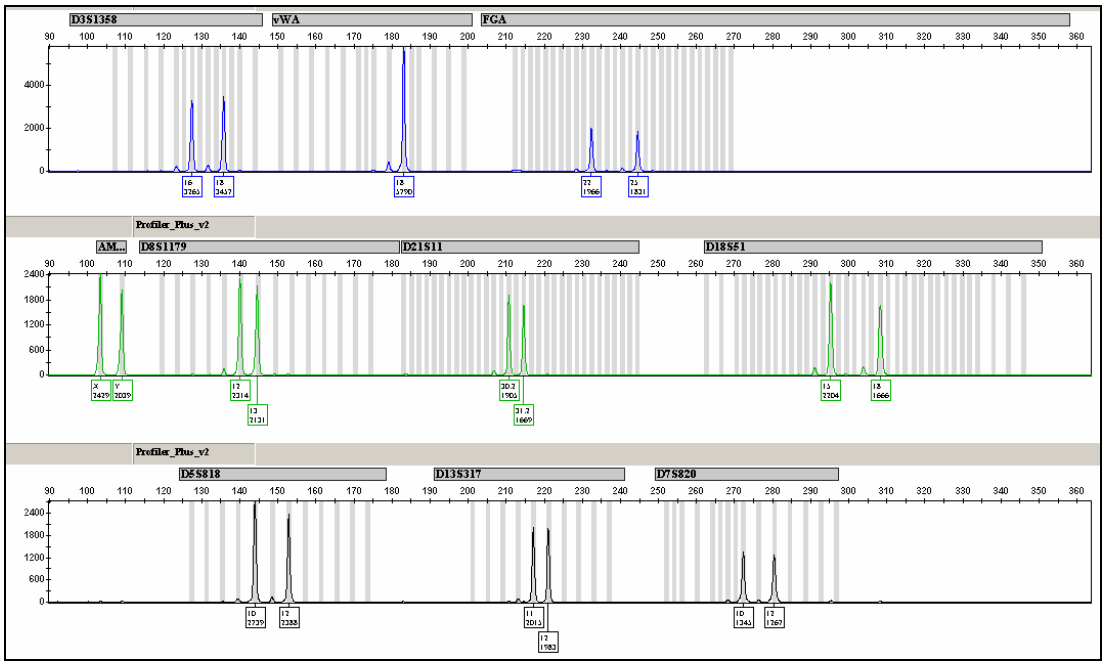
A number of forensically relevant biological matrices, including blood, saliva, and bone, as well as single-source genomic DNA, were investigated for the effects of exposure to gamma radiation. Initial research examined the effect of gamma radiation on these matrices at a number of doses up to 1000 Gy, taking into consideration that the human

LD_{50} for gamma radiation is approximately 4 Gy [4 Sv]. These results did not demonstrate any significant effects on the DNA profiles produced from the irradiated samples (data not shown). Doses were then investigated up to 1,000,000 Gy, and were selected to identify a maximum dose at which DNA profiling could provide information and demonstrate the qualitative and quantitative effects of high gamma radiation doses on various biological matrices. A minimum of six samples were analysed for each dose and these were treated and examined in triplicate. Analysis on each was performed 1-day, 1-week and 4-weeks post-irradiation to further investigate sample degradation, if any, associated with the delay between exposure and DNA extraction.

In Figures 2.3 to 2.14, loci have been presented along the x-axis of each graph in order from smallest to largest (Amelogenin < D3S1358 < D8S1179 < D5S818 < vWA < D21S11 < D13S317 < FGA < D7S820 < D18S51) to demonstrate the relationship between degradation and size of locus. The data points indicate average peak heights (RFU) as a percentage of those of the control. Results from genomic DNA are presented first to establish a baseline for data, as an indicator of damage in the absence of a cellular matrix, and this is followed by results from the forensically relevant biological matrices blood, saliva and bone.

Due to the number of sample replicates analysed, the following sections do not include presentation of the individual DNA profiles. However, Figure 2.2 is provided to illustrate examples of both a control profile and a degraded profile.

a)



b)

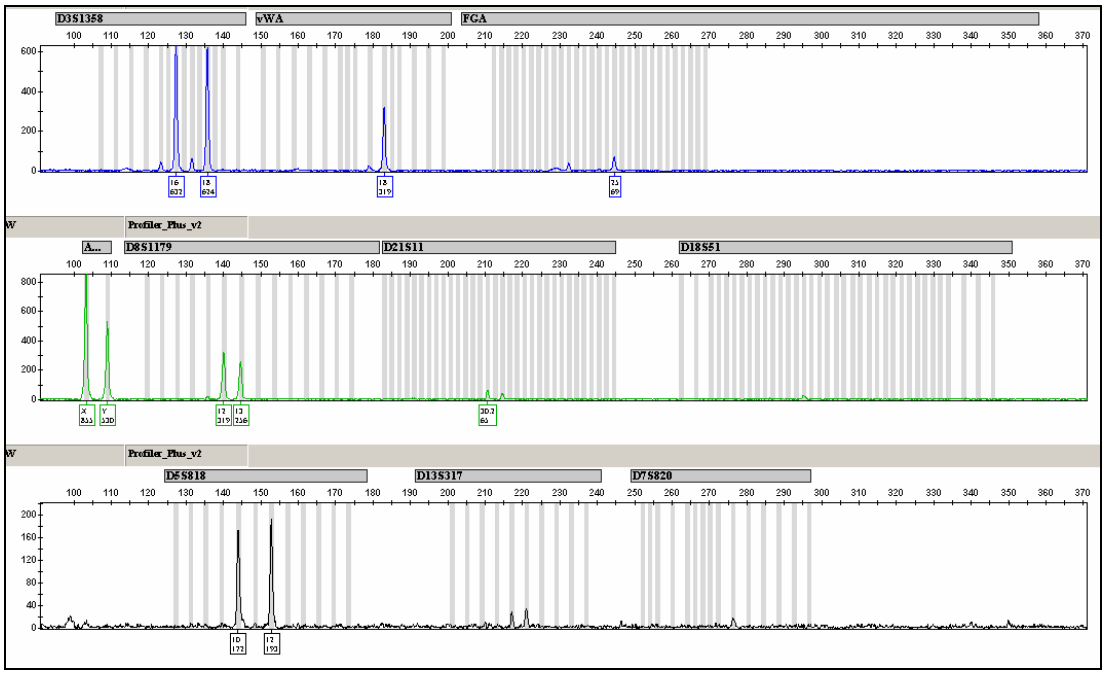


Figure 2.2: Example DNA profiles for a control sample (a) and bone irradiated at 100,000 Gy (b)

2.4.2.1 Gamma irradiation of genomic DNA standard

1-day post-irradiation

The results of the genomic standard at 1-day post-irradiation demonstrated a gradual decrease in peak height, relative to the control, with increasing dose (Figure 2.3). For doses between 50 and 1,000 Gy, peak heights were within the standard error of the control at all loci analysed, indicating no considerable effect as a result of exposure to these doses (see Table A1 in Appendix). At and above the next highest dose of 5,000 Gy, more substantial differences are exhibited. While full profiles were obtained up to 50,000 Gy, loss of the higher molecular weight loci FGA, D7S820 and D18S51 occurred at 100,000 Gy. Within the DNA profiles where peak heights approached the detection limit of the instrument (50 RFU), heterozygote imbalance was observed. This is consistent with profiles produced from degraded DNA and occurs across all matrices.

It should be noted that doses of 500,000 and 1,000,000 Gy did not produce DNA profiles in the genomic samples and all other matrices, therefore these doses will not be discussed further (data not shown).

In this dataset, the averaged peak heights (represented as a percentage of the control) exceed the control value i.e. produce values above 100%. This variation around the control is largely introduced by both the quantitation and CE fragment detection systems. Quantitation was conducted using real-time PCR, which provides an estimate of the DNA quantity based on comparison to known standards. The DNA quantity is then used to calculate a volume to be added to the STR amplification reaction. Imprecision in quantitation can be introduced through, for example, the small volume of DNA extract (2.5 µL) used in the analysis, as the sample taken may not represent the true amount of DNA present, in addition to variation around the standard curve used to generate concentration. In addition, DNA quantitation systems provide an estimate for the sample and in general benefit from analysis in triplicate.

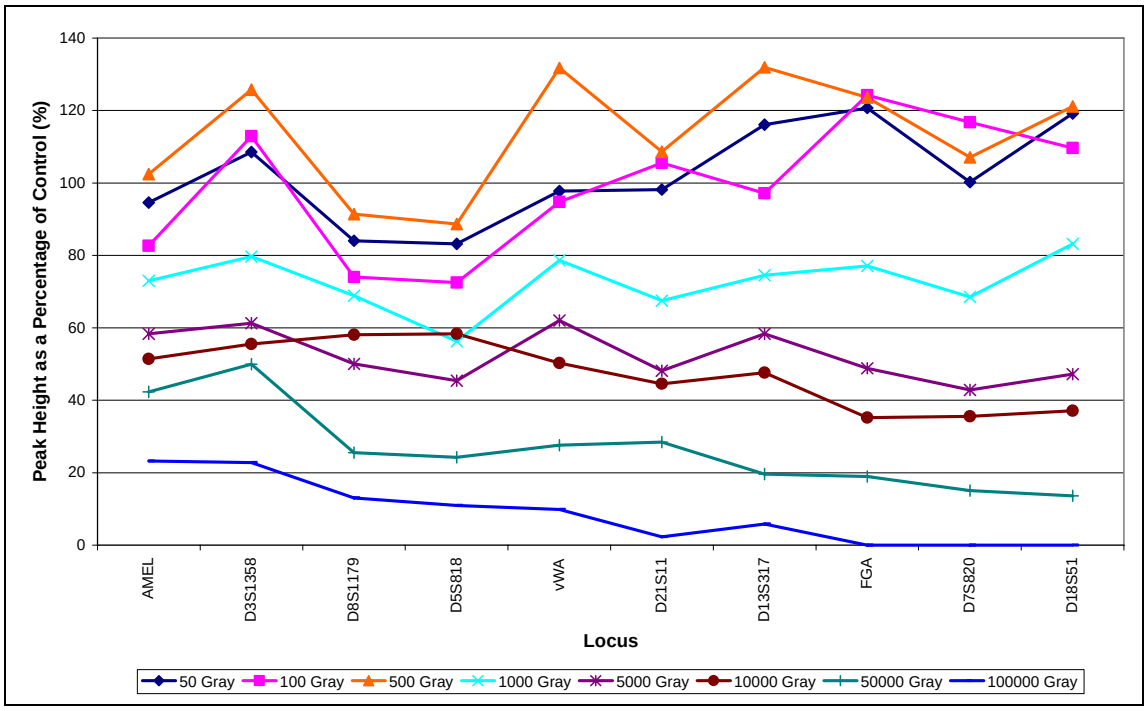


Figure 2.3: The effects of gamma radiation on single-source genomic male DNA (analysed 1-day post-irradiation; extracted with Chelex ® 100)

CE analysis may also contribute to this variation. Each time a sample is analysed by CE, either freshly prepared or from a previous run, different peak heights will be produced. This may be due to, for example, differences in the amount of STR fragments taken up during injection. Therefore, there is a degree of variability inherent to the analysis technique that may result in values above the control, especially at doses where there is no apparent effect. Please note that this variation around the control was present throughout the datasets.

1-week post-irradiation

Analysis of the 1-week post-irradiation samples demonstrated a similar response to those of the 1-day, however, the lower molecular weight amelogenin (AMEL), D3S1358 and D8S1179 loci of the samples exposed to 5,000 Gy also fell within the standard error of the control (Figure 2.4 and Table A2 in Appendix). The higher molecular weight loci values fell outside the control values, supporting the notion that larger loci are more susceptible to damage.

A dose-response relationship becomes more apparent at and above 5,000 Gy, with a gradual decrease in peak height response with increasing dose. While full profiles were obtained up to 50,000 Gy for the 1-day samples, allelic/locus dropout was observed at the D18S51 locus, thus limiting the generation of a full profile to the 10,000 Gy dose. Dropout was again seen at the FGA, D7S820 and D18S51 loci for 100,000 Gy, in addition to the D21S11 locus.

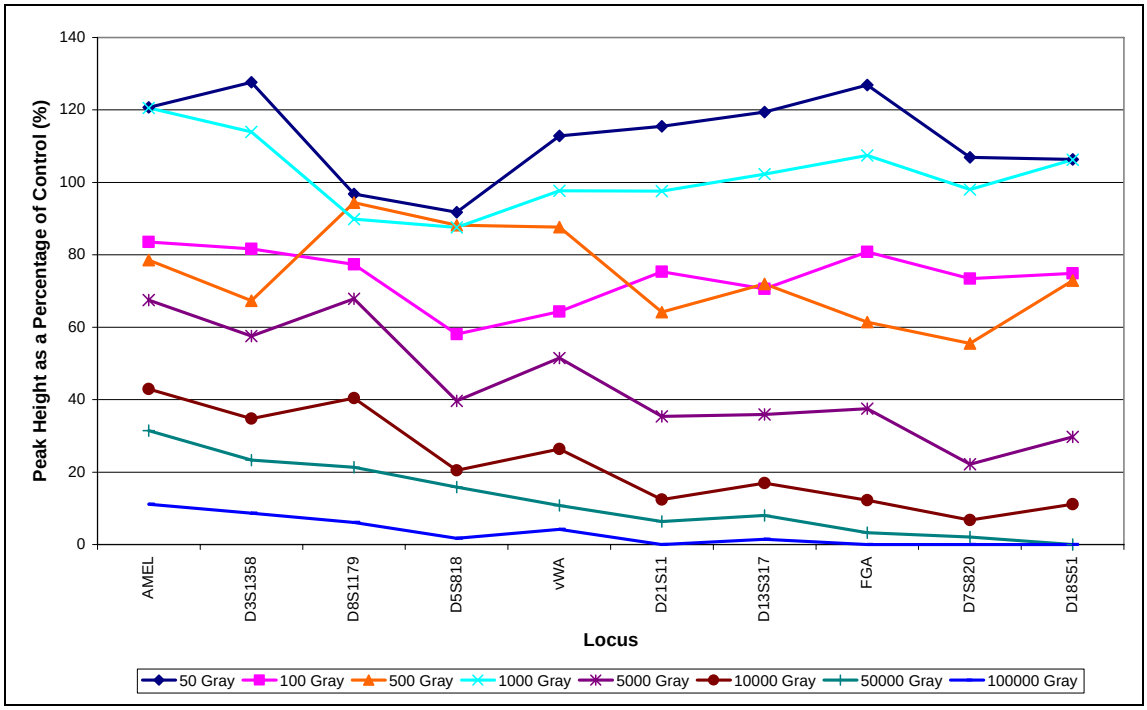


Figure 2.4: The effects of gamma radiation on single-source genomic male DNA (analysed 1-week post-irradiation; extracted with Chelex ® 100)

4-weeks post-irradiation

At 4-weeks post-irradiation, similar trends were noted to those observed for the 1-day and 1-week samples, including complete profiles up to the 10,000 Gy dose (Figure 2.5). In this case, however, it can be seen that the results for doses up to 5,000 Gy are within the standard error of the control (Table A3 in Appendix), which was not observed in the 1-day and 1-week samples. In addition, the findings also contrast previous results at the higher dose exposure of 50,000 Gy, as peak response is now lost at the D13S317, FGA, D7S820 and D18S51 loci, as well as the D21S11 locus for the 100,000 Gy dose.

These differences highlight the need for a conservative interpretation of enhanced degradation due to increased time-to-analysis. However, it should be noted that the overall peak heights of this dataset were less than those of the 1-day and 1-week. This may be due to variation in sampling or pipetting, or possibly due to other mechanisms of DNA degradation such as exposure to heat during irradiation. In addition, variation in instrument precision may have contributed to the overall reduction in peak height.

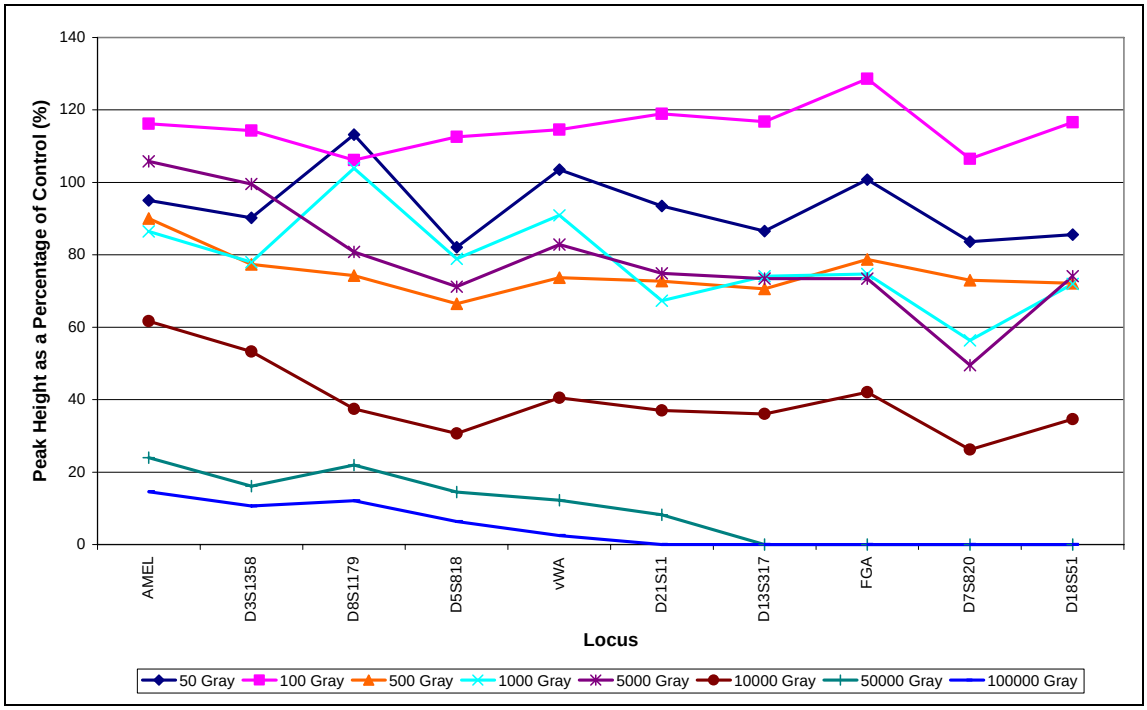


Figure 2.5: The effects of gamma radiation on single-source genomic male DNA (analysed 4-weeks post-irradiation; extracted with Chelex ® 100)

Summary

Overall, it can be seen that there is a significant effect occurring across the targeted loci typically at and above 5,000 Gy, with a partial loss of profile at the 50,000 and 100,000 Gy doses. Loss of signal is observed at the higher molecular weight loci, typically with D13S317, FGA, D7S820 and D18S51. It is difficult to discern any further degradation as a result of the time-to-analysis due to variation in overall peak heights over the measured time periods. However, general trends over the three time periods may suggest a reduction in response particularly for the samples irradiated at higher doses as

demonstrated by the 50,000 Gy and 100,000 Gy samples in Figures 2.3 to 2.5. This variation may be due to small sample size, instrumental variance, for example from quantitation or fragment detection, or from sample collection and extraction. Irrespective of this, the trend of DNA degradation being proportional to dose was clearly evident across the three experimental time intervals.

2.4.2.2 Gamma irradiation of blood

1-day post-irradiation

The results from the male blood samples at 1-day post-irradiation demonstrated a decrease in peak height at doses greater than 10,000 Gy (Figure 2.6). At doses up to 10,000 Gy, peak heights were within the standard error of the control (Table A4 in Appendix) therefore indicating that exposure at these doses did not have a demonstrable effect on the DNA profiles. Above 10,000 Gy, a clearer dose-response relationship is observed, however, unlike the results of the genomic standard, where allelic dropout was observed at 100,000 Gy (Figure 2.3), a complete DNA profile was observed up to this dose. From Figure 2.6, it can also be seen that there is a greater reduction in peak height occurring at the higher molecular weight loci.

In comparison to the genomic DNA standard, the blood appears to be more resistant to the effects of gamma radiation. This may be due to the presence of cellular proteins, a higher DNA concentration, or instrumental variations.

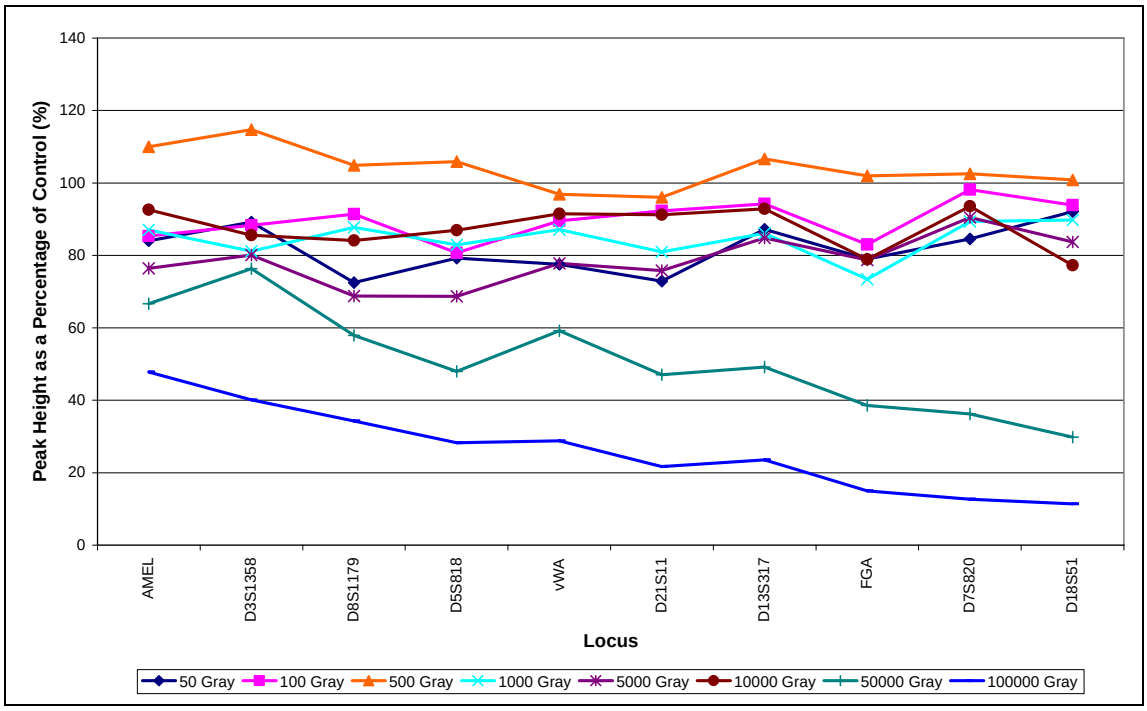


Figure 2.6: The effects of gamma radiation on male blood (analysed 1-day post-irradiation; extracted with Chelex ® 100)

1-week post-irradiation

At 1-week post-irradiation, the samples exhibited a similar response to those at 1-day; however, only doses up to 5,000 Gy fell within the standard error of the control (Figure 2.7 and Table A5 in Appendix). Furthermore, while samples exposed to 10,000 Gy fell outside this threshold, a reduction in the peak heights of higher molecular weight loci did not appear until 50,000 Gy. A complete profile was obtained up to 50,000 Gy; however, unlike 1-day post-irradiation, locus and allelic dropout was observed at 100,000 Gy. Specifically, locus dropout was observed at the D7S820 and D18S51 loci, in addition to the larger alleles at each of the heterozygote loci D21S11, D13S317 and FGA. It should be noted that when compared to the 1-day and 4-week samples, all samples at 1-week post-irradiation demonstrated an overall reduction in peak heights and this may have been a factor influencing the increased number of allele drop-outs in the sample.

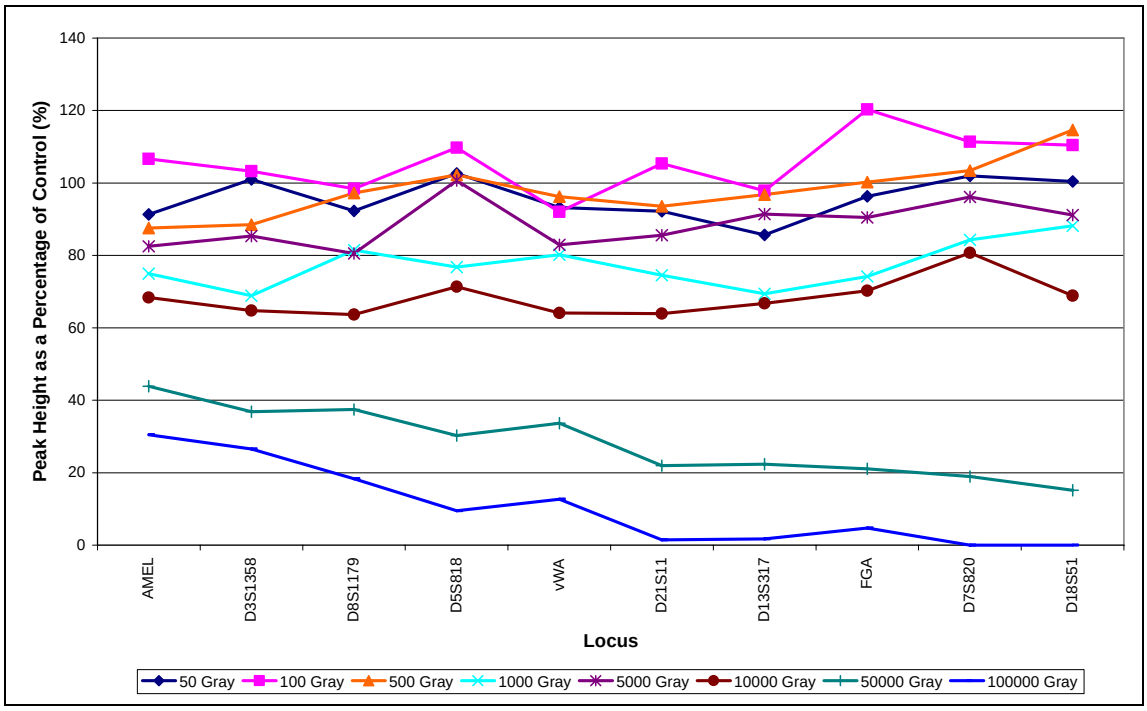


Figure 2.7: The effects of gamma radiation on male blood (analysed 1-week post-irradiation; extracted with Chelex ® 100)

4-weeks post-irradiation

At 4 weeks post-irradiation, the DNA profiles exhibited similar trends to the 1-day samples, where peak heights of doses between 50 and 10,000 Gy fell within the standard error of the control (Figure 2.8 and Table A6 in Appendix). At the higher dose of 50,000 Gy, the three lower molecular weight loci remained within the standard error, with degradation of the profile causing the higher molecular weight loci to drop below this threshold. Similar to 1-week post-irradiation samples, locus dropout occurred at the D7S820 and D18S51 loci at 100,000 Gy; however, the allelic dropout that was observed in the larger alleles of the heterozygote loci D21S11, D13S317 and FGA at 1 week was not observed in these samples. It was therefore not possible to conclusively identify any trend associating degradation with an increased time-to-analysis. This may possibly be due to the inter-day precision of the instrument, as samples were run when extracted, i.e. at 1-day, 1-week and 4-weeks. In addition, the peak heights of the highly degraded samples (100,000 Gy) were extremely low, and in some cases approached the limit of

detection of the instrument. Above 50,000 Gy, the higher molecular weight loci demonstrated a greater reduction in peak height relative to the control.

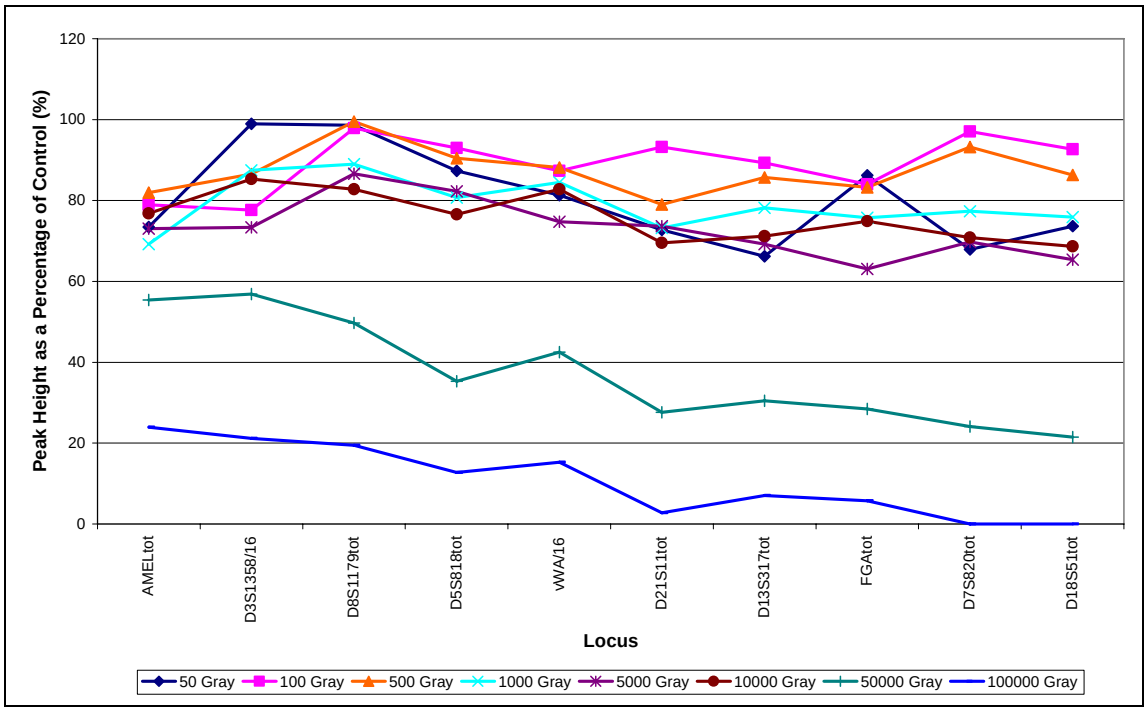


Figure 2.8: The effects of gamma radiation on male blood (analysed 4-weeks post-irradiation; extracted with Chelex ® 100)

Summary

Degradative changes have been demonstrated to occur from 10,000 Gy, with locus drop out occurring at the D7S820 and D18S51 loci at 100,000 Gy. As previously explored, there are inherent differences that occur as a result of the quantitation and fragment detection systems as well as inter-day precision of the instrument that may contribute to variable result. This may also have precluded any obvious demonstration of continued degradation over the measured time-to-analysis. Therefore, a general trend with respect to time-to-analysis has not been observed. In comparison to the genomic standard, there appears to be a less distinct dose-response relationship for the lower doses demonstrating an overall robustness of DNA to the effects of ionising gamma radiation, possibly due to the quantity of the DNA present or the nature of the blood matrix.

2.4.2.3 Gamma irradiation of saliva

1-day post-irradiation

At 1 day post-irradiation, exposure of saliva to gamma radiation produced a similar result to exposed blood, in that the profiles exhibited a decrease in peak height at doses greater than 10,000 Gy (Figure 2.9). In support of this, the peak heights of doses up to 10,000 Gy are within the standard error of the control, indicating there was not a considerable effect at these levels of exposure (see Table A7 in Appendix). At the higher doses of 50,000 Gy and 100,000 Gy there was a clear decrease in peak height response with an increase in the molecular weight of the locus, indicating that a greater degree of degradation was occurring. Locus dropout was observed at D7S820 and D18S51 in samples exposed to a dose of 100,000 Gy. It should be noted, however, that the saliva samples for all time periods exhibited inherent degradation, resulting in very low peak heights at the higher molecular weight loci for all samples tested. As a result, the peak heights as a percent of the control (illustrated in Figures 2.9 though 2.11) appear more variable, particularly for the lower dose samples as small changes in RFU values are reflected as larger percentage changes in the figures below. In addition, the combined effects of inherent degradation and radiation-induced degradation may be influencing the pattern of results.

As the saliva used in this study was collected by expectoration, the sloughed cells from the buccal cavity may have already undergone apoptotic events or exposure to degradative processes from bacterial contaminants, resulting in fragmentation of the DNA molecule.

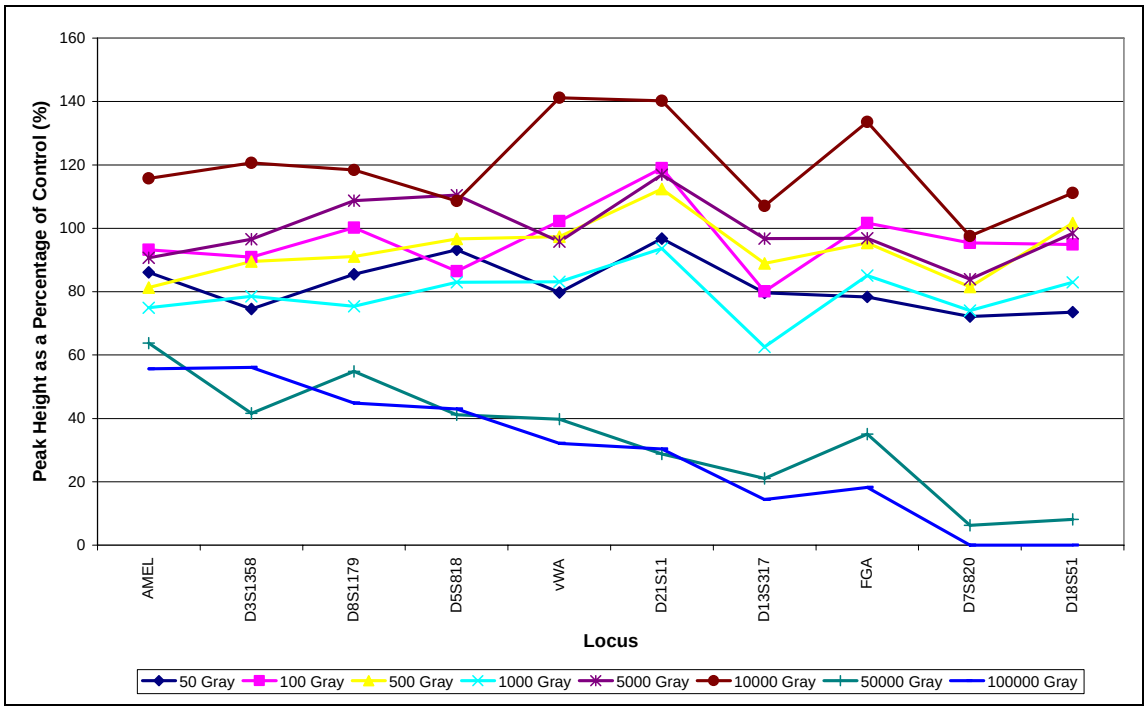


Figure 2.9: The effects of gamma radiation on male saliva (analysed 1-day post-irradiation; extracted with Chelex ® 100)

1-week post-irradiation

The samples extracted at 1-week post-irradiation, demonstrated a comparable response to the 1-day samples, where at doses up to 10,000 Gy, the profiles exhibit peak heights within the standard error of the control (Figure 2.10 and Table A8 in Appendix). In addition, at doses at and above 50,000 Gy, a reduction in peak heights of higher molecular weight loci is observed, with locus dropout at D21S11, D13S317, D7S820 and D18S51 for samples irradiated with a dose of 100,000 Gy. Interestingly, the FGA locus did not demonstrate locus dropout even though D13S317 and D21S11 are of lower molecular weight. As shown in Table 2.1, the DNA profile for the saliva matrix possesses homozygote alleles at the FGA locus (21,21), and heterozygote alleles at the lower molecular weight loci. In an electropherogram, homozygote alleles are represented as a single peak that is typically double the RFU value of a heterozygote peak (presented as two peaks). Therefore, the homozygote alleles at the FGA locus presented a peak height above the 50 RFU threshold value.

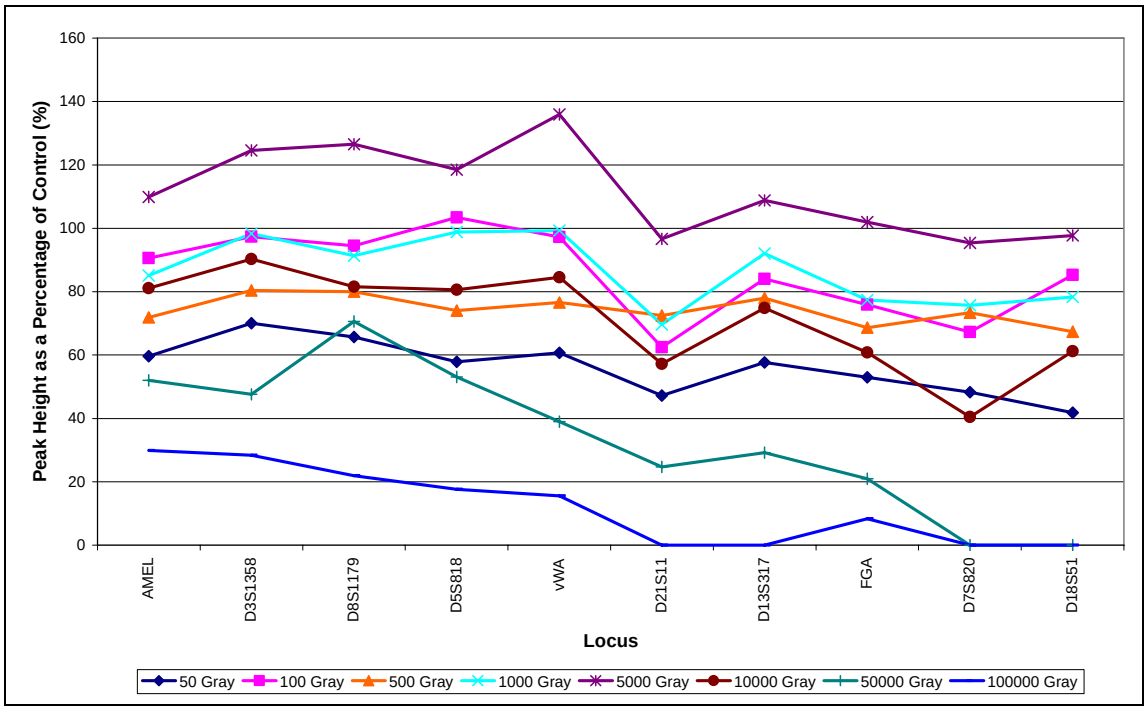


Figure 2.10: The effects of gamma radiation on male saliva (analysed 1-week post-irradiation; extracted with Chelex ® 100)

4-weeks post-irradiation

At 4-weeks post-irradiation, the DNA profiles exhibited similar results to the 1-day and 1-week samples, where the peak heights from doses between 50 and 10,000 Gy fell within the standard error of the control (Figure 2.11 and Table A9 in Appendix). At higher doses, there was a general decrease in peak height response with increasing dose, with dropout occurring at the D7S820 after exposure to 50,000 Gy and at D7S820 and D18S51 after exposure to 100,000 Gy.

It was noted, that these results appeared to be the highly affected by the inherent DNA degradation. The low RFU values presented a skewed dose-response relationship with regard to the control. It was therefore not possible to identify any trends associated with degradation and increased time-to-analysis.

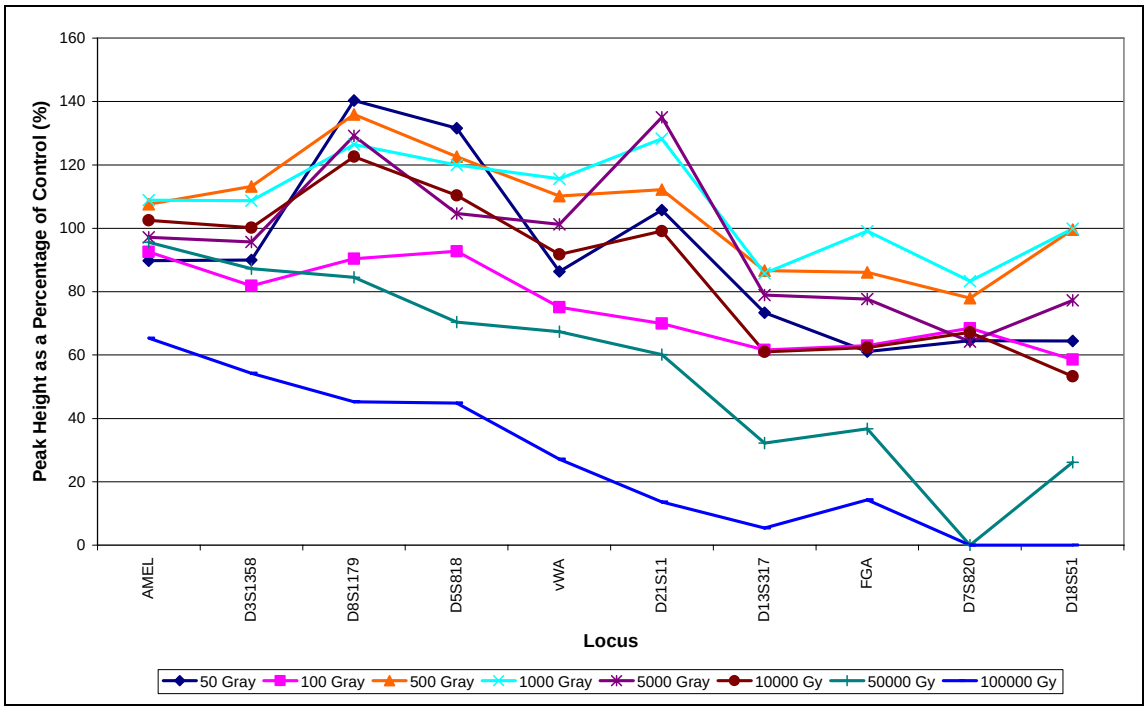


Figure 2.11: The effects of gamma radiation on male saliva (analysed 4-weeks post-irradiation; extracted with Chelex ® 100)

Summary

Overall, degradative changes were demonstrated to occur from around 10,000 Gy in the saliva samples. However, data from across all three time periods, particularly at 1-week and 4-weeks, exhibited signs of sample degradation prior to irradiation, which may have influenced the data collected for a dose-response relationship. The results demonstrated that the DNA from the saliva samples were degraded at the higher molecular weight loci irrespective of exposure to radiation. As a result, the peak heights detected for the controls were very low, particularly at the larger loci and this had affected the interpretation of the percentage difference from the control. Degradation of DNA over the measured time-to-analysis periods, was not able to be determined for saliva due to the factors listed above.

As previously mentioned, the saliva may have contained mostly sloughed cells that had already undergone apoptotic fragmentation or degradation by bacteria. This may have further implications for the use of saliva collected by expectoration for DNA profiling

and the nature of the expectorated saliva should be considered prior to its use in DNA profiling studies. In addition, inhibitory substances, such as proteins or ions, may have been present and affected the efficiency of the PCR amplification for all samples.

2.4.2.4 Gamma irradiation of bone

1-day post-irradiation

The results from the bone samples at 1-day extraction post-irradiation demonstrated a gradual decrease in peak height relative to the control (Figure 2.12). Peak heights for doses between 50 and 1,000 Gy fell within the standard error of the control (Table A10 in Appendix). This suggests that gamma radiation doses from a Cobalt-60 source up to 1,000 Gy do not cause any observable degradation in the DNA profile.

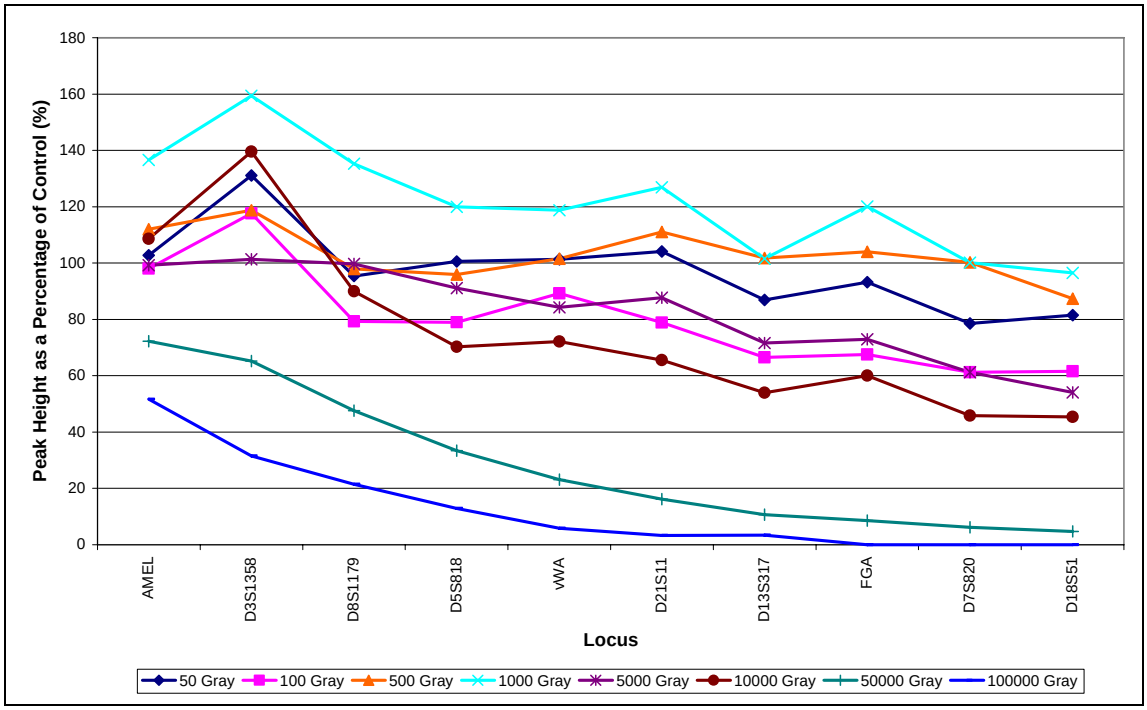


Figure 2.12: The effects of gamma radiation on male bone samples (analysed 1-day post-irradiation; extracted with Chelex ® 100)

At higher doses of 5,000 and 10,000 Gy, the lower molecular weight amelogenin (AMEL), D3S1358, D8S1179 and D5S818 loci were also within the standard error of

the control. The peak height reduction in the higher molecular weight loci demonstrates that these larger loci are more susceptible to damage.

While full DNA profiles are obtained up to 50,000 Gy, a clear degradative effect is observed, particularly for the higher molecular weight loci. At a dose of 100,000 Gy, alleles at the FGA, D7S820 and D18S51 loci had dropped out and peak heights for the larger alleles at the D13S317 and D21S11 loci were nearing non-detectable values.

1-week post-irradiation

With respect to 1-week post-irradiation, Figure 2.13 and Table A11 (see Appendix) demonstrate a similar response to those of the 1-day, however, at 5,000 Gy, all but the two largest markers, D7S820 and D18S11, fall within the standard error of the control. In contrast to the samples extracted at 1 day post-irradiation, the samples irradiated with 10,000 Gy were below the standard error threshold for all loci analysed. This reduction after a 1 week period may suggest additional degradation with time to extraction.

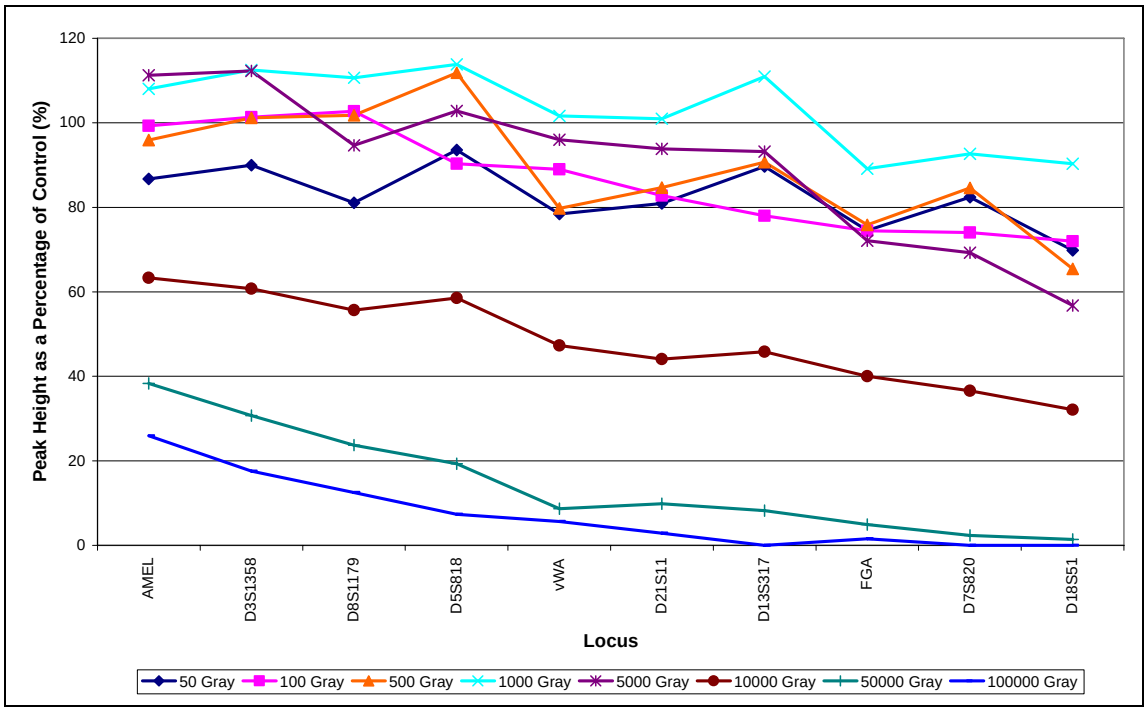


Figure 2.13: The effects of gamma radiation on male bone samples (analysed 1-week post-irradiation; extracted with Chelex ® 100)

As exhibited in the 1-day results, peak heights for samples irradiated at 50,000 Gy produced a full profile and demonstrated a decrease in response with increasing molecular weight. At 100,000 Gy, allele dropout is observed in the D13S317, D7S820 and D18S51 loci.

4-weeks post-irradiation

At 4 weeks post-irradiation, there appears to be an overall decrease in peak response for all doses examined, with an overwhelming majority of the DNA profiles falling outside the standard error of the control (except for 100 Gy irradiation; Figure 2.14 and Table A12 in Appendix). This has demonstrated a general trend suggesting that the DNA exposed to doses at or below 10,000 Gy degraded further over the four weeks from irradiation to extraction. This distinctive degradative pattern was not observed in any other matrix previously tested and it is possible that components unique to bone have influenced this result. For example, the hydroxyapatite structure of bone acts to protect DNA from undergoing degradation, however, interaction with gamma radiation may have accelerated the degradation of the bone matrix (as a result of time) thus accelerated the DNA degradative process. Furthermore, the interaction of gamma radiation with the bone matrix may have resulted in the release of contaminants that could affect the PCR amplification process. Moreover, this matrix was processed using an organic DNA extraction technique that was labour intensive and additional time was required to complete the procedure. The possible effects of the chemicals utilised in this protocol, such as phenol/chloroform/isoamyl alcohol, was also considered, however, this seemed unlikely as organic techniques are preferentially used for the extraction of DNA from aged and degraded bone samples (Hagelberg and Clegg 1993; Schmerer *et al.* 1999; Alonso *et al.* 2001; Ye *et al.* 2004). Other influencing factors may include the precision of the instrument over the samples tested as well as the small sample size.

However, consistent with the previous time periods, full DNA profiles were obtained up to 50,000 Gy. It can also be seen that at the two highest doses, degradation increases with increasing molecular weight of the loci. After exposure to 100,000 Gy, allelic

dropout occurs at the D7S820 and D18S51, and with peak heights at FGA and D13S317 nearing the instrument's limit of peak detection.

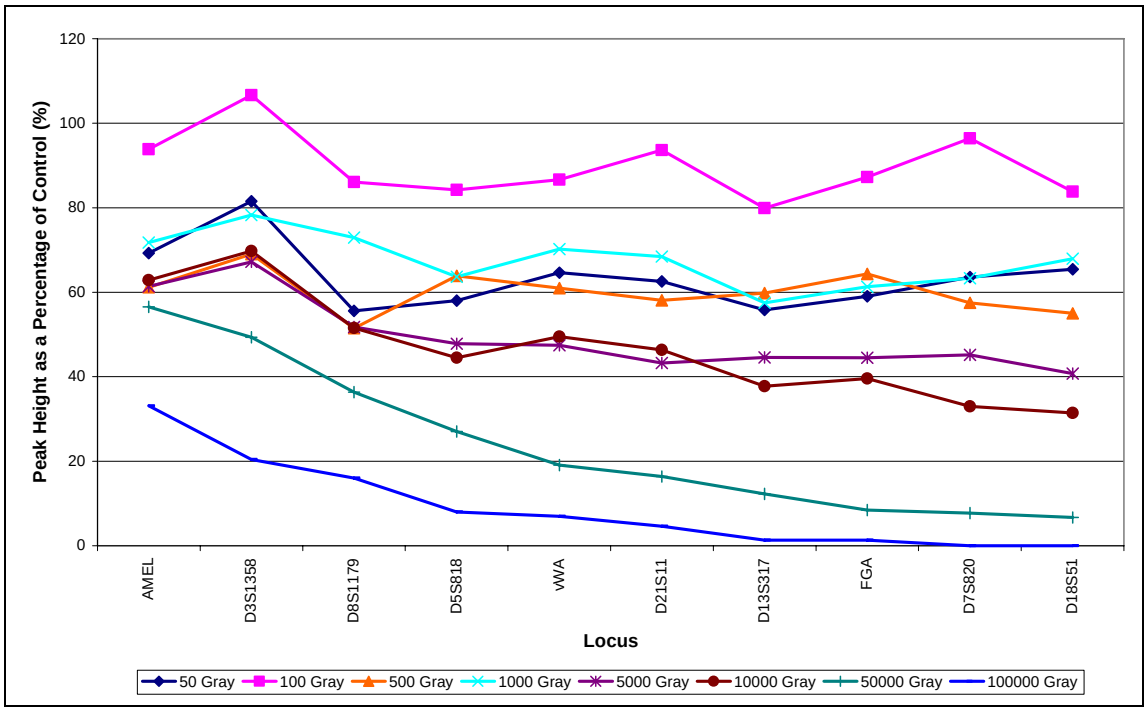


Figure 2.14: The effects of gamma radiation on male bone samples (analysed 4-weeks post-irradiation; extracted with Chelex ® 100)

Summary

Overall, it can be seen that there is a significant effect occurring across all the targeted loci at and above 5,000 Gy, with a partial loss of profile at 100,000 Gy. Loss of signal is observed at the higher molecular weight loci, with alleles disappearing at D13S317, FGA, D7S820 and D18S51. In contrast to the results from the genomic DNA and the blood samples, there appears to be a clear decrease in peak response with increased time between irradiation and analysis. This was most clearly demonstrated at 4-weeks post-irradiation, where there appeared to be distinct decrease in the peak heights relative to the control, particularly at the lower doses. These observations indicate that the DNA may have undergone further degradation over the 4-week period. As previously mentioned, there may be other factors affecting this relative decrease aside from degradation over time, such as the increase in time required for the extraction procedure,

influencing factors from the degradation of the hydroxyapatite matrix as well as sample size and instrument precision.

In addition, while the peak responses from the blood and saliva matrices did not demonstrate significant degradation up to 5,000 Gy, the bone samples exhibited a slightly lower threshold, indicating that the stability of the DNA is maintained up to 1,000 Gy, for 1-day and 1-week samples, and at less than 50 Gy for the 4-week samples. Nevertheless, full profiles were still obtained at a dose of 50,000 Gy.

2.4.2.5 General discussion of the effects of gamma radiation on biological matrices

The pattern of results obtained illustrated a progressive loss of the higher molecular weight loci as the extent of the DNA degradation increased, which was particularly evident at the 50,000 and 100,000 Gy doses. This resulted in the characteristic 'ski slope' effect seen in the DNA profiles of degraded DNA, where loss of signal is typically observed with the larger-sized STR products (Figures 2.3 to 2.14) (Bär *et al.* 1988; Clayton *et al.* 1995a; Clayton *et al.* 1995b; Whitaker *et al.* 1995; Butler *et al.* 2003). In addition, heterozygote imbalance was observed, particularly at loci with peak heights approaching the limit of detection for the instrumentation.

As demonstrated in Figures 2.3 to 2.14, the smallest target fragment, Amelogenin (109 bps), was successfully analysed up to 100,000 Gy for all matrices investigated. Conversely, one of the largest target fragments, D18S51 (264-344 bps), was typically absent at both the 50,000 and 100,000 Gy doses. As stated above, this reflects the typical pattern of degraded DNA where, within the DNA molecule, the longer fragments are statistically better "targets" than the shorter fragments (i.e. larger "cross section") for interaction with ionising radiation. Based on previous research by Hutchinson (1985) and Irwin *et al.* (2007), it is proposed that failure of PCR amplification is a result of the degradation of the DNA molecule due to fragmentation of the DNA strand, in addition to inter-strand cross-linking, deamination and dimer formation, resulting in insufficient

intact target molecules for primers to bind. It is therefore evident from these results that a correlation exists between the average length of alleles at a locus and the successful typing at that locus.

These data are therefore consistent with a degradation model based on the average molecular weight as an index for successful typing, and with DNA typing issues associated with compromised samples. Of note is that the radiation-induced DNA fragmentation did not lead to the appearance of additional/artefact peaks in the profiles of the samples analysed. This is in direct contrast to Whitaker *et al.* (1995) who demonstrated the presence of three consistent artefact peaks in their degraded samples. The authors attributed the presence of these peaks to the possibility that, due to degradative breaks in the DNA, short lengths of complementary regions in the DNA were exposed that allowed binding with the STR primers; i.e. there was partial homology to the primers in their flanking sequences resulting in amplification of those regions (Whitaker *et al.* 1995). It is likely that these artefacts were not observed in the current study as, unlike the STR primers used by Whitaker *et al.* (1995) (HUMVWFA31, HUMTHO1, HUMF13A01 and HUMFES/FPS), the Profiler Plus® system is comprised of robust primers that are optimised and highly specific for the region of interest on the human genome. In addition, the samples analysed were unlikely to contain any extraneous DNA (bacterial or otherwise) that may have shown complementarity to the primers.

Where alleles were able to be scored, the allele designation was always identical between replicates and the control, except where allele or locus dropout occurred. Similarly, the alleles present in the DNA profiles did not change at any dose for any matrix examined. This was anticipated as the cells under analysis were not living, and as a result would not undergo DNA repair or replication during mitosis that could lead to mutations and/or the appearance of different alleles in the profile.

Time-to-analysis

Time-to-analysis was investigated to discern the possible implications that a lengthy investigation may have on the ability to collect useful DNA evidence. While it was not possible to definitively establish the effects of time, the findings indicate a possible reduction in peak height response over the three time periods, particularly evident in the bone samples. This may reflect an ongoing instability in the DNA molecule as a result of gamma irradiation, however further investigation is required before any further conclusions can be reached.

2.4.3 Statistical comparison/certainty for DVI

For DVI, identification of an individual requires comparison of the victim's DNA profile with an antemortem sample. Typically, this can be achieved through direct comparison with DNA profiles obtained from personal items attributable to that individual (e.g. toothbrush, razor etc). For a direct comparison, an example of the statistical implications from radiation exposure is presented below for bone samples (Table 2.8). In this case the control represents an antemortem sample and the 100,000 Gy dose represents a DVI sample. Table 2.8 demonstrates a four-order of magnitude reduction from the control to the 100,000 Gy gamma irradiated sample.

With the loss of allelic information, the statistical certainty is reduced, however, according to Buckleton (2005); the statistical weighting would still be verbally described as very strong to extremely strong evidence (100,000 to 1,000,000+).

Table 2.8: Comparison of statistical certainty of DNA profiles from irradiated and non-irradiated bone samples (Profiler Plus® loci)

Dose (Gy)	Profiler Point Estimate (θ = 0.03): 1 in	Upper Confidence Limit: 1 in	Lower Confidence Limit: 1 in
0[1]	7.1×10^{10}	4.6×10^{10}	1.1×10^{11}
100,000[2]	1.3×10^{6}	9.6×10^{5}	1.9×10^{6}

Direct comparison to an antemortem sample however is not possible in all circumstances and therefore an alternative analysis comparing the victim with as many first degree relatives (i.e. father, mother or siblings) as practical is conducted. This type of comparison, kinship or pedigree analysis, is a very complicated statistical process that benefits greatly from as much DNA profiling information as possible. It is a very locus hungry process; in other words, the more information available from the DNA profile, the more accurate the identification will be. It is, however, beyond the scope of this study to expand on the complexities of statistical estimates in a DVI situation.

2.4.4 Estimation of DNA template size (bps) from allelic dropout

The PCR product yield for the larger sized loci began to decrease as the template DNA degraded. Based on results from Chung *et al.* (2004), it is possible to estimate template size based on the correlation between known locus sizes and observed allelic/locus dropout. For example, after bone samples were exposed to 100,000 Gy, there is complete allele dropout at the FGA, D7S820 and D18S51 loci. It is possible to estimate the size of the degraded template to be between the largest remaining allele (Allele 12 at D13S317) and the smallest dropout allele (allele 22 at the FGA locus). Based on their approximate base pair lengths of 221 and 232 respectively, the estimated template size is

[1] Statistical calculations were conducted using the AFP Profiler Plus® Confidence Interval Calculation Sheet against the Australian Capital Territory Caucasian Database. Calculations were based on the male bone DNA profile provided in Table 2.1

[2] Statistical calculations were conducted using the AFP Profiler Plus® Confidence Interval Calculation Sheet against the Australian Capital Territory Caucasian Database. Calculations were based on the male bone DNA profile 16,18 18,18 nr,nr 12,13 30.2,31.2 nr,nr 10,12 nr,nr nr,nr (nr = not reportable)

between these two values. This suggests that 100,000 Gy of gamma radiation causes a reduction in DNA template length to a minimum of 221 base pairs. It is important to note, however, that the interaction of ionising radiation with the DNA may not necessarily result in fragmentation of the strand but could also be increasing the amount of cross-linking, oxidation or hydrolysis within the DNA molecule, preventing elongation of the strand by the *Taq* polymerase (Lindahl 1993). This data could be extrapolated to predict the success of profiling with a miniSTR system, such as Amp*Fl*STR® MiniFiler™ PCR amplification kit, which analyses the higher molecular weight loci using primers that bind closer to the region of interest therefore requiring less intact target DNA (see Section 2.6 for further discussion).

2.5 Summary and conclusions

Prior to investigating the effects of gamma radiation on bone, three techniques were examined for their robustness and reliability for DNA extraction. The techniques included the AFP organic extraction protocol, protocol A from Alonso *et al.* (2001), and a modified DNA IQ™ solid-phase extraction system. Results suggest that the Alonso *et al.* (2001) organic extraction method produced a consistently high yield for extracting DNA from bone. This method was therefore selected for the subsequent bone sample analyses in this study.

2.5.1 Dose and time effects of gamma radiation on biological matrices

The findings from this research have demonstrated that gamma irradiated biological matrices are particularly robust for DNA analysis using commercially available STR systems, such as Amp*Fl*STR® Profiler Plus®. In general, there appears to be little observable degradation up to 1,000 Gy for all samples tested, and it is possible to obtain a full DNA profile at doses at least up to 10,000 Gy. This suggests that there is a threshold dose of between 1,000 and 10,000 Gy for degradation in the DNA profile to be observed, depending on the matrix tested. Marked decreases in peak height relative to

the control were typically observed at 10,000, 50,000 and 100,000 Gy, and complete loss of profile occurred at 500,000 Gy.

Overall, the results illustrate that allelic dropout first occurs at the higher molecular weight loci for doses at and above 50,000 Gy. Loss of signal was observed at the D13S317, FGA, D7S820 and D18S51 loci. In addition, the degradative effects of gamma radiation on DNA appear to be comparable with other degradative processes, such as exposure to humidity or bacterial colonisation, and therefore current standard operating procedures used in the analysis and interpretation of degraded DNA can be equally applied to DNA that has undergone exposure to ionising radiation.

While it is difficult to discern any further degradation as a result of the time-to-analysis, some trends over the three time periods suggest a reduction in response for the samples, particularly within the bone sample analysis. However, further investigation into the effects of time-to-analysis is required, to include a greater sample size and possibly longer time periods (such as extracting samples three or six months from irradiation) in order to clarify these findings.

2.5.2 Implications for operations

It is possible to apply outcomes of this research to operational scenarios either within a domestic crime scene or DVI setting. In light of such high dose thresholds for all matrices examined and apparently minor degradation with time, operational decisions regarding evidence collection would need to be based largely on potential radiation exposure to the crime scene personnel. The potential effects on DNA would be unlikely to warrant great urgency to retrieve evidentiary items from the scene.

Furthermore, the high dose thresholds observed for bone and other matrices suggest that, within a DVI setting involving ionising radiation, the primary influence on DNA integrity might logically come from other sources in addition to radioactive materials. Other contributing factors that have demonstrated accelerated DNA degradation include

post-mortem decay, as well as associated environmental conditions, such as bacterial colonisation, temperature, humidity, interfering organic compounds, and incineration by fire. In addition, other issues such as co-mingling and loss of body fragments may also impact on the successful identification and repatriation.

2.6 Future directions

The findings presented here suggest that DNA from blood, saliva and bone that has undergone irradiation with a cobalt-60 gamma source is very robust, and it is possible to obtain a full DNA profile at doses up to at least 10,000 Gy and over a 4-week period from exposure. Nevertheless, additional studies may be warranted to further explore the impact of gamma radiation on DNA evidence.

In this series of experiments, gamma irradiation was conducted with a cobalt-60 source that produces two gamma rays with energies of 1.17 MeV and 1.33 MeV. Further research may include investigation of alternative sources of gamma radiation, such as the gamma/beta-emitter caesium-137 which has a single gamma ray energy of 662 KeV, to investigate the impact of energy levels on threshold doses for degradative effects.

In this research, the starting amount of DNA template was kept consistent to ensure that a full profile would be produced and to limit variations that would be introduced by other factors such as low levels of DNA. Concurrent experiments conducted in collaboration with the Australian Nuclear Science and Technology Organisation (Abbondante 2007) has supported and extended this research to include saliva/epithelial cell deposits on cigarette butts, anagen hairs, and epithelial cells in nitrile gloves. Further investigations examining trace DNA on clothing and other items commonly found in crime scenes would be recommended.

Additional investigations may also seek to show the potential impact of radiation on the substrates on which the biological materials are found. For example, at high exposure cigarette butts and nitrile gloves become very brittle (Abbondante 2007). This has the

potential to release additional contaminants into the DNA extract and pose new sampling challenges for DNA analysis. This may further support the use of solid-phase extraction, such as the DNA IQ™ system, to assist in removing any non-biological material from the extract. Contamination and extraction are discussed further in Chapters 4 and 5.

Significant allelic/locus dropout was not observed until doses reached 50,000 Gy; however, there are alternative methods of analysis that may be applied to retrieve information from degraded samples of this nature.

Butler *et al.* (2003) stated that the loss of information from a DNA profile is "exacerbated when large multiplex PCR reactions are used due to the wide size range of PCR products produced". To overcome this, a number of research groups have produced reduced size STR primer sets, or "miniSTRs". They were able to decrease the size of the target sequence and therefore increase the chance of obtaining a successful profile from degraded DNA. The miniSTR primers work by binding as close to the repeat region as possible and therefore potentially recover information from an additional two or three loci depending on the template size of the target DNA molecule.

These miniSTR primer sets have demonstrated concordance with Profiler Plus® (Applied Biosystems, USA) or PowerPlex16® (Promega, USA) depending on the primer design specifications, as well as a number of additional non-traditional primer sets. The miniSTRs have been successfully applied to the analysis of a number of naturally and artificially degraded DNA samples (Butler *et al.* 2003; Chung *et al.* 2004; Coble and Butler 2005; Opel *et al.* 2006; Meissner *et al.* 2007; Opel *et al.* 2007; Hill *et al.* 2008). It should be noted, however, that miniSTR analysis does have limitations and other issues arise when the quantity of DNA template is minimal or sample throughput is a concern, as there is an increased number of amplification reactions and electrophoresis analyses that must be performed to obtain a profile for all loci of interest (Balogh *et al.* 2003; Irwin *et al.* 2007; Phipps and Petricevic 2007).

While the relatively small target size of conventional STRs makes them ideal for analysing degraded samples, there are various alternative methods of analysis (other than the miniSTRs discussed above) that may be applied. These include:

- Mitochondrial DNA analysis, which is tailored for degraded samples due to the high number of mitochondria present in a single cell, may provide information on the maternal lineage (Seo *et al.* 2000);
- YSTR analysis, which utilises STRs located on the Y chromosome, and may provide additional information on the paternal lineage (Hanson and Ballantyne 2005);
- Single Nucleotide Polymorphisms (SNPs), which utilise variations at single nucleotides in the genome for identification, with the small target sequence making them very useful for application to degraded DNA (Petkovski *et al.* 2004); and
- Low Copy Number (LCN) STR typing, which increases the DNA signal of aged or degraded DNA by increasing the number of amplification cycles (Hanson and Ballantyne 2005; Ballantyne *et al.* 2007; Irwin *et al.* 2007).

Beyond forensic analysis, single-cell gel electrophoresis (comet assay) can be utilised to investigate degradation of the whole chromosome (Johnson and Ferris 2002) and gas chromatography-mass spectroscopy has been shown to be a useful tool in identifying and quantifying modifications in structure of the DNA molecule (Dizdaroglu 1992; Höss *et al.* 1996).

During this research, the analysis of bone samples presented a number of areas that could be further explored. Further study and development of the DNA IQ™ system methodology for bone extraction may be required. Due to limits related to the binding capacity of the beads, it is unlikely that the DNA IQ™ system will surpass the organic DNA extraction methodologies for overall DNA yield. However, changes to methodology could be employed to increase DNA yields, such as utilising larger quantities of beads or conducting successive extractions on a single supernatant (for example, after the beads have been added, the DNA bound and the beads recovered,

additional beads may be added to the supernatant to capture any remaining DNA in the sample). The resulting increase in manual manipulations and the potential risk of exposure/contamination would need to be taken into consideration.

In addition, during the analysis of gamma irradiated bone, the quantitation of DNA produced extremely high values of total DNA for the higher dose irradiations. This can be explained by the fact that the real-time PCR quantitation target sequence is 106 bps long. This is one of the limitations of using a real-time PCR process for DNA quantitation (Swango *et al.* 2006; Swango *et al.* 2007). This further demonstrates the stability that smaller molecular weight fragments have over the larger target fragments and the quantitation technique's potential for misrepresenting the true quantity of total DNA available for PCR. While there may appear to be significant amounts of DNA template present, it is highly degraded with only low molecular weight DNA present. Recent publications by Swango *et al.* (2006; 2007) presented research into multiplex qPCR (quantitative PCR) for the assessment of DNA degradation in forensic samples. The method utilises two targets on the human genome, a longer target from the TH01 locus (170-190 bp) and a shorter target from the CSF1PO locus (67 bp) (Höss *et al.* 1996). The use of a multiplex system that has a target sequence more representative of the STR targets under analysis, may give a more accurate representation of the true quantity of useful DNA in the sample, the quality of the sample, and the likely success of obtaining a DNA profile, especially when analysing degraded DNA.

The level of complexity of the human identification process is largely dependent upon the extent of body fragmentation, the number and type of remains that can be recovered from the incident scene, and the condition of the remains. With the added difficulties associated with radiological contamination, new issues arise with the collection, storage and repatriation of remains. Such contamination may complicate analytical processes and raise issues that need to be addressed regarding occupational health and safety for both scene examiners and laboratory analysts. These issues are further discussed in Chapters 4 and 5.

Chapter 3: Effects of Alpha Radiation on DNA Profiles Obtained from Forensically Relevant Biological Matrices

al. 2004). A clear example of the damaging nature of alpha radiation on biological systems is the poisoning and subsequent death of ex-KGB agent Alexander Litvinenko with the alpha emitter polonium-210 (Harrison *et al.* 2007). From review of the available literature, however, the effect of alpha radiation on forensic DNA evidence has not been previously reported.

3.1.2 Biological matrices

As mentioned in previous chapters, blood and saliva are typical matrices analysed for DNA by the forensic science community (Section 2.1.2). Blood and saliva are comprised of a number of proteins and other cellular material in addition to the cells containing DNA for analysis.

In addition to blood and saliva, a cultured human cell line, HEp-2, was included in this experimental research. HEp-2 is an immortal cell line commonly used in virus research. The HEp-2 cell line was selected to produce a single layer of human cells on a heat-conductive surface specific for alpha irradiation in a particle accelerator. In addition, the HEp-2 cells were cultured *in vitro* and did not contain any other biological components to influence the effects of the alpha radiation.

3.1.3 Time-to-analysis

Examining a crime scene for forensic evidence has the potential to take prolonged periods of time, especially for larger incidents or complicated scenes. Within a scene involving radioactive materials, time-to-analysis will impact on the dose the material receives and the potential stability of the material over time (Section 2.1.3).

For this research, a particle accelerator was employed to reduce the risks associated with handling radioactive materials emitting alpha radiation and to increase the repeatability of dose and throughput of samples for analysis. The Small Tandem Accelerator for Research (STAR) is located at the Australian Nuclear Science and Technology

3.1 Introduction

Chapter 2 investigated the effects of gamma radiation on the DNA profile of biological matrices and established threshold values for appreciable decreases and/or complete loss in profiling response. In addition to gamma radiation, it is also necessary to investigate an alternative such as alpha radiation, as this will allow for the examination of potential differences in effects resulting from different emission characteristics.

3.1.1 Alpha radiation

Unlike gamma radiation, alpha radiation consists of a particle in the form of a helium atom (two protons and two neutrons), and therefore has two units of positive charge. A consequence of the particle's large size and charge is higher LET, i.e. significant ionisation or damage occurs within a specified distance; however, it also limits both the particle's ability to penetrate materials and the distance it can travel from the source. Damage is largely influenced by this distance from the source and the make-up of the material being irradiated. For example, cell traversal of an alpha particle (energy of 5.5 MeV) is approximately 40 μm in skin (cell width is typically 50 μm), while, with less resistance, this particle will travel approximately 40 mm in air.

As stated in Chapter 1, americium-241 has been mentioned in open source literature as a radioactive material of potential use in a radiological dispersion device (Ferguson *et al.* 2004; Zagorin and Shannon 2004; Colella *et al.* 2005). Americium-241 is usually found as an oxide powder in industrial moisture/density gauges or as a metal component in smoke detectors. During americium-241 decay to neptunium-237, two primary alpha particles are emitted at 5.486 MeV and 5.449 MeV, as well as a low energy gamma ray at 59.5 KeV (NNDC 2009).

Research has explored the biological consequences of exposure to alpha particles in living systems through, for example, the appearance of chromosomal aberrations with analysis using fluorescent *in-situ* hybridisation (Bauchinger *et al.* 1997; Barquinero *et*

Organisation (ANSTO), NSW, and was used to produce a particle of energy 5.5 MeV, consistent with americium-241 (Figure 3.1). In addition, certain criteria were established: the source was a 3.7×10^{11} Bq (10 Ci) americium-241 source, the biological materials were made as thin as possible (to form a single layer of cells), and, to formulate dose, it was assumed that the source and sample were in contact.

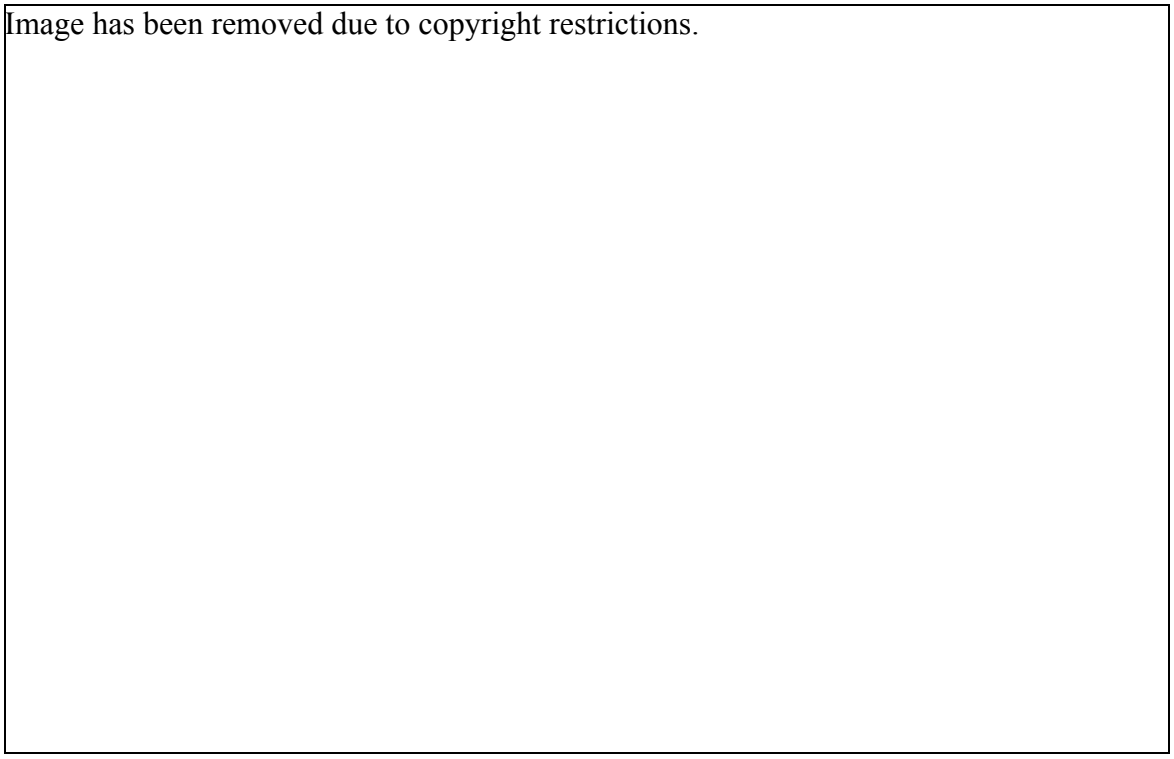


Figure 3.1: The Small Tandem Accelerator for Research at the Australian Nuclear Science and Technology Organisation NSW (ANSTO 2008)

3.1.4 Aims

The effects of alpha radiation on biological materials, particularly those relevant to forensic investigations, require additional research. Therefore, the objectives of the studies described in this chapter were to:

1) Investigate the effects of alpha particles from a particle accelerator (particle energy of 5.5 MeV) on DNA from biological matrices of blood, saliva and a human epithelial cell line.
2) Investigate the potential impact of time-to-analysis on the qualitative and quantitative aspects of a DNA profile.
3) Establish limits of exposure for the analysis of alpha particle-irradiated biological samples.
4) Compare the effects of gamma and alpha radiation on DNA profiles obtained from blood and saliva.

3.2 Experimental

3.2.1 Biological matrices

Human blood, saliva and a HEp-2 (epithelial) cell line were used for this study. Donated human male blood was obtained from the Australian Federal Police standards collection and stored at -20 ^{o}C until required. Donated human male saliva was collected by expectoration and stored at -20 ^{o}C until use. The *in vitro* human epithelial cell line, HEp-2, was provided by the Faculty of Applied Science at the University of Canberra.

Protocols for DNA extraction utilised Chelex® 100 resin purchased from Bio-Rad Laboratories (USA). Real-Time PCR analysis was utilised for quantitation of DNA extracts. Primers and probes were purchased from Sigma-Aldrich (USA), with additional reagents from Invitrogen (USA).

DNA profiling analysis was conducted using Amp*Fl*STR® Profiler Plus® PCR Amplification Kit (Profiler Plus®) from Applied Biosystems (USA). All reagents associated with the DNA profiling and capillary electrophoresis analysis were purchased from Applied Biosystems (USA). The DNA profiles produced from each of the three matrices using Profiler Plus® are shown in Table 3.1.

Table 3.1: Profiler Plus® DNA profiles for the selected matrices of blood, saliva, and HEp-2 cells

Locus	Blood	Saliva	HEp-2 cell line
Amelogenin	X,Y	X,Y	X,X
D3S1358	16,16	14,15	15,18
vWA	16,16	17,17	16,18
FGA	21,22	21,21	18,21
D8S1179	12,13	12,13	12,13
D21S11	30,31	30,31.2	27,28
D18S51	16,17	16,17	16,16
D5S818	12,13	11,13	11,12
D13S317	8,12	8,13	12,13.3
D7S820	7,11	9,11	8,12

3.2.2 Preparation of blood, saliva and HEp-2 cells for irradiation

The use of the STAR accelerator for irradiation placed restrictions on sample preparation. The samples were required to be mounted on a conductive surface to prevent charge and heat build-up; therefore, traditional substrates such as glass, plastic or fabric could not be used. Aluminium is a common substrate used for this purpose.

Aluminium baking trays (<152.4 μm thick) were cut into 3 x 5 cm pieces and pressed to provide a visually uniform surface. An adhesive binder reinforcement ring with a 5 mm hole in the centre was placed on each piece of aluminium to centralise the location and standardise the size and shape of the biological sample added. Each piece of aluminium

was wiped with 10% bleach and 70% ethanol, and then sterilised with ultraviolet radiation for 45 minutes prior to sample addition.

5 µL of 1:4 and 1:3 diluted blood[3]:TE Buffer, 1:1 diluted saliva:TE Buffer or the HEp-2 cell suspension was added to the 5 mm ring in the centre of the sticker and allowed to air-dry in a Class II laminar flow hood (laminar flow hood). Samples were then placed in a desiccating jar at room temperature until required.

3.2.2.1 HEp-2 cell culturing and sample preparation

For cell culturing, a sterile work area within a laminar flow hood (with ultraviolet irradiation for 10 min and treatment with 70% ethanol), the use of aseptic techniques, sterile disposable plastic pipettes, culture vessels, disposable gloves and clean laboratory coats were required.

The HEp-2 cell line was obtained from the Centre for Biomolecular and Chemical Studies, University of Canberra. The cells were cultured in Opti-MEM® culture medium (a modified Eagle's minimum essential medium; Invitrogen, USA) supplemented with 3% newborn bovine serum (Invitrogen, USA), penicillin (50 U/ml) and streptomycin (50 µg/ml) at 37 ^{o}C in a humidified 95% air / 5% CO_2 atmosphere. After the cells became 80-90% confluent, the culture medium was decanted, the cells were rinsed with 10mM Phosphate-Buffered Saline (PBS) without calcium or magnesium (Gibco, USA), and released from the flask using a sterile cell scraper or 0.25% Trypsin (incubated at 37 ^{o}C in 95% air / 5% CO_2 for 10 mins). Trypsinisation was discontinued by the addition of Opti-MEM® culture medium supplemented with 3% newborn bovine serum, and the cells were either subcultured and incubated at 37 ^{o}C in 95% air / 5% CO_2 or prepared for experimental use.

[3] Pilot studies investigated sample preparations of whole blood and less diluted concentrations, however, it was demonstrated that at these levels, the biological material became brittle and would not adhere to the metallic surface. This was particularly important as samples were to be vertically mounted for irradiation in the STAR.

For sample preparation, the released HEp-2 cells were decanted from the culture vessel into a 10 mL screw-cap tube and centrifuged at 2000 rpm for 10 min at 4 ^{o}C. After centrifugation, the supernatant was decanted, with the pellet remaining at the bottom of the tube and 1 mL PBS was added. The HEp-2 cells were then resuspended and counted using a haemocytometer to establish approximate cell numbers. For counting, 20 µL of the cell sample was added to 20 µL of PBS and Trypan blue (Sigma-Aldrich, USA) and the haemocytometer placed under a microscope. Using a manual counter, total cell number was estimated at 3.4×10^6 cells/mL.

3.3 Experimental procedures

3.3.1 Irradiation of samples

For this series of experiments, the Small Tandem for Applied Research (STAR) particle accelerator at ANSTO was used to generate the alpha particles. The STAR accelerator is a compact two million volt tandem particle accelerator flooded with helium ions.

Irradiation with the STAR particle accelerator required the samples to be vertically mounted, less than 9 mm in diameter to accommodate beam size, completely dry, a single layer of cells (where possible), and fixed on a conductive mount (i.e. aluminium).

Prepared blood, saliva and HEp-2 samples were irradiated with 5.5 MeV helium ions using the STAR particle accelerator. The beam diameter was measured at approximately 9 mm and the target current maintained at 40 nA to reduce electric discharge from the plastic reinforcement rings and to prevent the samples from overheating faster than the aluminium could dissipate the heat.

Samples were irradiated for different time periods of 0, 3, 10, 20, 60, 180, 600 and 1200 seconds. The converted doses were calculated as 0, 66,000, 220,000, 440,000, 1,320,000, 3,690,000, 13,200,000, and 26,400,000 grays. An example calculation is presented below.

The conversion requires calculation of the charge (nC'), activity (Bq), dose (MeV/kg) and finally dose (Gy). The following example calculates the dose of a 3 second irradiation at a current of 40 nA.

Charge (as nC'):

$$nC' = t(s) \times c(nA)$$
$$nC' = 3s \times 40nA \qquad \text{[Equation 1]}$$
$$nC' = 120$$

where *t* is the time in seconds exposed to the beam and *c* is the current (nA) of the beam. Using the charge (nC'), it is possible to determine the activity (Bq).

Note that, for the following equation, nC' must be converted into C' by multiplying by 1×10^{-9}.

Activity (as Bq):

$$Bq = \frac{Ch(C')}{Ch_{Particle}(J)}$$
$$Bq = \frac{120 \times 10^{-9}}{3.204 \times 10^{-19}} \qquad \text{[Equation 2]}$$
$$Bq = 3.75 \times 10^{-11}$$

where *Ch* is the charge in coulomb (C') and $Ch_{particle}$ is the elementary charge of the particle, i.e. alpha particle (He^{2+}) charge is 3.204×10^{-19} J (i.e. $2 \times 1.602 \times 10^{-19}$ J).

Dose (as MeV/kg):

$$MeV/kg = \frac{Energy(MeV)}{Mass(kg)}$$
$$MeV/kg = \frac{Activity(Bq) \times Energy_{Particle}(MeV/Bq)}{Mass(kg)}$$
$$MeV/kg = \frac{3.75 \times 10^{-11} Bq \times 5.5 MeV/Bq}{5 \times 10^{-6} kg}$$
$$MeV/kg = 4.12 \times 10^{-17}$$

[Equation 3]

Dose is given by energy (MeV) divided by mass (kg), where energy (MeV) is activity of the sample (Bq) multiplied by the energy of the particle (MeV). Dose in MeV/kg is then converted into grays (Gy) by the following equation.

Dose (as Gy):

$$Gy = 4.12 \times 10^{-17} MeV/kg \times 1.602 \times 10^{-13} J$$
$$Gy = 6.6 \times 10^{4}$$
$$Gy = 66000$$

[Equation 4]

Dose (MeV/kg) is converted to dose (Gray), where 1 MeV is equal to 1.602×10^{-13} J.

3.3.2 Sample collection and Chelex® 100 extraction of DNA from blood, saliva and HEp-2 cells.

Dried biological fluids on the aluminium substrate were collected with a cotton swab moistened with distilled water for blood samples and 70% ethanol for the saliva and HEp-2 cell samples.

Extraction was conducted using a 5% Chelex® 100 resin solution. Further experimental details can be found in Section 2.3.2.2.

3.3.3 DNA quantitation, amplification and profiling using CE

The DNA extracts were processed using the Rotor-Gene™ 3000 real-time PCR system (Corbett Research, Australia) for DNA quantitation, amplified using the Amp*Fl*STR PCR amplification kit (Applied Biosystems, USA), and profile using the ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems, USA) in conjunction with the ABI PRISM® 3100 Data Collection Software V1.2, GeneScan® Analysis Software Version 3.7, and Genotyper® Version 2.0 with Kazam® Macros (Applied Biosystems, USA). Further details are provided in Sections 2.3.4 through 2.3.7. DNA typing protocols were conducted as described as in Section 2.3.8.

3.4 Results and discussion

Dried male blood, saliva and epithelial (HEp-2) cells were irradiated at seven doses, including 66,000, 220,000, 440,000, 1,320,000, 3,690,000, 13,200,000, and 26,400,000 Gy to examine a broad range of exposures on these matrices. After irradiation, the DNA was extracted at three time periods (1-day, 1-week and 4-weeks) to assess any increase in degradative effects as a result of delay to extraction. A minimum of six samples were analysed for each dose at each time point and these were treated and examined in triplicate.

In Figures 3.2 to 3.11, loci have been presented along the x-axis of each graph in order from smallest to largest (Amelogenin < D3S1358 < D8S1179 < D5S818 < vWA < D21S11 < D13S317 < FGA < D7S820 < D18S51) to demonstrate the relationship between degradation and locus size. The data points indicate average peak heights in relative fluorescent units (RFU) as a percentage of the control.

3.4.1 Alpha irradiation of HEp-2

1-day post-irradiation

The results of the HEp-2 cells at 1-day post-irradiation demonstrated a distinct dose-response relationship as there was a gradual decrease in peak height, relative to the control, with increasing dose (Figure 3.2).

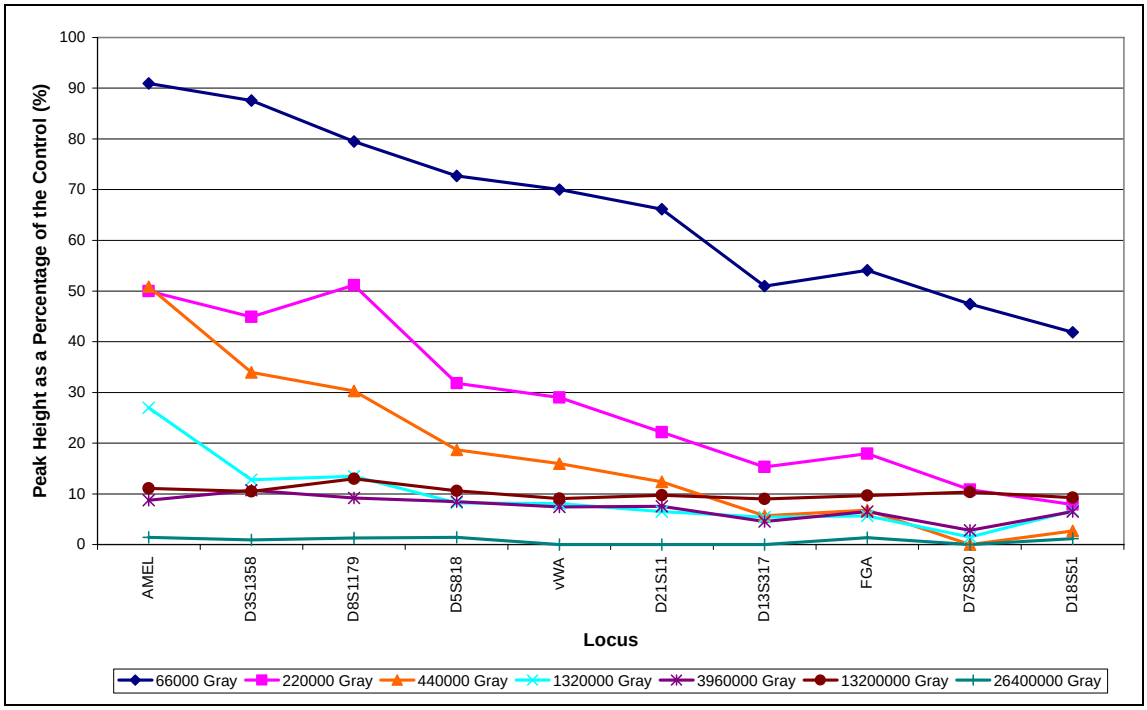


Figure 3.2: The effects of alpha radiation on DNA from HEp-2 cells (analysed 1-day post-irradiation; extracted with Chelex® 100)

Immediate degradation of the DNA profile was observed at the first dose examined (66,000 Gy), with only the first three loci exhibiting peak heights within the standard error of the control (Table A13 in Appendix). Within this dose of 66,000 Gy, a considerable decrease in peak height response occurs from the lowest (AMEL at 91% that of the control) to highest molecular weight locus (D18S51 at 42% of the control). This trend clearly demonstrates the susceptibility of larger molecular weight loci to the degradative effects caused by alpha radiation.

From Figure 3.2 and Table A13 (Appendix), it can also be seen that the next highest dose of 220,000 Gy fell outside the standard error of the lower dose. This further demonstrates a clear pattern associating the dose and the peak height response. A greater than 50% reduction in peak height relative to the control is observed at doses at or above 220,000 Gy. Full profiles were obtained up to 13,200,000 Gy, with loss of higher molecular weight loci vWA, D21S11, D13S317 and D7S820 at 26,400,000 Gy. It should be noted, however, that while a partial profile is obtained at the highest dose of 26,400,000 Gy, the peaks detected were close to the limit of detection for the instrument and would not have contributed to a DNA profile for evidential purposes in a criminal investigation. In a similar fashion to the 1-week post-irradiation results from the gamma irradiation of saliva (Section 2.4.2.3), the larger D18S51 locus did not dropout before the lower molecular weight D7S820 locus. As shown in Table 3.1, the DNA profile for the D18S51 locus of the HEp-2 cell line is homozygote 16,16, with heterozygote alleles at the lower molecular weight locus. As previously explored, the homozygote alleles will appear in an electropherogram as a single peak at approximately twice the RFU value of a heterozygote peak. Therefore alleles from the D18S51 were able to be detected due to the greater peak height generated for the homozygote alleles at that locus.

It should be noted that the HEp-2 DNA profiles exhibited the variant allele 13.3, detected at 228 bps in the D13S317 locus. This variant has been previously reported for this type of cell line (Butler and Reeder 1997).

1-week post-irradiation

At 1-week post-irradiation, the samples exhibited a similar trend as described above, with a decrease in peak height response with increasing dose. However, at this time period, peak heights from all doses fell outside the standard error of the control (Figure 3.3 and Table A14), demonstrating a considerable effect to the DNA profiles as a result of exposure to these doses.

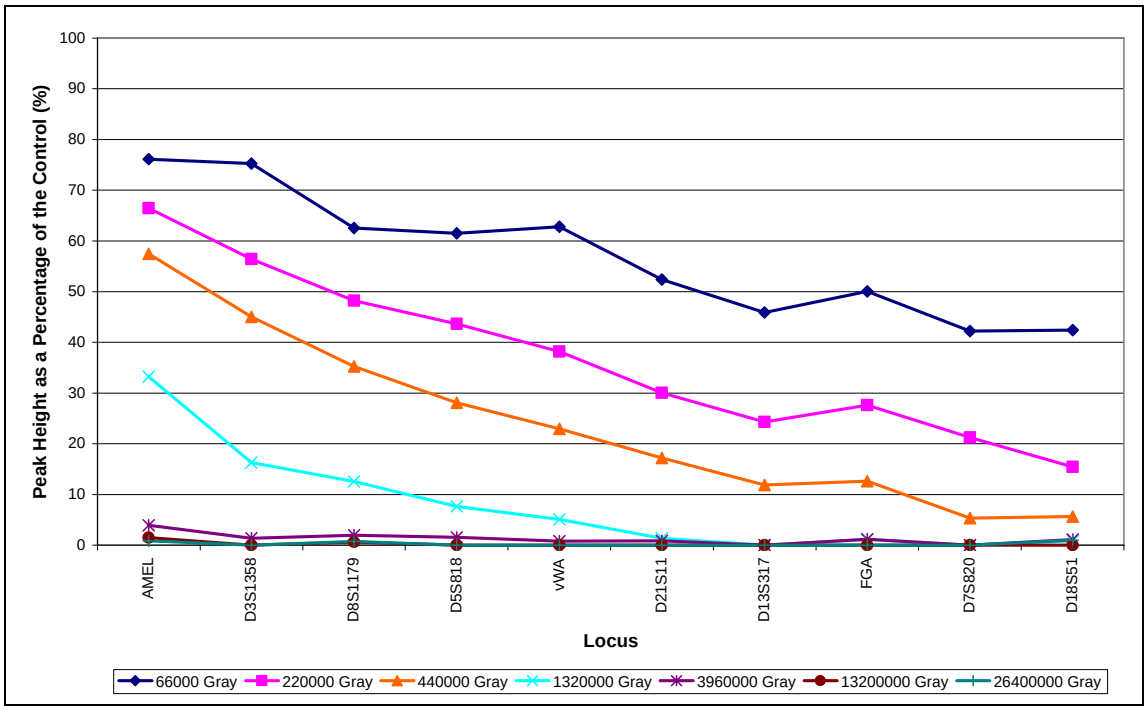


Figure 3.3: The effects of alpha radiation on DNA from HEp-2 cells (analysed 1-week post-irradiation; extracted with Chelex® 100)

Unlike the 1-day post-irradiation samples, a complete profile was only obtained up to 440,000 Gy, and allelic/locus dropout was observed at the D13S317, FGA and D7S820 loci after irradiation with 1,320,000 Gy. Above 13,200,000 Gy, a complete loss of the DNA profile occurred at all except the amelogenin locus. This trend suggests some degree of degradation in the delay from 1-day to 1-week.

4-weeks post-irradiation

At 4 weeks post-irradiation, a similar pattern was observed to those generated for the 1-day and 1-week post-irradiation samples, as marked degradation was observed from the lowest dose of 66,000 Gy. However, unlike the 1-week post-irradiation samples, the AMEL and D3S1358 loci for this dose fell within the standard error of the control with peak heights decreasing as the molecular weight of the locus increases (Figure 3.4 and Table A15 in Appendix).

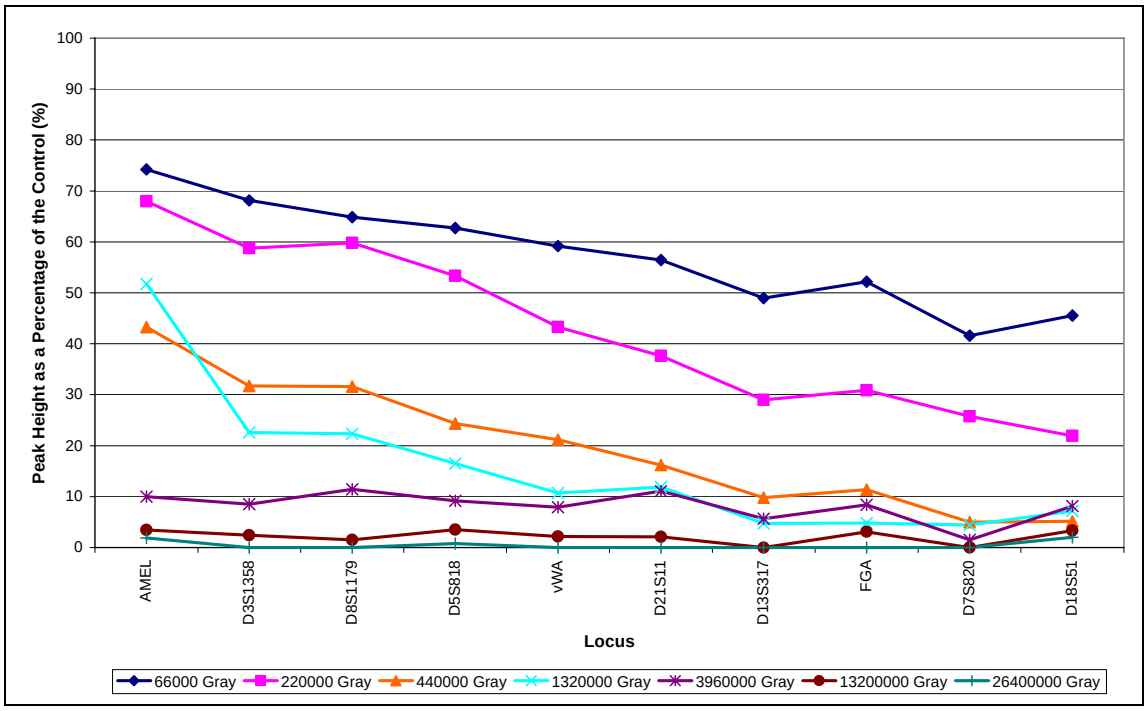


Figure 3.4: The effects of alpha radiation on DNA from HEp-2 cells (analysed 4-weeks post-irradiation; extracted with Chelex® 100)

In contrast to the 1-week post-extraction, full profiles were able to be obtained up to the higher dose of 3,960,000 Gy, with locus dropout occurring at D13S317 and D7S820 of the 13,200,000 Gy dose. Almost complete loss of profile occurs at 26,400,000 Gy, with detectable peaks only present for AMEL, D5S818 and D18S51.

Summary

Figures 3.2 to 3.4 demonstrate that the likelihood of obtaining a DNA profile from epithelial (HEp-2) cells decreases as the dose increases, with almost a complete loss of the DNA profile at the highest dose. This dose dependency pattern does not alter over time, as there are no discernable trends from extraction at 1-day, 1-week or 4-weeks post-irradiation.

Overall, the results indicate that a significant effect is occurring across the targeted loci at and above 66,000 Gy, with a partial loss of profile first observed at the 1,320,000 Gy dose. Loss of signal is observed at the higher molecular weight loci, typically with

D13S317 and D7S820, but also with vWA and D21S11. There is some indication from the time-to-analysis delay of 1-day to 1-week that further DNA degradation occurred over this period.

3.4.2 Alpha irradiation of blood

Two dilutions of blood were examined. Group 1 consisted of a 1:4 dilution and group 2 consisted of a 1:3 dilution.

1-day post-irradiation (Group 1)

At 1-day post-irradiation, the blood exhibited a decrease in peak response as exposure to alpha radiation increased (Figure 3.5).

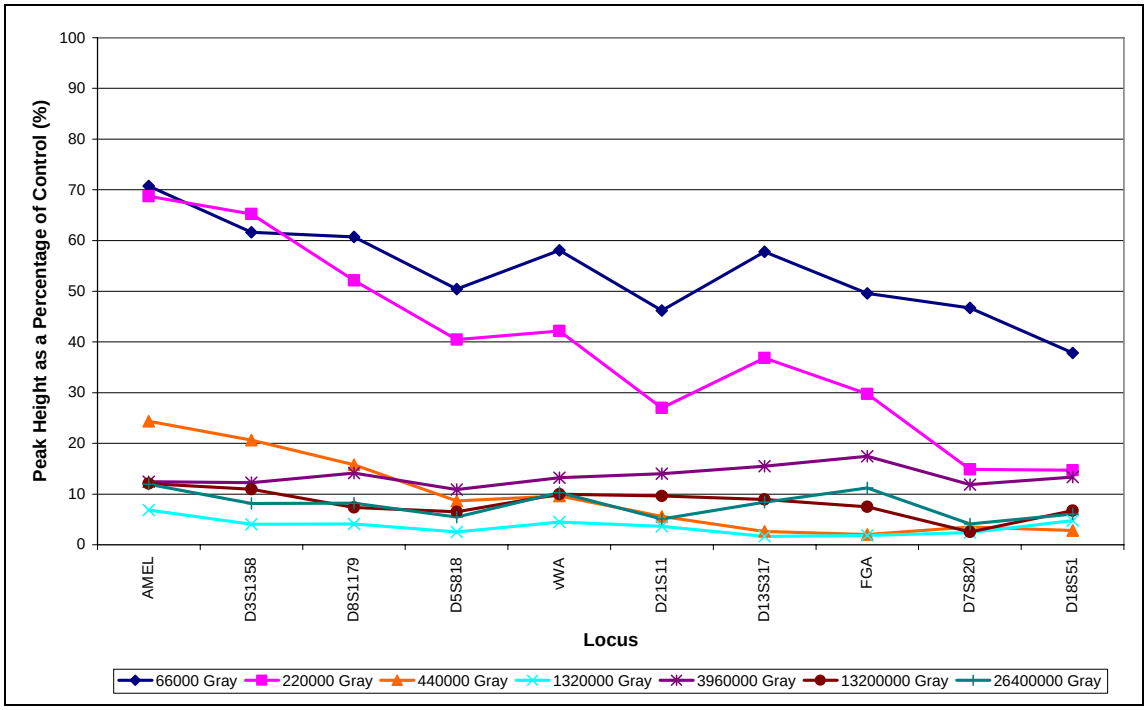


Figure 3.5: The effects of alpha radiation on DNA from 1:4 diluted male blood (analysed 1-day post-irradiation; extracted with Chelex® 100)

For doses 66,000 and 220,000 Gy, peak heights at the AMEL and D3S1358 loci fell within the error of the standard control. However, the larger molecular weight loci fell

outside this threshold and demonstrated a substantial difference from the control (Table A16 in Appendix). This is consistent with the concept that due to their size, larger molecular weight loci are more likely to interact with alpha particles and therefore exhibit a greater effect. In addition, full profiles were observed at all doses, showing a degree of robustness not observed in the HEp-2 cells, however it should be noted that the higher doses produced peaks very close to the threshold value for detection with this system.

1-week post-irradiation (Group 1)

At 1-week post-irradiation, the male blood samples demonstrated a decrease in peak height as the dose increased (Figure 3.6 and Table A17 in Appendix). As observed at 1-day post-irradiation, a reduction in peak height is observed at a dose of 66,000 Gy, with a greater impact on the peak heights of the higher molecular weight loci. For example, the lower doses (66,000 – 440,000 Gy) exhibited peak heights within the standard error of the control at the first two loci.

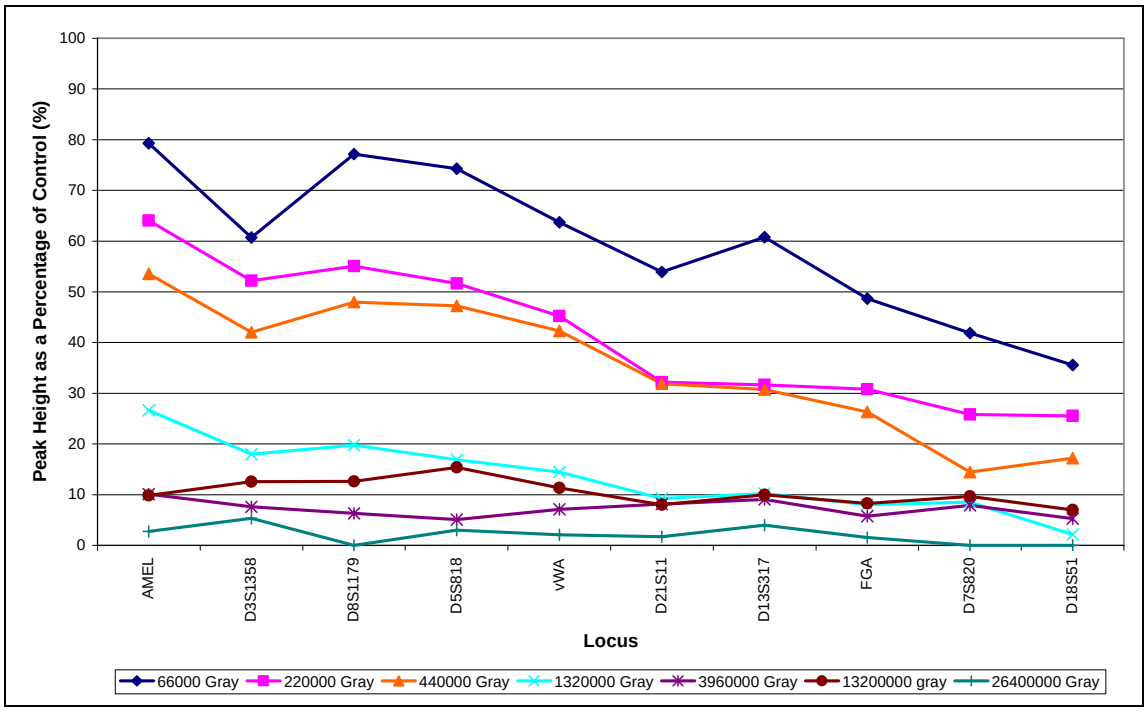


Figure 3.6: The effects of alpha radiation on DNA from 1:4 diluted male blood (analysed 1-week post-irradiation; extracted with Chelex® 100)

Full DNA profiles were obtained up to 13,200,000 Gy, with allele dropout occurring at the D7S820 and D18S51 loci as well as the D8S1179 for the 26,400,000 Gy dose. Similar to the 1-day results, the peak height values for the higher doses bordered on the limit of detection for the instrument.

4-weeks post-irradiation (Group 1)

At 4 weeks post-irradiation, a similar pattern was generated as for the 1-day and 1-week post-irradiation samples. However, allele dropout occurred at the lower dose of 440,000 Gy, and almost complete loss of profile is observed above this dose (Figure 3.7 and Table A18). At 440,000 Gy, peak response is lost at the D7S820 and D18S51 loci, and at higher doses, locus dropout is observed at the D5S818, D21S11, D13S317, and FGA loci. The increase in locus dropout demonstrated across the three time points, may demonstrate an increase in degradation over time.

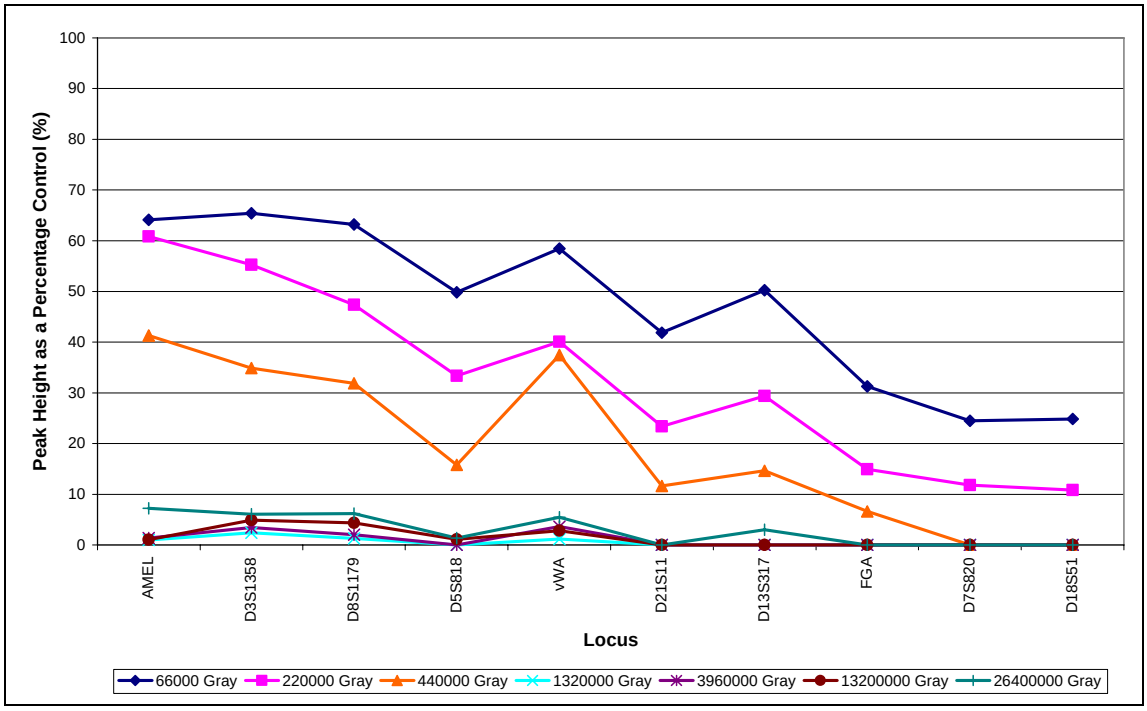


Figure 3.7: The effects of alpha radiation on DNA from 1:4 diluted male blood (analysed 4-weeks post-irradiation; extracted with Chelex® 100)

Summary

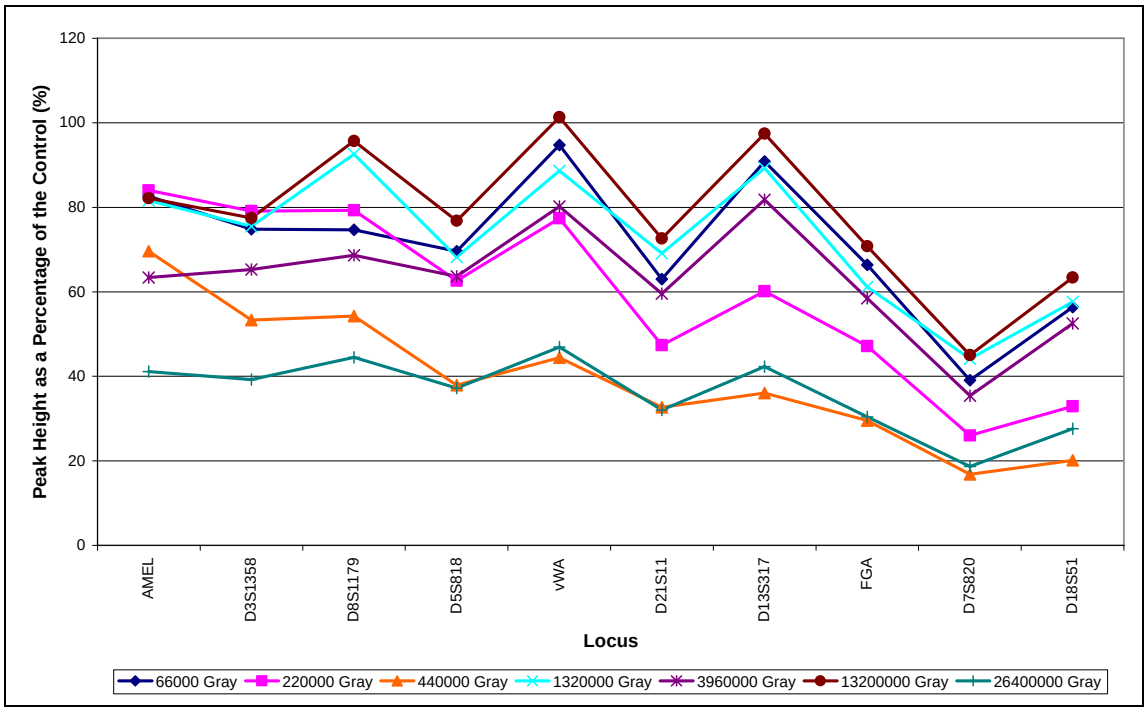
Overall, there appears to be a consistent trend whereby DNA degradation is first observed at 66,000 Gy, with a dose-dependant decrease in profiling success. For all time periods, degradation occurred preferentially at the higher molecular weight loci, with a correlation between locus size and degradation.

It is difficult to discern any further degradation as a result of the time-to-analysis, particularly as a result of the possible variation produced by the consistency/viscosity of the blood matrix (see below) and the penetrability of the alpha particles, in addition to the sample size and instrumental variation. However, there is a general trend over the three time periods indicating a reduction in response over time (Figures 3.5 to 3.7). This is demonstrated by full profiles obtained at all doses for 1-day post-irradiation as compared to some locus dropout at 1-week post-irradiation and complete loss of DNA profile information at doses above 1,320,000 Gy for 4-weeks post-irradiation.

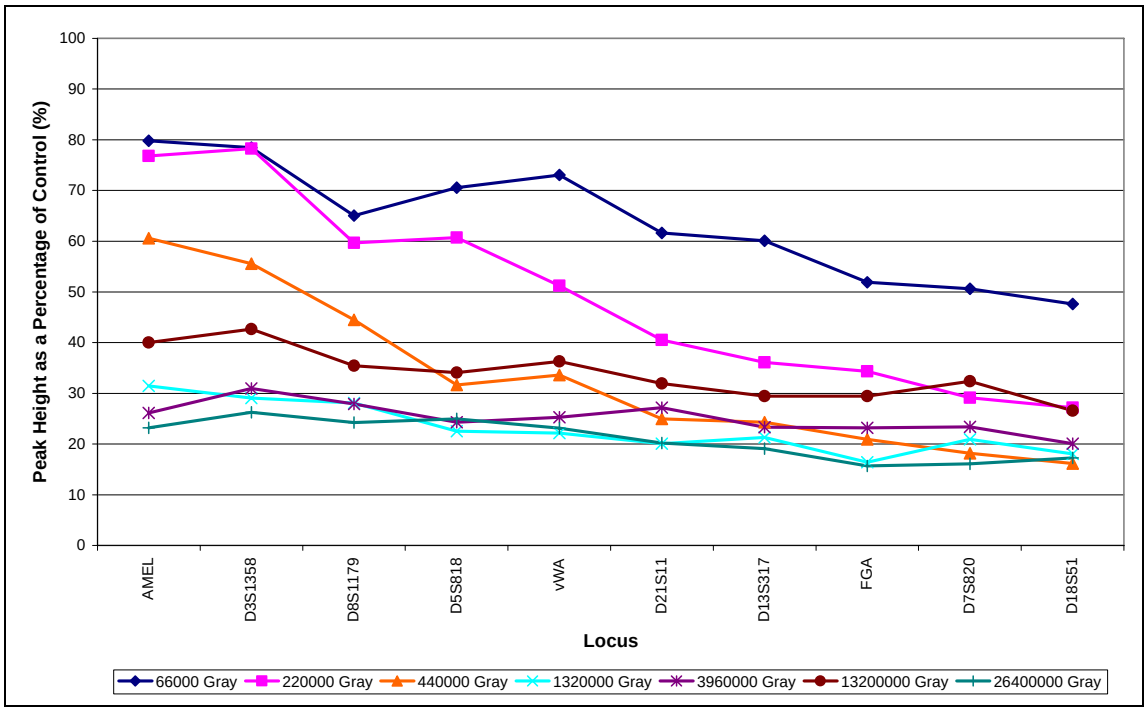
1-day, 1-week and 4-weeks post-irradiation (Group 2)

Figure 3.8 illustrates findings obtained from 1:3 diluted blood at 1-day, 1-week and 4-weeks post-irradiation. There was a higher degree of variability for this series (Table A19 through A21 in Appendix), however, this does not detract from the general trend of a reduction in profiling success for DNA in human blood due to an increase in alpha radiation dose. This deviation may be explained in the difficulties in preparing and analysing samples for alpha radiation, e.g. ensuring a single layer of cells on the aluminium mount which can influence the penetrability (approximately 0.04 mm) and therefore the effect of the alpha particles.

(a)


Peak Height as a Percentage of the Control (%)
120
100
80
60
40
20
0
AMEL
D3S1358
D8S1179
D5S818
vWA
D21S11
D13S317
FGA
D7S820
D18S51
Locus
66000 Gray
220000 Gray
440000 Gray
1320000 Gray
3960000 Gray
13200000 Gray
26400000 Gray

(b)


Peak Height as a Percentage of Control (%)
100
90
80
70
60
50
40
30
20
10
0
AMEL
D3S1358
D8S1179
D5S818
vWA
D21S11
D13S317
FGA
D7S820
D18S51
Locus
66000 Gray
220000 Gray
440000 Gray
1320000 Gray
3960000 Gray
13200000 Gray
26400000 Gray

(c)

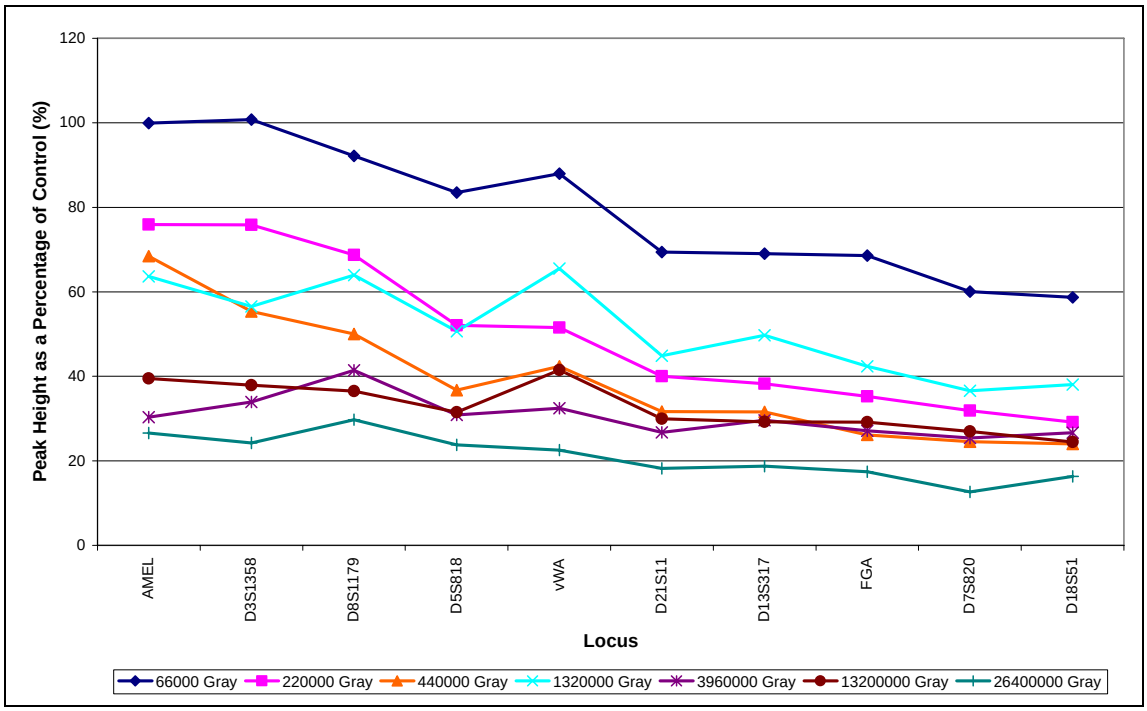


Figure 3.8: The effects of alpha radiation on DNA from 1:3 diluted male blood analysed (a) 1-day, (b) 1-week and (c) 4-weeks post-irradiation (extracted with Chelex® 100)

In contrast to the epithelial cells and 1:4 diluted blood, the DNA in the 1:3 diluted blood appears more stable at the highest doses, as over 20% of the starting amount of DNA remains after exposure. This may suggest that the constituents of the blood, such as the white blood cells, red blood cells and the plasma, afforded some protection by shielding the DNA from the alpha particles. As such, peak response may not be exhibiting the typical dose-response pattern due to variations in the layering of the cells within the samples. Therefore, slight changes in blood consistency may result in changes to the ability of alpha particles to penetrate and impact the DNA. Further analysis of a less concentrated sample e.g. 1:5 may confirm the impact of consistency/viscosity on DNA profiling success.

In addition, the DNA exhibits increased stability when extracted at 1-day post-irradiation, as a greater degradation effect was observed as the time to extraction

increases to 1-week. However, it can be seen that there is no further degradation from one week to four weeks post-irradiation.

3.4.3 Alpha irradiation of saliva

1-day post-irradiation

The results from saliva at 1-day post-irradiation demonstrated a similar result to other exposed matrices, in that the profiles exhibited a decrease in peak height from the initial dose of 66,000 Gy (Figure 3.9 and Table A22 in Appendix). A complete profile was only observed at this initial dose, with locus dropout occurring at D7S820 and D18S51 for the 220,000 Gy dose group. Peak heights continued decreasing with increasing dose, with greater reduction occurring at the higher molecular weight loci.

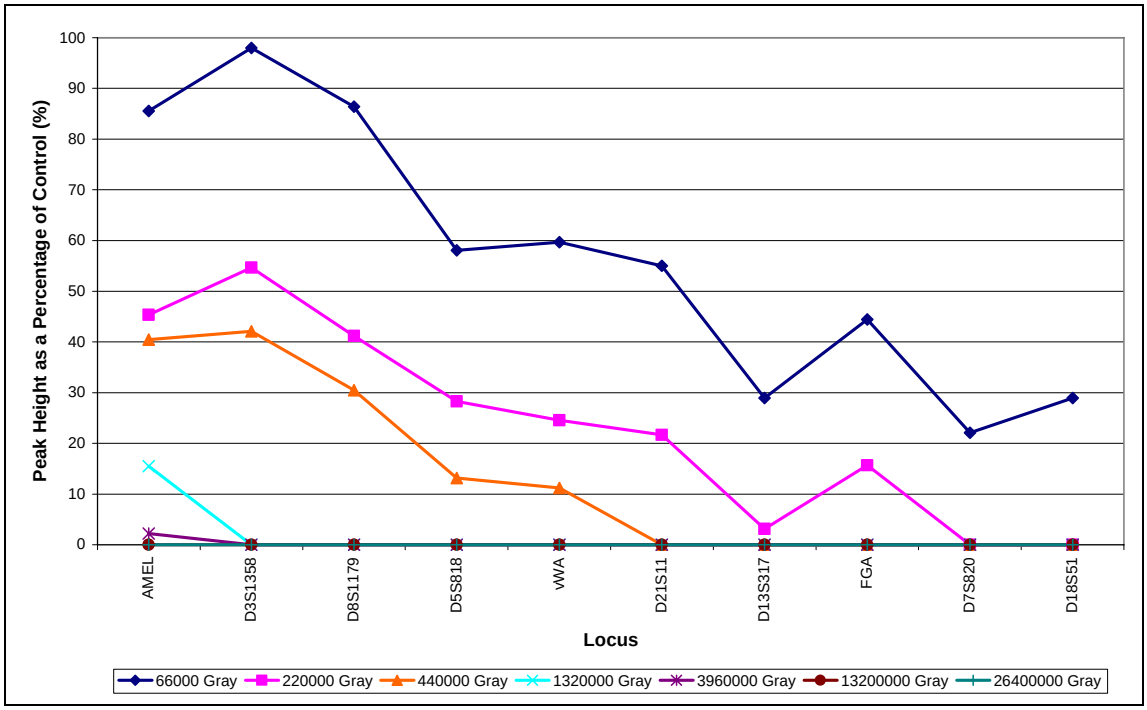


Figure 3.9: The effects of alpha radiation on DNA from male saliva (analysed 1-day post-irradiation; extracted with Chelex® 100)

No profiles were obtained at or above 1,320,000 Gy. This is in direct contrast to results obtained from alpha irradiation of HEp-2 cells and blood, where full profiles were obtained up to 220,000 Gy in all instances (Figures 3.2 to 3.7).

As noted in Chapter 2, the saliva samples for all time periods demonstrated inherent degradation, resulting in low peak heights at the higher molecular weight loci for all samples tested. As a result, there appears that the combined effects of the inherent degradation and exposure to alpha radiation may have produced a more marked decrease in the peak heights, resulting in profile drop-out at lower doses.

1-week post-irradiation

The results from the 1-week extraction of the saliva samples demonstrated a similar degradative effect at doses above 66,000 Gy, with larger molecular weight loci dropping below 50% of the peak height with respect to the control (Figure 3.10 and Table A23 in Appendix).

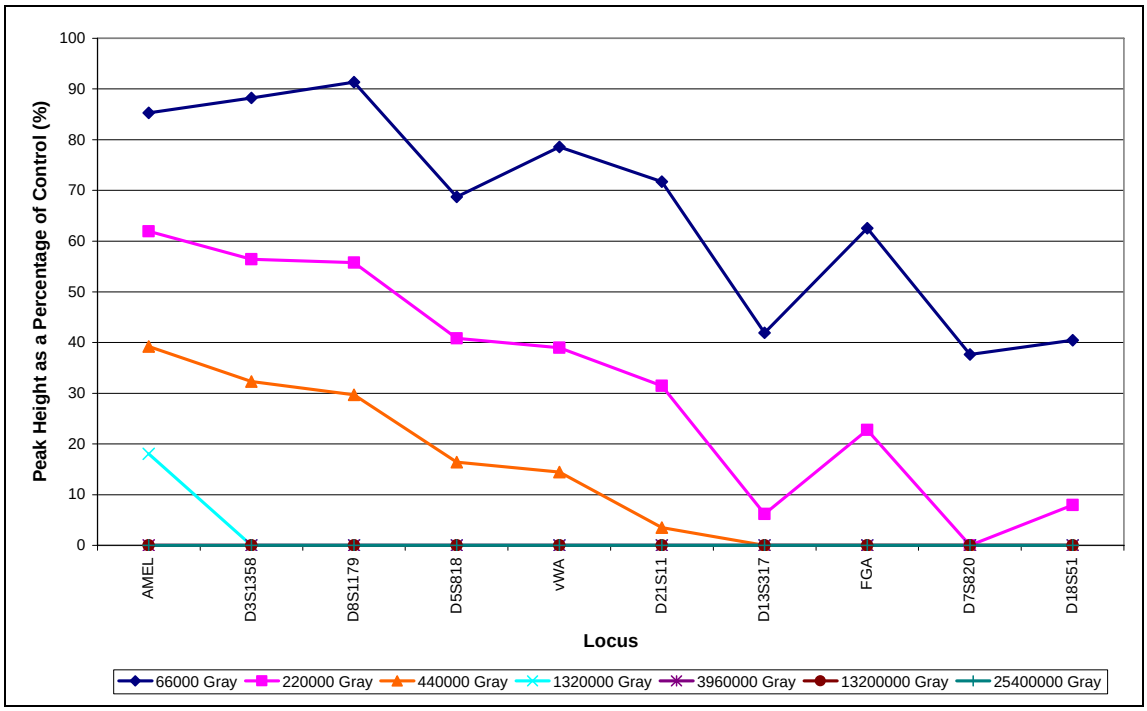


Figure 3.10: The effects of alpha radiation on DNA from male saliva (analysed 1-week post-irradiation; extracted with Chelex® 100)

As with the 1-day samples, full DNA profiles were only obtained at 66,000 Gy, with locus dropout at the D7S820 locus for the 220,000 Gy dose, and complete loss of profile observed above 1,320,000 Gy.

4-weeks post-irradiation

At 4-weeks post-irradiation, a similar pattern is observed to the 1-day and 1-week samples, as there was degradation across all doses and a full profile was demonstrated only at the 66,000 Gy dose (Figure 3.11 and Table A24 in Appendix). Locus dropout first occurs at the D7S820 locus at a dose of 220,000 Gy, and a gradual decrease in peak height is observed with an increase in locus dropout. At 3,960,000 and 13,200,000 Gy, only peaks at the amelogenin locus were detected and no profile was obtained at 26,400,000 Gy.

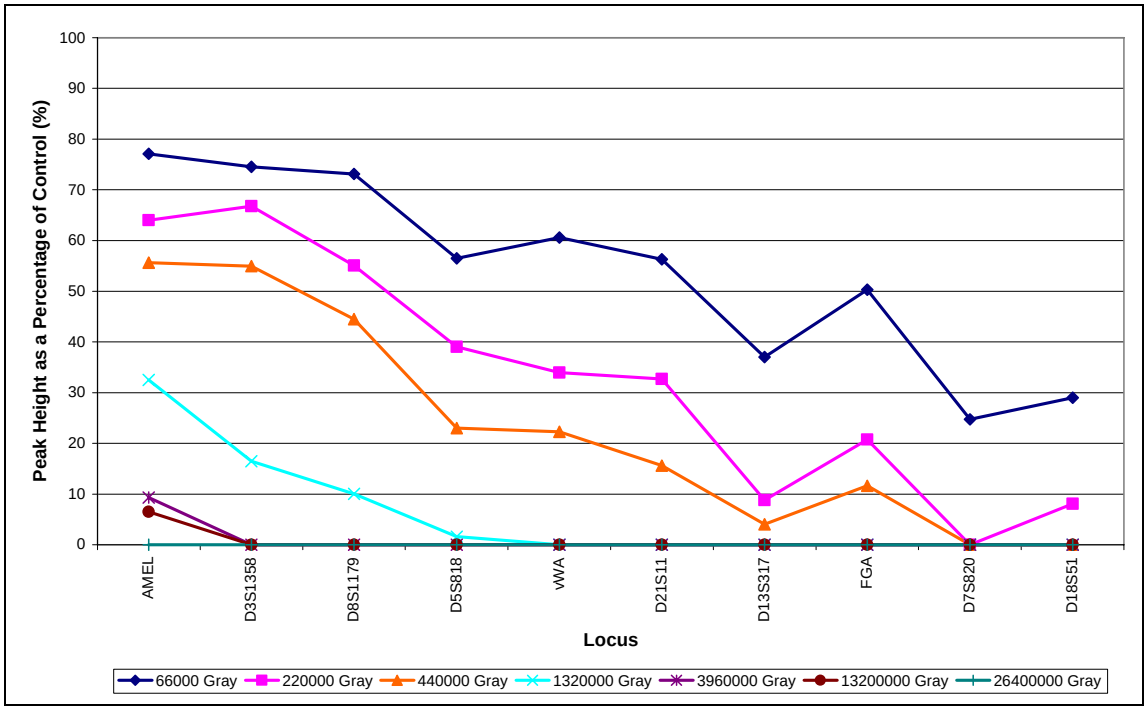


Figure 3.11: The effects of alpha radiation on DNA from male saliva (analysed 4-weeks post-irradiation; extracted with Chelex® 100)

Summary

Overall, alpha irradiation of male saliva has shown DNA degradation across all doses, with a decrease in peak height with increase in dose. Degradation is first observed at the 66,000 Gy dose across all time periods, with higher molecular weight loci affected preferentially over the lower molecular weight loci (Figures 3.9 to 3.11).

There does not appear to be a consistent trend generated from the time-to-analysis study on the saliva samples. Compared to the HEp-2 and blood matrices, the saliva appears more susceptible to DNA degradation from alpha radiation. However, as previously mentioned, the saliva used in this study was likely to have been contaminated with bacteria or other components that could potentially cause degradation to the sample independent of the radiation. As a result, the peak heights at the higher molecular weight loci were relatively low prior to irradiation (see Section 2.4.2.3). This phenomenon is therefore more likely a combination of existing quality of the sample and the exposure to alpha radiation, and demonstrates the importance of other factors influencing the successful generation of a DNA profile from a sample of unknown quality and quality.

3.4.4 General discussion of the effects of alpha radiation on biological matrices

Consistent with data reported for gamma radiation, the results from alpha irradiation demonstrate that allelic dropout first occurs at the higher molecular weight loci (i.e. loci at the right-hand-end of the x axis in Figures 3.2 to 3.11). This trend has been observed in other forms of DNA degradation and is due to the higher molecular weight loci providing a larger cross section for alpha particles to interact and cause damage. These ionisations cause single- and double-stranded breaks in the DNA molecule, as well as DNA cross-linking, deamination and dimer formation, which results in PCR amplification failure as there is insufficient intact template DNA for primers to bind.

Similarly, additional or artifact peaks were not observed and DNA profiles were identical between controls and replicates, except where allelic dropout occurred.

Time-to-analysis

The effects of a delay in the analysis of a sample after radiation exposure were investigated to determine possible impacts on the generation of useful DNA evidence. Based on these findings, it was not possible to definitively determine the effects of time-to-analysis, due to significant variation within and between sample sets. This variation could possibly be due to the consistency/viscosity of the biological matrix as well as the method of irradiation. While further research is required to establish any trends, these results may indicate a possible decrease in response over the three time periods and, as previously stated, this may reflect instability in the DNA molecule as a result of exposure to radiation.

3.4.5 Estimation of DNA template size (bps) from allelic dropout

As previously demonstrated in Section 2.4.4, it is possible to estimate the size of the DNA template based on allelic dropout. The results presented here show there is substantial variation present in the alpha irradiated samples, within and between matrices. As a result, it is not possible to estimate DNA template size for a particular dose of alpha radiation across all matrices. This, however, may indicate a matrix dependant threshold which can be useful for guiding analysis with miniSTR amplification kits.

3.5 Summary and conclusions

3.5.1 Dose and time effects

The experimental outcomes from this research have demonstrated that there is a general reduction in profiling success with an increased dose of alpha particles of energy 5.5 MeV. Across all matrices, DNA degradation was observed at the first dose of 66,000 Gy, and a characteristic degradation pattern was generated where allelic dropout first occurs at the higher molecular weight loci. Degradation caused by radiation appears to be consistent with other degradative processes; therefore, as stated for gamma

radiation, the current standard operating procedures used in the interpretation of degraded DNA can be applied to the interpretation of DNA irradiated with alpha particles.

While each matrix exhibited effects in the DNA profile due to the incident radiation, the extent of these effects was variable across the matrices and over the dose range tested. This variability was likely due to the physical make-up of the various tissues and, in the case of lower dilution blood, made it difficult to ascertain a dose-effect relationship, particularly at 1-day post-irradiation.

The proteins and other cellular materials in blood may have absorbed and/or blocked a portion of the ionising radiation. While it was hypothesised that like the blood, the accompanying constituents within the saliva would afford some protection to the DNA, the saliva matrix exhibited a higher degree of degradation. This may again be due to DNA degradation that occurred prior to irradiation, most likely as a result of contaminating bacteria or other components that may have contributed to the observations made (see Section 2.4.2.3).

Similar to the time-to-analysis effects observed for gamma radiation, it is difficult to discern any further degradation linked to this parameter. However, general trends over the three time periods suggest a reduction in response for the samples, which was observable in the HEp-2 cells and the blood (Figures 3.2 to 3.8).

3.5.2 Implications for forensic investigations

As discussed previously, doses were based on a 3.7×10^{11} Bq (10 Ci) americium-241 source that is in contact with the sample, and this provided a basis for estimating potential dose from a sealed source. In this research, it was not possible to estimate activity of a source dispersed by an RDD. As such, a hypothetical scenario, where samples were directly in contact with the source was defined to assist in the experimental design.

While the doses examined are extremely high, there was a remarkable resilience shown by the DNA in excess of that demonstrated with the examination of gamma radiation effects (see Chapter 2). Possibly the most significant factor influencing this phenomenon is the limited penetrability of the alpha particle and the composition of the biological matrices being analysed.

Due to the short penetration distance, unless the sample is in direct contact with the radioactive material there should not be any detrimental effects on the DNA from alpha radiation. In addition, the matrix composition will play a significant role in protecting the sample. Further experimentation should seek to examine co-mingling of radioactive material and the biological matrix to address the effects of contaminating alpha emitting materials on the DNA profiling success.

Unlike the gamma radiation study, bone was not investigated. While it was not possible within the confines of the experimental design to irradiate bone in the particle accelerator, there were additional factors that ruled against further exploration of this matrix, particularly with regard to a DVI scenario. The structure of bone allows for adequate protection from the limited penetrability of the alpha particles. This is particularly relevant as samples are usually collected from within the specimen (e.g. bone marrow, deep muscle), and therefore any external damage caused by the alpha particles will not impact on the analysis. Furthermore, if a DVI sample were externally contaminated, it may be possible to implement strategies to remove the contaminating material prior to analysis. For example, sonication may be employed to remove contaminating radioactive materials from the exterior surface and further reduce exposure[4].

Another consideration is that access to a scene will not be impeded by concerns regarding external exposure to alpha radiation. However, precautions should be utilised to protect from internal contamination, such as use of filtered breathing apparatus, to

[4] Sonication of a contaminated sample must be used with caution and steps taken to limit accumulation of excessive contaminated liquid waste.

prevent inhalation and ingestion, and protective clothing, to prevent uptake by wounds and absorption though the skin.

3.6 Future directions

The STAR particle accelerator was used to demonstrate the effect of alpha irradiation on biological matrices; however, the format of the instrument and the associated sample requirements imposed limitations on the experimental design. For example, all matrices needed to be on a conductive surface (aluminium) to dissipate the electrical charge and heat, the samples needed to be completely dry to not disrupt the formation of the accelerator vacuum, and the biological matrices needed to be as flat as possible and in a single layer of cells to receive an even exposure. In addition, blood dilutions needed to be at least at 1:3 to prevent the dried blood flaking from the aluminium substrate.

To confirm and further explore the results obtained from this investigation, future research would benefit from the use of the radioactive source of interest, e.g. americium-241. By using a true source, certain experimental design limitations can be addressed, such as alternative dilutions of biological materials, alternative substrates for the biological materials, additional biological matrices for examination (including a genomic standard), and the effects from different dose activities. In addition, increasing the sample sizes at all doses may reduce the variation observed in this study.

In addition, further studies using alternative alpha sources with different alpha particle energies, such as plutonium-238 (5.499 MeV and 5.456 MeV), would allow for characterisation of exposure effects for other sources of concern (NNDC 2009).

Furthermore, additional research previously mentioned in Chapter 2 may be extended to alpha radiation experiments. These include possible use of alternative primer set termed miniSTRs to gain additional profile information, as they require less intact DNA template for analysis.

Additionally, to further elucidate the effects of time-to-analysis, exploring greater time periods (such as three or six months from irradiation) may assist in illustrating greater or more defined effects.

Chapter 4: Extraction and Decontamination of DNA Using DNA IQ™, ChargeSwitch® and Chelex® 100 Resin / Microcon® Filtration Protocols

4.1 Introduction

The previous chapters have focused on the investigation of gamma and alpha radiation and their effects on biologically relevant forensic samples, in addition to defining dose limits for such samples. Chapters 4 and 5 will address the issue of decontamination of the biological sample from both a representative non-radioactive material, caesium-133, and its radioactive counterpart, caesium-137. A comprehensive investigation of available procedures is achieved using both existing and emerging DNA extraction and purification methodologies.

It has been established that prolonged exposure of any material to radioactive emission can result in a high cumulative dose and gradual and/or continual degradation (see Chapters 2 and 3). Therefore, removal of a radioactive contaminant is paramount to preserving the integrity of the evidence. This would allow for storage of forensic evidence for prolonged periods of time, as future scrutiny of the evidence may be required. This may be necessary if a perpetrator is not identified for some time or defence council requires time to undertake its own investigation, and also where future technology might provide an opportunity for more information through a reanalysis of the evidence. In addition, prompt removal of a radioactive contaminant is also desirable to ensure the safety of the forensic analysts handling the evidence.

4.1.1. Processing of biological evidence

There are six key areas comprising biological evidence processing for forensic DNA analysis: (i) search/screening and sample collection; (ii) extraction; (iii) quantitation; (iv) amplification; (v) fragment separation; and, (vi) data interpretation. This series of experiments focuses on the areas including and subsequent to extraction (see Chapters 1 and 6 for more discussion regarding search/screening and collection of evidence for DNA processing).

After the sample is collected, DNA is extracted from the biological material using liquid or solid-support extraction systems. The DNA extract is then subjected to quantitation, where the amount of DNA is estimated and the appropriate amount can be determined for addition to the amplification reaction. This step allows specific areas on the genome to be amplified. The amplification product is then analysed using electrophoretic instrumentation for separation of the various fragments of DNA. The separation of these fragments provides an electropherogram (DNA profile) that can be compared to other profiles obtained from individuals or crime scene exhibits.

Numerous factors can interfere with the DNA analysis of forensic samples. The presence of certain contaminants can cause degradation of the DNA molecule and/or interfere in downstream amplification processes. Factors causing a degradative effect may include environmental conditions and the presence of chemical contaminants. Examples include exposure to heat, bacteria, soil and damp environments, in addition to the presence of fabric dyes, haemoglobin, humic acid, fulvic acid, hydroxyapatite, or tannins that may inhibit the amplification reaction (Kalamár *et al.* 2000). Furthermore, there may be issues with contaminating DNA or degraded DNA in the biological sample that interfere and can influence the analysis. For example, insufficient DNA template will prevent the generation of a full DNA profile (Kalamár *et al.* 2000). In any case, subsequent DNA profiling steps are inconsequential if the extraction process is not effective, although modern profiling kits, such as Profiler Plus® (Applied Biosystems, USA) are very robust and will function in the presence of some contaminants.

Given the criticality of the extraction step, this stage is the most practical of the DNA analysis process in which to target the removal of specific contaminants. In addition, for contaminants such as a radioactive material, removal at this step prevents downstream exposure to both the sample and the analyst. Traditional DNA extraction for many forensic laboratories is conducted with Chelex® 100 resin (Walsh *et al.* 1991). While the Chelex® 100 technique is a quick and simple method for the release of DNA from cells, it does not allow for the removal of contaminants from the DNA, other than divalent cations such as magnesium. For the removal of soluble contaminants, the

supernatant must be purified through filtration, such as with Microcon Y-100 filtration devices, or by use of solid-phase extraction (SPE) methods. However, this can significantly increase analysis time; conducting the extraction as a lengthy two-step process would result in more sample manipulations, which for radioactive contaminants will lead to longer exposure times for the analyst (see Section 1.7.1).

To overcome these issues, extraction techniques were investigated for their potential to simultaneously decontaminate/purify and extract a contaminated sample. From this research, two novel technologies emerged as being suitable: DNA IQ™ and ChargeSwitch® extraction systems. These systems provide advantages over other extraction methodologies in that they allow for the removal of known and unknown PCR inhibitors, are relatively easy to employ, and are amenable to automation (using robotic workstations, for example).

A number of papers have been published comparing the success of profiling after the application of different extraction methods (Vandenberg *et al.* 1997; Hoff-Olsen *et al.* 1999; Prado *et al.* 2002; Smith *et al.* 2003; Barbaro *et al.* 2004b; Staiti *et al.* 2004) (Komonski *et al.* 2004; Lazzarino *et al.* 2008). Chelex® 100 extraction, while generally viewed as the most rapid, simple and inexpensive procedure available in the field of forensic biology, has proven to be severely limited when applied to soft tissue or bone extractions. This is in addition to requiring prior and/or subsequent purification steps to remove any soluble contaminants that might be present (as previously discussed).

The DNA IQ™ extraction system has been successfully applied to a range of biological matrices, including fresh and frozen blood, bloodstains (on various substrates including blue and black denim, cotton, FTA® paper, leather), buccal cells (on cotton swabs, cigarette butts, envelopes, toothbrushes, chewing gum), as well as various fixed and fresh tissues, hair, bone, antler and differential extractions for semen (Vandenberg *et al.* 1997; Hoff-Olsen *et al.* 1999; Prado *et al.* 2002; Smith *et al.* 2003; Barbaro *et al.* 2004b; Staiti *et al.* 2004). The manufacturer also reports that bloodstains in soil and urine, and

on rayon, and CEP paper buccal swabs are able to be extracted with high efficiency, although these matrices have not yet been independently tested (Promega 2002b).

The manufacturer of ChargeSwitch®, Invitrogen Inc., claim that the system works for dried blood spots on paper and clothing, hair follicles and hair shafts, cigarette butts and paper, envelopes, chewing gum, sperm head cells, vaginal epithelial cells, drinking vessel swabs, door handle swabs, strip removed cells (e.g. hats, coats and gloves), touch DNA (e.g. tools, mobile phones, and microscopes) and dyed denim (Invitrogen 2005). However, in-house laboratory testing of dyed denim at Forensic Services, Australian Federal Police, has lead to conflicting results (Stone 2008). Wilkinson (Personal communiction, 2009) stated that the negatively-charged contaminants, such as the anionic dyes in denim and some contaminants in soil, are attracted to the positively charged resin of the ChargeSwitch® system. It was identified that once attached to the resin these competing anions could not be removed and therefore inhibited the effective extraction of the DNA. The interference of negatively-charged ions on DNA extraction efficiency will present issues for the working laboratory, where pristine forensic samples are rare and the unknown nature of contaminants presents a potential for poor extraction efficiency. Of particular relevance to this research, certain isotopes, such as iodine-131, are negatively-charged and therefore have the potential to interfere with DNA extraction.

The DNA IQ™ and ChargeSwitch® systems vary in their mechanism for DNA binding. As previously mentioned in Chapter 1, DNA IQ™ utilises high ionic strength solutions and positively-charged ions to bind the DNA molecule to the silica beads (Melzak *et al.* 1996; Smith and York 2000; Tian *et al.* 2000; Smith and York 2002; Tereba *et al.* 2004). The ChargeSwitch® system utilises a pH sensitive moiety on the beads, which under low pH exhibit a positive charge to bind DNA (Baker 2002). Both systems can be described by the following procedural steps, including binding of DNA to paramagnetic beads, washing and removal of contaminants from the immobilised DNA, and elution of DNA from the beads (Figure 4.1).

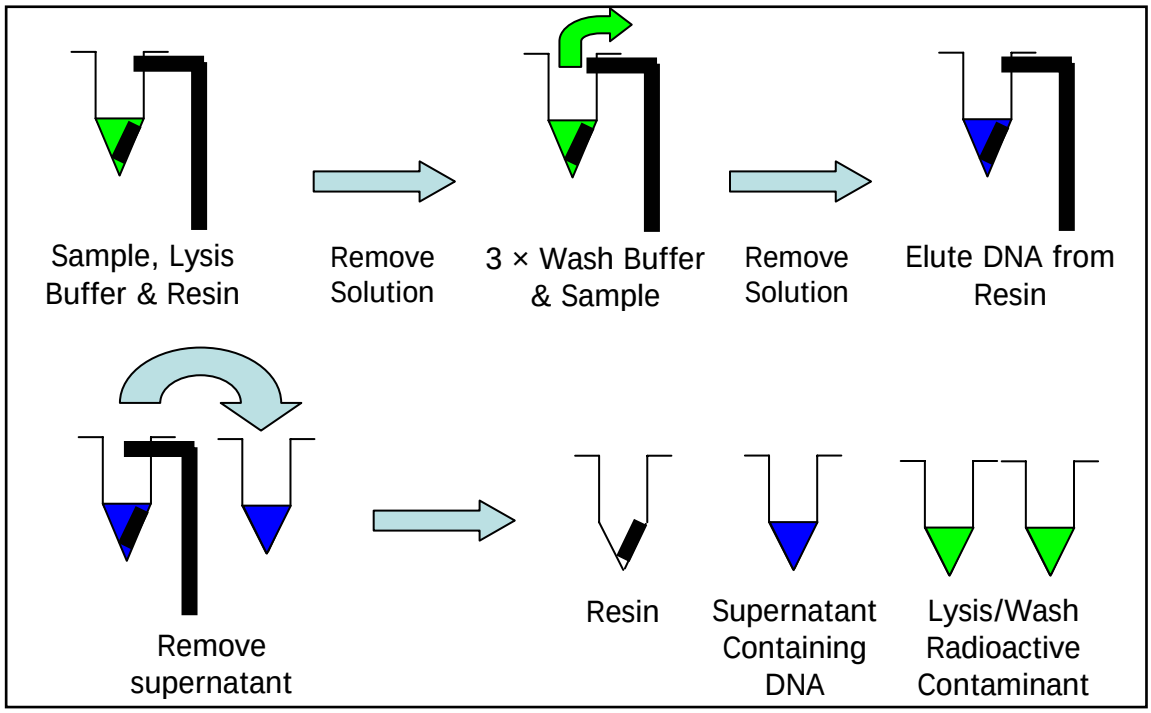


Figure 4.1: Generalised extraction procedure for the DNA IQ™ and ChargeSwitch® DNA extraction systems

As discussed in Chapter 1, caesium-137 was identified as a representative radioactive material in this research for a number of reasons, including the following:

- its potential as a source for the radioactive component in a radiological dispersion device (RDD);
- its physical properties as a soluble salt (i.e. more likely to find caesium in salt form than other materials such as cobalt-60);
- its prevalence/availability in the Australian radiation workers' environment; and,
- its potential for interference in the DNA binding mechanism of the extraction procedures.

As a substantial amount of time and effort is required in assessing the efficiency of an extraction method, the non-radioactive isotope caesium-133 was used for experimental investigation. In this regard, exposure of the laboratory analyst and third-parties to

radioactive material was negated, and the available laboratory space and resources did not require special modification for the handling and storage of radioactive agents.

4.1.2. Aims

The DNA extraction step is critical for the successful forensic processing of biological evidence. With special consideration for biological samples contaminated with radioactive material, this step is capable of both effective decontamination of the sample and recovery of purified DNA for downstream profiling. Therefore, the objectives of these experiments were to:

1) Investigate the usefulness of the DNA IQ™ and ChargeSwitch® extraction systems for the removal of contaminants from biological samples;
2) Compare these novel systems against the conventional Chelex® 100 resin extraction procedure;
3) Quantify the efficacy of the extraction systems to remove a contaminating caesium-133 salt;
4) Investigate the quantity of recovered DNA from each extraction system; and,
5) Examine the quality of the subsequent DNA profiles.

4.2 Experimental

4.2.1 Samples and standards

Blood for these experiments were sourced from a healthy male volunteer. Blood was collected by venipuncture and stored in a polypropylene sample tube. Samples were stored at -20 °C until analysis. A genomic DNA standard (9948 Male DNA [10ng/uL]) was obtained from Promega Corp, USA.

A quantity of caesium-133 nitrate salt (FLUKA, USA) was obtained from ANSTO, Lucas Heights. The caesium-133 nitrate salt was diluted using deionised water to

prepare working stock concentrations of 0.035 M, 0.0175 M, and 3.5×10^{-3} M, for DNA IQ™ and Chelex® 100, and 0.26 M, 0.13 M and 0.026 M for ChargeSwitch®. This allowed for variations in starting volumes for each system to produce starting concentrations of 0.01 M, 0.005 M and 0.001 M respectively. These stock solutions were stored at room temperature prior to analysis.

The levels of radioactive contamination expected to be encountered in a radiological incident is dependant on a range of variables, and therefore these concentrations were selected to reflect a 10-fold range of doses/concentrations. For example, the derived gamma dose rates from the "contaminated" DNA IQ™ samples would be expected to give approximately 231 μSv/h, 117 μSv/h and 24 μSv/h, respectively, at 50 cm from the sample.

4.2.2 The DNA IQ™, ChargeSwitch®, and Chelex® 100 Resin extraction materials

The DNA IQ™ extraction system, including reagent solutions, was obtained from Promega Corporation, USA. The ChargeSwitch® extraction system, including reagent solutions, was obtained from Invitrogen, USA. Chelex® 100 resin was obtained from Sigma-Aldrich, USA. The solvents used in the Chelex® 100 extraction were reagent grade. Microcon® YM-100 filtration units for post-Chelex® 100 sample clean-up were purchased from Millipore, USA.

4.2.3 Instrumentation

This experimental series utilised an Inductively Coupled Plasma-Mass Spectrometer (ICP-MS) unit, a HP4500 Quadrupole ICP-MS (Hewlett-Packard, Germany), for the elemental analysis of recovered caesium-133, in addition to Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) for the supplementary analysis of the DNA IQ™ resin. The ICP-AES unit was a Vista Simultaneous Inductively Coupled Plasma-Atomic Emission Spectrometer (Varian Inc., USA).

Instrumentation details for DNA analysis, including quantitation, amplification and capillary electrophoresis and DNA typing can be found in Section 2.3.4 through 2.3.8.

4.2.4 Procedures

4.2.4.1 Contamination of samples with caesium-133 salt

Stock solutions of non-radioactive caesium-133 nitrate salt were introduced to TE-buffered biological samples, including male human blood and the genomic DNA standard, to give final caesium-133 concentrations of 0.01 M, 0.005 M and 0.001 M.

The efficacy of caesium-133 removal was investigated utilising a 5% w/v suspension of Chelex® 100 resin (followed by Microcon® filtration), the DNA IQ™ system, and the ChargeSwitch® system. For the DNA IQ™ and ChargeSwitch® extraction protocols, the Lysate, Wash and Eluate components, and the Filtrate and Eluate from the Chelex® 100 / Microcon® filtration process, were each measured for the amount of caesium-133.

This investigation also included testing to determine if there were any deleterious effects to the amplification process or the subsequent DNA profile in the presence of caesium-133 salt at the working stock concentrations (as listed in Section 4.2.1). In these experiments, 40 µL aliquots of each separate caesium stock concentration were added to samples of male genomic DNA and 1:4 dilution of blood:TE buffer, and extracted with Chelex® 100. This dilution was selected to enable comparison of the results across the three systems with an appropriate yield of DNA and without inhibiting the PCR process.

4.2.4.2 DNA IQ™ System extraction procedure and optimisation

The DNA IQ^{TM} system extraction was performed according to the manufacturer's protocol (Promega 2002b). The methodology is described in the following paragraphs.

The following steps were performed in preparing the DNA IQ™ system for the extraction experiments:

- Preparation of 1x Wash Buffer: 35 mL of ethanol (95-100%) and 35 mL of isopropyl alcohol was added to the 2x Wash Buffer provided with the DNA IQ™ System Kit 400 and labelled appropriately (enough for 400 samples).
- Preparation of Lysis Buffer: Before addition of the Lysis Buffer to the sample, dithiothreitol is added at 1 µL to 100 µL Lysis Buffer and mixed thoroughly; for liquid samples, 200 µL Lysis Buffer was required for each sample.

For extraction, a stock solution of Resin and Lysis Buffer was prepared using the ratio of 7 µL of Resin to 93 µL of prepared Lysis Buffer per sample. The Resin container was thoroughly mixed by vortex at high speed before being added to the Lysis Buffer. The Resin:Lysis Buffer stock suspension was thoroughly mixed by vortex for three seconds at high speed, and 100 µL of the stock suspension was added to the tube containing the 1:4 diluted blood:TE Buffer or genomic DNA (note: the Resin:Lysis Buffer mixture was mixed again if the resin began to settle while dispensing aliquots). The mixture was then vortexed at high speed for three seconds and incubated at room temperature for 5 minutes.

Samples were then mixed at high speed and placed immediately on the MagneSphere® Technology Separation Stand (magnetic stand) to separate the beads from the solution. All liquid was removed with care so as not to disturb the resin collected at the side of the tube. The tube was then removed from the stand, 100 µL of the prepared Lysis Buffer was added to each sample, and then mixed by vortex for two seconds.

The tube was then returned to the magnetic stand and the Lysis Buffer collected in an eppendorf tube and stored at 4 °C prior to analysis by ICP–MS. The tube was removed from the magnetic stand, 100 µL of the prepared 1 × Wash Buffer was added, and then mixed by vortex for two seconds. The tubes were returned to the magnetic stand for separation. The wash step was repeated a further two times (three times in total),

ensuring that all the solution had been removed after the last wash. The wash solutions from a single sample were combined in a single eppendorf tube and stored at 4 °C prior to analysis by ICP–MS.

The resin was then air-dried for 5 minutes while still on the magnetic stand. After air-drying, 100 µL of the Elution Buffer was added to each sample, mixed by vortex, and placed in a 65 °C heating block for 5 minutes. The tube was then removed from the heating block and mixed for two seconds at high speed on a vortex. The sample was immediately placed on the magnetic stand and the eluate was transferred into a clean 1.5 mL microcentrifuge tube. The DNA solution was stored at -20 °C.

In an effort to measure the improvements in recovery, if any, from changes to the protocol, several variations to the published methods were explored. These variations included:

- binding time (5 and 10 minutes);
- vortex elution time (2, 10, 20 and 60 seconds); and
- elution volume (100 µL and 50 µL + 50 µL).

It should be noted, however, that these efforts do not reflect an exhaustive list of method optimisation options, only those thought likely to affect the process.

4.2.4.3 ChargeSwitch® System extraction procedure

The ChargeSwitch® extraction procedure was previously optimised by staff of the Biological Criminalistics Laboratory of the Australian Federal Police (AFP 2008b). Due to the improved recovery demonstrated through this optimisation, a modified extraction procedure developed by the AFP was employed for this series of experiments.

For cell lysis, a lysis mix stock solution consisting of 1 mL ChargeSwitch® Lysis Buffer (L13) and 10 µL Proteinase K was prepared in a microcentrifuge tube (per sample). A 25 ng sample of gDNA was then added to the tube, followed by 1 mL of the

Lysis Mix, and the solution thoroughly mixed by vortex for 10-15 seconds. The samples were then incubated for 1 hour at 55 °C until cell lysis was complete.

In preparation for DNA binding, the stock ChargeSwitch® Magnetic Beads were mixed by vortex for 10-15 seconds to fully resuspend the beads (note: once resuspended, the beads remain evenly distributed in suspension for up to 45 mins). A 200 µL aliquot of the low pH ChargeSwitch® Purification Buffer (N5; approximately pH 4) was then added to the sample and mixed by gentle pipetting (approximately 5 times). 20 µL aliquots of the resuspended ChargeSwitch® Magnetic Beads were added to each sample and mixed gently by pipetting 5 times. Samples were then incubated at room temperature for 20 minutes to allow the DNA to bind to the ChargeSwitch® Magnetic Beads.

After the incubation period, the microcentrifuge tube containing the sample was placed in the MagnaRack™ until the beads bound with DNA formed a tight pellet on the side of the tube. The supernatant was then carefully removed without disturbing the pellet, collected in an eppendorf tube and stored at 4 °C prior to caesium-133 analysis. If the pellet was disturbed then the sample was mixed again by gently pipetting 5 times and returned to the MagnaRack™.

To remove cellular debris, etc, sample tubes were removed from the MagnaRack™ and 500 µL of ChargeSwitch® Wash Buffer (W12) was added to each tube. The ChargeSwitch® Magnetic Beads were then resuspended by gently pipetting 5 times, and then the tube was placed back on the stand until a tight pellet was formed. The supernatant was then carefully removed without disturbing the pellet, collected in an eppendorf tube and stored at 4 °C prior to caesium-133 analysis. This wash step was repeated twice more for a total of 3 washes, ensuring that all the supernatant was removed after the last wash.

For elution, the sample tubes were removed from the MagnaRack™ and 100 µL Elution Buffer (E5) was added to each. The magnetic beads were resuspended by gently

pipetting 10 times and the tubes incubated at 55 °C for 5 minutes. Following this incubation, the samples were mixed by gently pipetting 10 times to resuspend the beads and to assist with elution of the DNA from the beads.

The tubes were returned to the MagnaRack™ until a tight pellet was formed. The supernatant containing the DNA was then removed and transferred to a new labelled microcentrifuge tube (note: if the eluate was discoloured, the eluate was placed back on the MagnaRack™ until a tight pellet formed and the supernatant transferred into a new tube). The used magnetic beads were discarded and the sample DNA solutions were stored at -20 °C until further analysis.

4.2.4.4 Chelex® 100 Resin extraction procedure (with Microcon® filtration)

The Chelex® 100 extraction procedure was carried out as presented in Section 2.3.2.2. This was then followed by filtration using Microcon® YM-100 units (Millipore, USA).

Microcon® YM-100 filtration units are designed to desalt, purify and concentrate DNA samples. The filtration units contain a low-binding anisotropic hydrophilic regenerated cellulose membrane that allows molecules smaller than 100 kDa to pass (Millipore 1998). The cellulose membrane requires rinsing prior to use to equilibrate the membrane and to assess the flow of liquid through the filters (Millipore 1998).

The Microcon® YM-100 filtration units were placed in microcentrifuge tubes and washed using 400 µL of TE Buffer (10mM Tris-HCl, 0.1mM EDTA, pH8.0) followed by centrifugation at 2500 rpm for 5 minutes. This wash process was repeated twice more.

Each sample of DNA extract was added to a separate filtration unit. The samples were then spun through the filter using a microcentrifuge at 2500 rpm for 10 minutes. Each

filter was then washed twice using 200 µL of deionised H_2O and centrifugation at 2500 rpm for 10 minutes or until approximately 10 µL remained above the filter.

Approximately 50 µL of distilled deionised H_2O was added to each tube and left to stand for 5 minutes at room temperature to allow the DNA to release from the matrix of the filter. The filtration units were then inverted into a new tube and centrifuged at 2500 rpm for 5 minutes. Samples were stored at -20 ^{o}C prior to use.

4.2.4.5 Sample preparation for analysis with ICP-MS

ICP-MS analysis was conducted with assistance from staff of the Institute of Environmental Research of the Australian Nuclear Science and Technology Organisation (ANSTO).

In preparation for ICP-MS analysis, an external calibration series was prepared containing all elements to be analysed (chromium, iron, copper, zinc, molybdenum, cadmium, tin, caesium, barium, mercury, thorium, and uranium). A series of concentrations were used that were designed to cover the concentration range potentially present in the samples (0.5 ppb to 100 ppb).

An internal standard was added to all samples and standards prior to analysis, which consisted of one or more elements that were not naturally present in the sample. In this series of experiments, Rhodium-103 was used to ensure quality in the system during analysis.

Due to the low volume of the samples (and potentially low concentrations contained therein), each sample was diluted to a larger volume with 5% v/v nitric acid (aq).

4.2.4.6 Analysis of DNA IQ™ resin by ICP-MS and ICP-AES

Prior to determining the efficiency of the extraction systems, it was of interest to determine the elemental composition of the resin (or bead) components. This would allow a measure of any level of background elemental caesium present that may interfere with determination of the extraction system recovery and the final caesium content of the resin and the associate Lysis, Wash and Elution buffers. Due to limited resources, only the DNA IQ™ system resin was selected for this targeted analysis.
To assist in determining the elemental composition of the DNA IQ™ beads, a 200 µL aliquot of the resin was prepared using microwave-assisted acid digestion (ANSTO 2004). Due to the hazards associated with this procedure (e.g. use of hydrofluoric acid), only certified individuals were permitted to perform the digestion. Therefore, the certified inorganic analysts at the Institute of Environmental Research of ANSTO performed the microwave digestion step.

In preparation for the digestion, 3 × 200 µL of resuspended DNA IQ™ resin aliquots were transferred into 1.5 mL eppendorf tubes and the accompanying buffer liquid carefully removed, resulting in approximately 0.05 g of the resin in each sample tube. The resin was placed in a TFM™ [tetrafluorrmethaxil™] (Hoechst) microwave vessel along with 2 mL of deionised water, and 3 mL of concentrated nitric acid to digest organics or carbonates that might be present in the resin. Concentrated hydrochloric acid (0.2 mL) and hydrofluoric acid (0.2 mL) was added; the vessel was then sealed appropriately and placed in a Milestone MLS-1200 Microwave unit (Milestone S.R.L, Italy). Microwave digestion settings are provided in Table 4.1.

Table 4.1: Microwave-assisted digest settings for the Milestone MLS-1200 Microwave unit (1000 W)

Step	Power (W)	Time (minutes)	Temperature (^{o}C)
1	250	5	75
2	400	10	120
3	550	15	150

Following the digestion, the vessels were vented (cooled) for 30 minutes, and then allowed to cool for a further 1-2 hours. After cooling, the vessel lids were removed and all condensate was rinsed into the vessels using reagent-grade water. The digest solution was then poured into a 10 mL vial. The vessel was rinsed 3 times with reagent-grade water and the rinsate poured into the vial. The digestate was made up to 8-10 mL with reagent-grade water, and the vials capped and stored at room temperature in the laboratory for analysis using ICP-MS and ICP-AES.

Note: Samples were weighed before digestion, after the addition of reagents, and after digestion to determine any loss of sample [due to loss of vessel integrity, use of excessive digestion time, too large a sample, or improper heating conditions]. Quality control was maintained by inclusion of a blank consisting of reagents only, and appropriate certified reference materials were digested with the samples.

In preparation for the ICP-MS and ICP-AES analysis, an external calibration series was prepared containing all elements to be analysed. For the ICP-MS analysis, the external calibration series consisted of the elements listed in Table 4.2. A series of several concentrations were used that were designed to cover the concentration range that might be present in the samples (0.001 mg/L to 100 mg/L, depending on the element).

Table 4.2 External calibration series elements for ICP-MS and ICP-AES

ICP-MS	ICP-AES
titanium, vanadium, cobalt, nickel, zinc, arsenic, selenium, rubidium, strontium, zirconium, molybdenum, silver, cadmium, tin, antimony, tellurium, caesium, lanthanum, tungsten, gold, mercury, thallium, lead, thorium and uranium	aluminium, barium, beryllium, bismuth, calcium, chromium, copper, iron, indium, potassium, lithium, lutetium, magnesium, manganese, sodium, phosphorous, scandium, silicon and yttrium

For ICP-AES, an internal standard was added to all samples and standards prior to analysis. In this series of experiments, caesium-133 was used to ensure quality in the system during analysis.

Prior to running the resin digestate on the ICP-AES, all samples were mixed by inversion before an aliquot was added to the analysis vial. As the resin contains silica, all samples were prepared in polypropylene tubes and diluted to an appropriate volume with 5% v/v nitric acid (aq).

4.2.4.7 DNA quantitation, amplification and profiling

DNA samples were processed as a way of determining the effects, if any, of excess caesium-133 on extraction recovery, particularly whether the presence of the contaminant affected the pH or ionic strength of the extraction systems. In addition, profiling provided a quantifiable measure of the effects, if any, on profiling success in the presence of the caesium-133 contaminant (i.e. DNA viability).

Procedures for the quantitation, amplification and profiling of DNA were outlined in Sections 2.3.4 through 2.3.8.

4.3 Results and discussion

Prior to examination of the extraction systems, it was of importance to establish the potential effect of caesium nitrate salt on DNA profiling in the absence of an effective removal system. The presence of the caesium nitrate salt at the three concentrations of interest (0.01 M, 0.005 M and 0.001 M) did not alter the effectiveness of the quantitation, amplification or separation by capillary electrophoresis, and subsequent DNA profile interpretation (data not shown).

4.3.1 Determination of caesium-133 as an existing component of DNA IQ™ System resin

Following the microwave-assisted acid digestion of the resin aliquot, it was determined by ICP-MS that the resin and the associated lysis, wash and elution buffers contained negligible background levels of elemental caesium (approximately 1000 ppb in the resin and 700, 20 and 60 ppb in the lysis, wash and elution buffers, respectively). This is important in that existing caesium leaching from the resin during extraction could be ruled out as interference when investigating the recovery and quantitation of caesium during the extraction procedure. In other words, this relative absence of caesium further supports the use of caesium-133 as the representative contaminant, as the determination of recovery efficiency can be closely monitored without confounding contribution from existing caesium. In addition, ICP-AES analysis confirmed, as expected, that the composition of the DNA IQ™ resin is largely silica and iron, in addition to a number of other trace elements (data not shown).

4.3.2 DNA IQ™ System optimisation studies

Optimisation studies were conducted on the DNA IQ™ system using 10 ng and 100 ng of genomic DNA, and demonstrated the effectiveness of the manufacturer's protocol in the three modifications investigated (see Figures 4.2 to 4.4). From these trials, the protocol was not improved by increasing the DNA binding time, increasing the vortex

elution time, or changing the single elution volume of 100 µL to two elutions of 50 µL (total 100 µL).

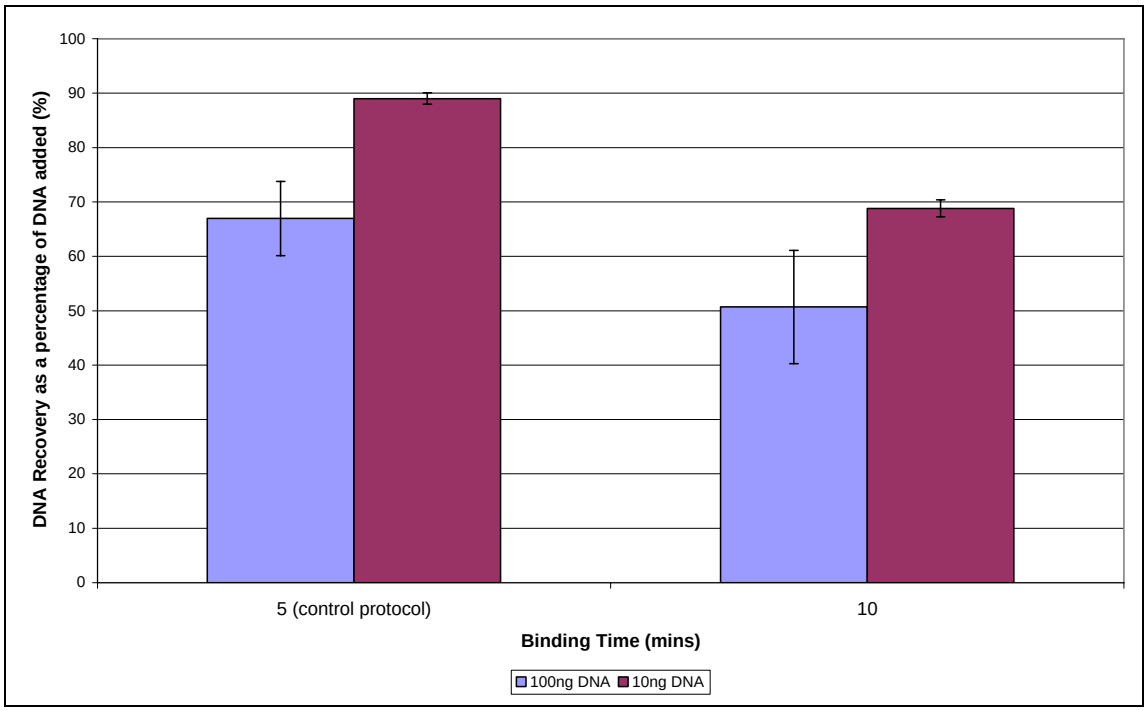


Figure 4.2: Comparison of DNA recovery (%) with changes to binding time for the DNA IQ™ System at 10 ng and 100 ng of DNA (n = 6)

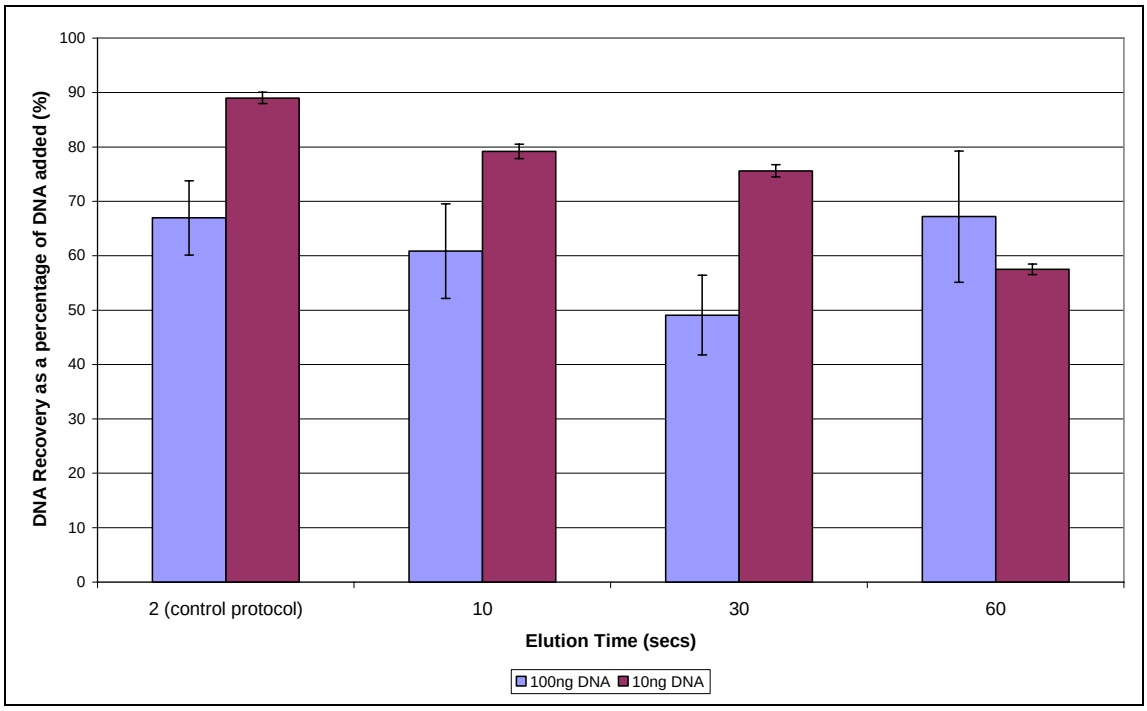


Figure 4.3: Comparison of DNA recovery (%) with changes to vortex elution time for the DNA IQ™ System at 10 ng and 100 ng of DNA (n = 6)

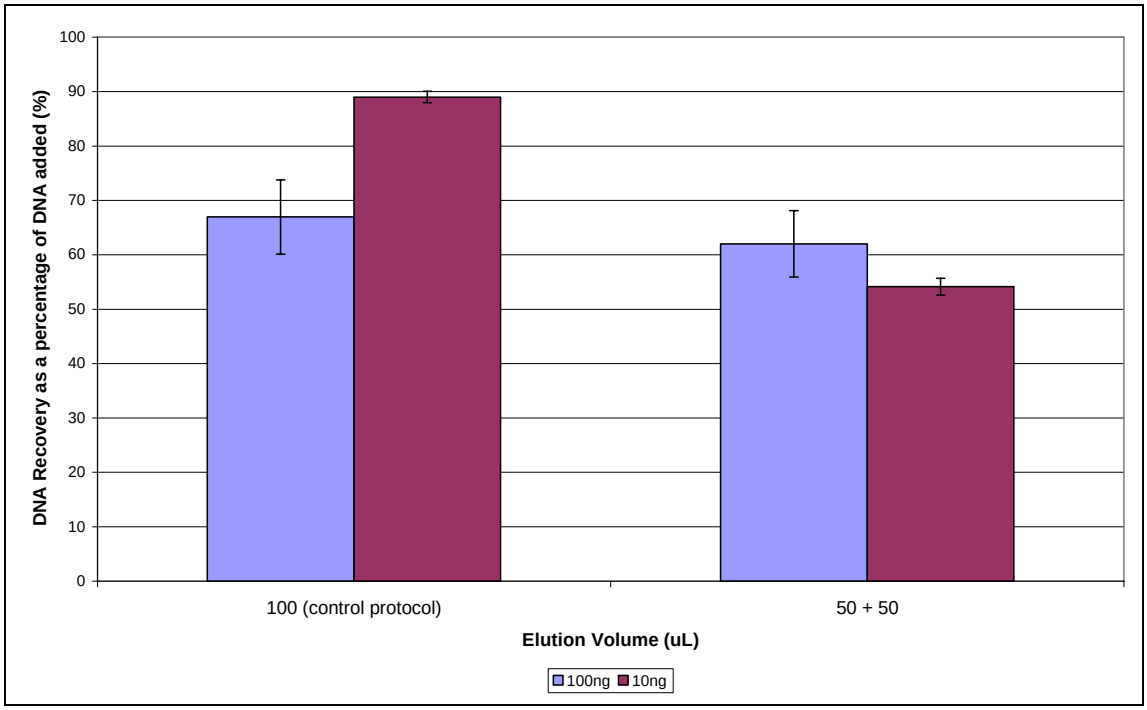


Figure 4.4: Comparison of DNA recovery (%) with changes to elution volume for the DNA IQ™ System at 10 ng and 100 ng of DNA (n = 6)

Increasing the time for the DNA to bind to the resin, from 5 minutes to 10 minutes, did not increase the amount of DNA binding to the resin. While observing the sample, it was noted that the resin settled out of solution within 5 minutes, and therefore prevented any further interaction with the sample (thus gentle resuspension of the resin after 5 minutes may be required if an increased binding time is desired). Patent information indicates that further optimisation of the system may benefit from targeting the DNA elution step as a portion of DNA remains bound to the resin possibly due to the stability of the bond formation (Smith and York 2000).

Additional optimisation investigations are warranted, however, to include a lysis and incubation step prior to addition of the resin. This may be particularly true with regard to biological matrices such as blood and epithelial cells to allow for complete cell lysis. Furthermore, future optimisation may include proteinase K in the incubation step for effective digestion of a forensic sample.

These studies also demonstrated that the addition of a lower DNA quantity provides a better percentage DNA yield, consistent with manufacturer recommendations, which may reflect steric hindrance and/or saturation at the surface of the bead.

4.3.3 Removal of caesium-133 using the DNA IQ™ System, ChargeSwitch® System and the Chelex® 100 Resin and Microcon® filtration

The analysis by ICP-MS included the determination of caesium removal by each extraction system and estimation of caesium loading onto the beads both with and without sample DNA (concentrations of caesium-133 tested were 0.001 M, 0.005 M and 0.01 M). Following the extraction procedure, the total caesium-133 recovered in the lysis buffer, wash solutions, and eluate was determined. The recovered caesium-133 from the lysis buffer and wash solutions were combined to reflect the total amount removed (recovered) in the DNA IQ™ and ChargeSwitch® systems. Results from the analyses are shown in Tables 4.3-4.5.

4.3.3.1 Removal of caesium-133 using the DNA IQ™ System

The DNA IQ™ system extraction procedure demonstrated an overall removal of ≥ 99.95% of contaminating caesium-133. Between 88-97% of the caesium-133 was removed in the initial lysis and wash steps of the extraction procedure (indicated by "% Recovered" in Table 4.3), with a significant portion (up to 12%) likely to have been adsorbed onto the silica extraction beads.

The analysis demonstrated that less than 0.05 ± 0.02% of the initial caesium-133 remained in the sample after the extraction was completed, equating to 0.058 ± 0.089 µg remaining in the eluate. Furthermore, the levels of caesium-133 remaining in the eluate were at a similar amount regardless of the original amount added and in the presence and absence of DNA/blood. Therefore, the addition of DNA in the form of a genomic standard or the biological matrix did not appear to affect the capacity of the system to remove caesium contamination.

Table 4.3: Results from the elemental analyses, indicating the DNA IQ™ System efficiency for removal of caesium-133 (n = 3 at each concentration)

Amount of caesium-133 added to the samples before extraction (µg)[a]	Average caesium recovered in Lysis and Wash (µg)	Standard Deviation (µg)	Average recovered in Lysis and Wash (%)	Average caesium remaining in eluate (µg)[b]	Standard Deviation (µg)	DNA Source
196	177	12	91	3.4×10^{-3}	3.4×10^{-3}	No DNA
100	91	4.5	92	1.3×10^{-3}	2.4×10^{-4}	
20	18	0.88	92	2.6×10^{-3}	2.7×10^{-3}	
196	186	3.4	95	5.8×10^{-2}	8.9×10^{-2}	DNA
100	95	2.1	95	4.8×10^{-3}	1.4×10^{-3}	
20	18	1.8	88	8.9×10^{-3}	1.3×10^{-2}	
196	182	7.3	93	1.5×10^{-2}	4.7×10^{-3}	Blood
100	97	9.4	97	2.0×10^{-3}	0.0	
20	18	0.93	91	2.3×10^{-3}	5.8×10^{-4}	

[a]Values derived from added volumes of 0.010 M, 0.005 M, and 0.001 M solutions, respectively.

[b]Note: at least one replicate in each batch returned results less than the 0.0011 µg cut-off value for quantitation. In generating averages, 0.0011 µg was used in these cases and therefore provides a higher estimate.

NOTE: µg values derived from results of ICP-MS analysis. For example, a replicate for the average caesium remaining in the eluate (line 1):

11 µg/L result on ICP–MS = 1.1×10^{-5} µg/µL = [1.1×10^{-5} µg/µL × 100 µL final volume] = 0.0011 µg remaining.

4.3.3.2 Removal of caesium-133 using the ChargeSwitch® System

The ChargeSwitch® system extraction procedure demonstrated an overall removal of ≥ 99.99% of contaminating caesium-133 (Table 4.4). Between 69-90% of the caesium-133 was removed in the initial lysis and wash steps of the extraction procedure, with a significant portion (up to 30%) likely to have adsorbed onto the silica extraction beads. The analyses demonstrated that less than 0.01% of the caesium-133 remained in the sample after the extraction was completed, equating to 0.04 ± 0.0066 µg remaining in the eluate. Furthermore, the levels of caesium-133 remaining in the eluate were at a similar amount (i.e. 0.04 ± 0.0066 µg) regardless of the original amount added or whether DNA was present or absent. Therefore, the addition of DNA in the form of a genomic standard or a biological matrix did not appear to affect the ability of the system to remove caesium contamination.

The data also indicate that a higher proportion of caesium-133 ions is adsorbed to the ChargeSwitch® beads compared to the DNA IQ™ system resin. There are several plausible explanations for this observation, such as the apparent larger volume of resin utilised in the ChargeSwitch® extraction, the longer incubation time, the structural or chemical makeup of the resin, or it may reflect the larger mass of caesium added to the samples to maintain the same starting concentration across systems (note: as the concentration of caesium increases, a higher percentage of caesium is recovered in the initial lysis step when the ChargeSwitch™ resin is present. This may indicate that only a certain amount of caesium salt can be adsorbed to the resin, however it was beyond the scope of this study to investigate this further.

Table 4.4: Results from the elemental analyses, indicating ChargeSwitch® System efficiency for removal of caesium-133 (n = 3 at each concentration)

Amount of caesium-133 added to the samples before extraction (µg)[a]	Average caesium recovered in Lysis and Wash (µg)	Standard Deviation (µg)	Average recovered in Lysis and Wash (%)	Average caesium remaining in Eluate (µg)[b]	Standard Deviation (µg)	DNA Source
1490	1429	81	90	3.1×10^{-2}	1.8×10^{-2}	No DNA
1100	880	12	80	7.0×10^{-3}	4.6×10^{-3}	
159	110	7.7	70	4.6×10^{-3}	3.8×10^{-3}	
1490	1441	87	90	4.0×10^{-2}	6.6×10^{-3}	DNA
1100	759	15	69	1.2×10^{-2}	1.1×10^{-3}	
159	113	9.6	71	1.5×10^{-3}	4.0×10^{-3}	

[a]Values derived from added volumes of 0.010 M, 0.005 M, and 0.001 M solutions, respectively.
[b]Note: at least one replicate in each batch returned results less than the 0.0011 µg cut-off value for quantitation. In generating averages, 0.0011 µg was used in these cases and therefore provides a higher estimate.
NOTE: µg values derived from results of ICP-MS analysis. For example, a replicate for the average caesium remaining in the eluate (line 1):
11 µg/L result on ICP–MS = 1.1×10^{-5} µg/µL = [1.1×10^{-5} µg/µL × 100 µL final volume] = 0.0011 µg remaining.

4.3.3.3 Removal of caesium-133 using the Chelex® 100 Resin and Microcon® filtration

The Chelex® 100 Resin and Microcon® filtration extraction procedure demonstrated an overall removal of ≥ 98.8% of contaminating caesium-133 (Table 4.5). Between 16-27% of the caesium-133 was removed in the Microcon® filtration step, with a significant portion (up to 80%) suspected to have been captured in the cellulose filter. The analysis demonstrated that less than 0.5% of the initial caesium-133 remained in the sample after the extraction was complete, with a slightly higher retention of caesium (up to 3.0%) in the eluate. These higher levels of caesium in the eluate of extracted blood samples may have been a result of additional larger molecular weight contaminants such as proteins and other cellular material blocking the filter.

Furthermore, unlike the DNA IQ™ and ChargeSwitch® extraction systems, the levels of caesium-133 remaining in the eluate more closely correlated with the starting concentrations, such that the higher starting concentration of 0.010 M yielded a proportionally larger amount of caesium remaining in the eluate (approximately 2.2 µg), the mid-range starting concentration of 0.005 M yielded approximately 1.2 µg caesium in the eluate, and the lowest starting concentration of 0.001 M yielded approximately 0.16 µg caesium in the eluate. This may be attributable to the requirement for the Microcon® filtration units to maintain a consistent volume of at least 10 µL of liquid above the filter at all times and this would therefore reflect the starting amount. This may represent a limitation of centrifugal filtration systems.

Table 4.5: Results from the elemental analyses, indicating the Chelex® 100 resin and Microcon® filtration efficiency for removal of caesium-133 (n = 3)

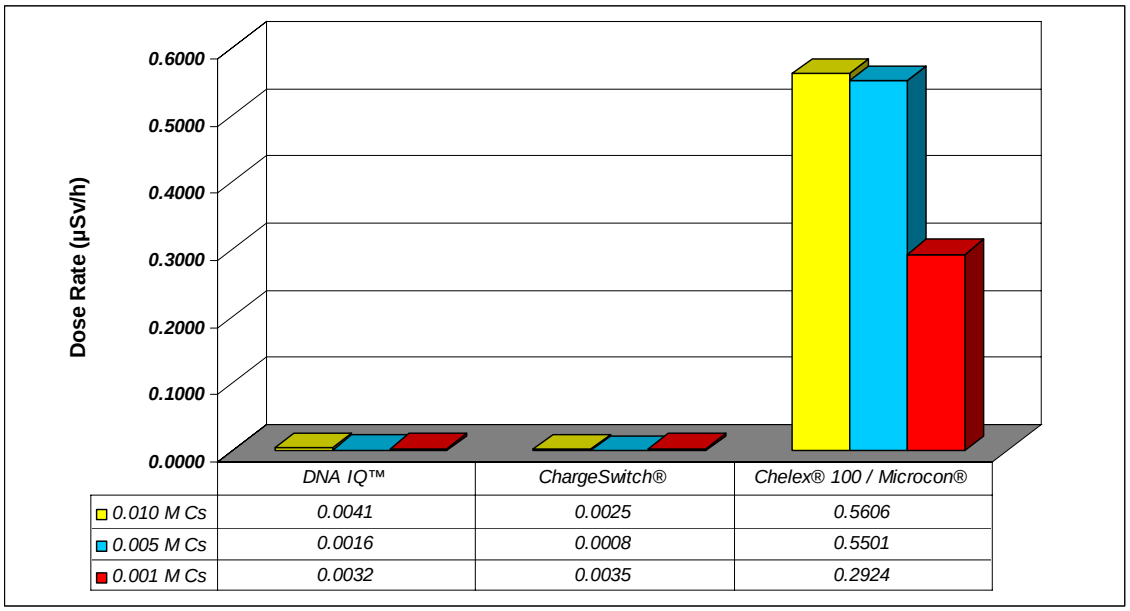
Amount of caesium-133 added to the samples before extraction (µg)[a]	Average caesium recovered in Lysis and Wash (µg)	Standard Deviation (µg)	Average recovered in Lysis and Wash (%)	Average caesium remaining in Eluate (µg)	Standard Deviation (µg)	DNA Source
481	125	2.2	26	2.3	0.17	No DNA
242	49	2.2	21	1.1	0.17	
48	7.8	1.5	16	0.16	0.13	
481	125	1.3	26	2.3	0.19	DNA
242	52	2.1	22	1.2	0.51	
48	7.8	1.6	16	0.14	0.12	
481	129	2.8	27	3.0	0.22	Blood
242	51	1.4	21	2.8	1.1	
48	9.6	4.4	20	0.39	0.40	

[a]Values derived from added volumes of 0.010 M, 0.005 M, and 0.001 M solutions, respectively.

NOTE: µg values derived from results of ICP-MS analysis. For example, a replicate for the average caesium remaining in the eluate (line 1):
27900 µg/L result on ICP–MS = 0.0279 µg/µL = [0.0279 µg/µL × 100 µL final volume] = 2.79 µg remaining.

4.3.4 Implications of remaining contaminant in a laboratory setting

Caesium-133 was used in this series of experiments as a safe, representative alternative to radioactive caesium-137, a gamma/beta emitter (see Chapter 1). To estimate the level of exposure to the analyst following sample extraction, the amounts remaining in the eluate were converted to dose rate (µSv/h) using an online calculator for Radiation Safety professionals (McGinnis 2008) and manual calculations. The comparative results are shown in Figure 4.5.



	DNA IQ™	ChargeSwitch®	Chelex® 100 / Microcon®
0.010 M Cs	0.0041	0.0025	0.5606
0.005 M Cs	0.0016	0.0008	0.5501
0.001 M Cs	0.0032	0.0035	0.2924

Figure 4.5: Estimated dose rates (µSv/h) following extraction using DNA IQ™, ChargeSwitch® and Chelex® 100 / Microcon® for the removal of representative caesium-133 (Cs)

Figure 4.5 indicates that the DNA IQ™ and ChargeSwitch® protocols produce a reduced final dose rate compared to the Chelex® 100/Microcon® procedure. Moreover, the efficiencies of the DNA IQ™ and ChargeSwitch® systems do not appear to be affected by the initial starting concentration tested under these conditions.

From these data, it can be seen that a contaminated sample extracted using either the DNA IQ™ or ChargeSwitch® would have a dose rate well below the derived

0.5 μSv/working hour (1 mSv per annum) limit designated for a non-radiation workers (ICRP 1991; ICRP 2007). This reduced dose rate allows for a safer working environment, higher sample throughput and facilitates more convenient storage and handling protocols for the laboratory. However, it should be noted that the dose rate of the sample prior to extraction and the number of samples analysed will need to be taken into account when determining dose rates and integrated doses for each analyst (see further discussion on laboratory protocols for handling radioactive samples in Chapter 5).

The DNA IQ™ and ChargeSwitch® systems have therefore been demonstrated to be preferential methods for the removal and purification of DNA after contamination with soluble caesium salt. This is true, particularly, as the Chelex® / Microcon® methods require greater sample handling and they are not amenable to automation. This reflects the potential for their successful application to traditional forensic DNA profiling following radiological contamination (continued in Chapter 5).

4.3.5 Examination of DNA viability for profiling

4.3.5.1 DNA extraction and profiling using the DNA IQ™ and ChargeSwitch® Systems

The effectiveness of the DNA IQ™ and ChargeSwitch® extraction procedures were investigated with regard to the DNA binding affinity of the beads, the beads' interaction with caesium-133, and the subsequent DNA profiles produced.

Following extraction with the DNA IQ™ system (single-source genomic DNA standard), samples were analysed for quantity of DNA and the quality of the DNA profile obtained with and without the presence of a caesium-133 contaminant. Quantitation of the DNA samples indicated approximately 60% recovery, regardless of the caesium concentration, which is consistent with the DNA IQ™ patent for higher concentrations of DNA [note: 100 ng DNA added] (Smith and York 2000; Smith and

York 2002; Tereba *et al.* 2004). The presence of the caesium did not alter the quantity of DNA in the final eluate (Figure 4.6).

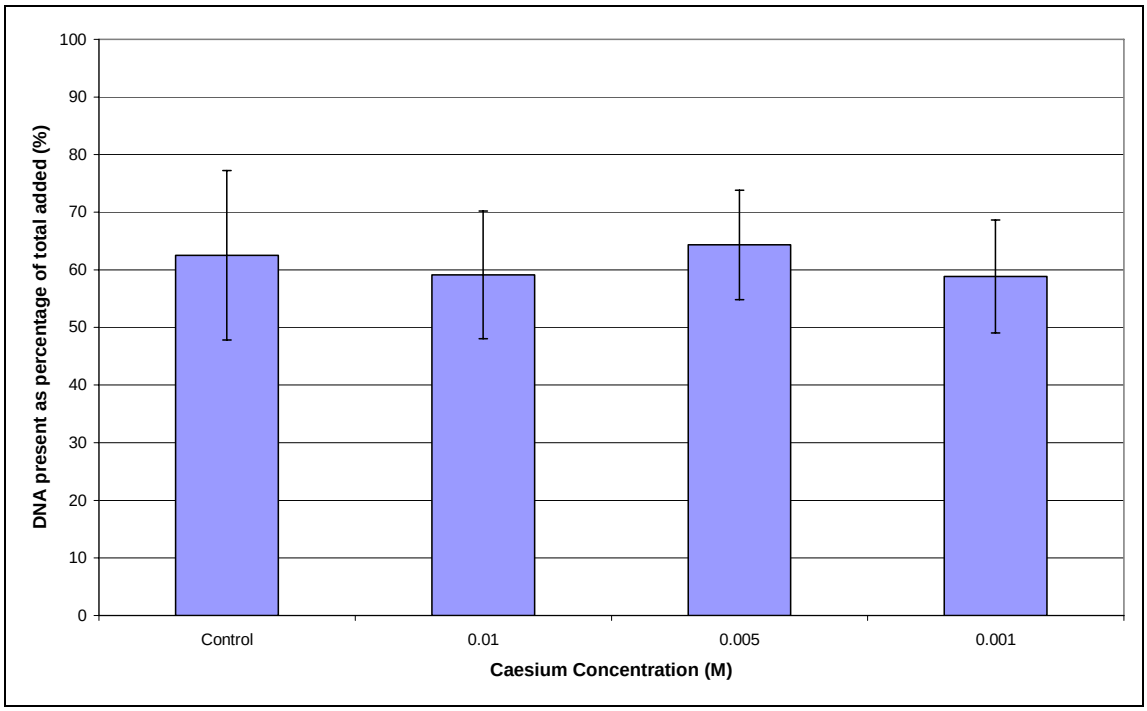
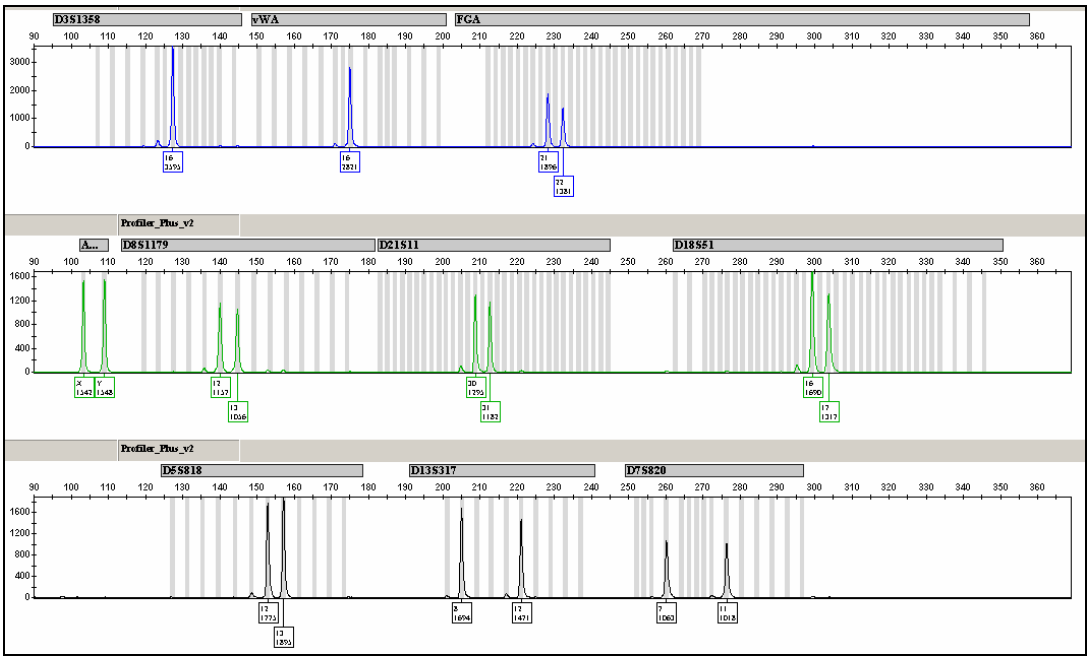


Figure 4.6: Yield of DNA extracted using the DNA IQ™ System in the presence of three concentrations of representative caesium-133 (n = 3)

The profiles produced from caesium-contaminated samples were consistent with those from control samples. Figure 4.7 (a) is an example of a DNA profile produced after the sample was contaminated with 0.01 M caesium nitrate and subsequently extracted with the DNA IQ™ system. As demonstrated, the sample has successfully produced a profile equivalent to that from an uncontaminated sample (Figure 4.7 b).

The DNA IQ™ system has therefore been demonstrated to be capable of removing a significant amount of the caesium salt contaminant while extracting the maximum amount of DNA from the biological sample. The efficient removal of the caesium-133 allowed PCR amplification to successfully occur; the caesium divalent ions did not interfere in the quantitation, amplification or capillary electrophoresis process.

a)



b)

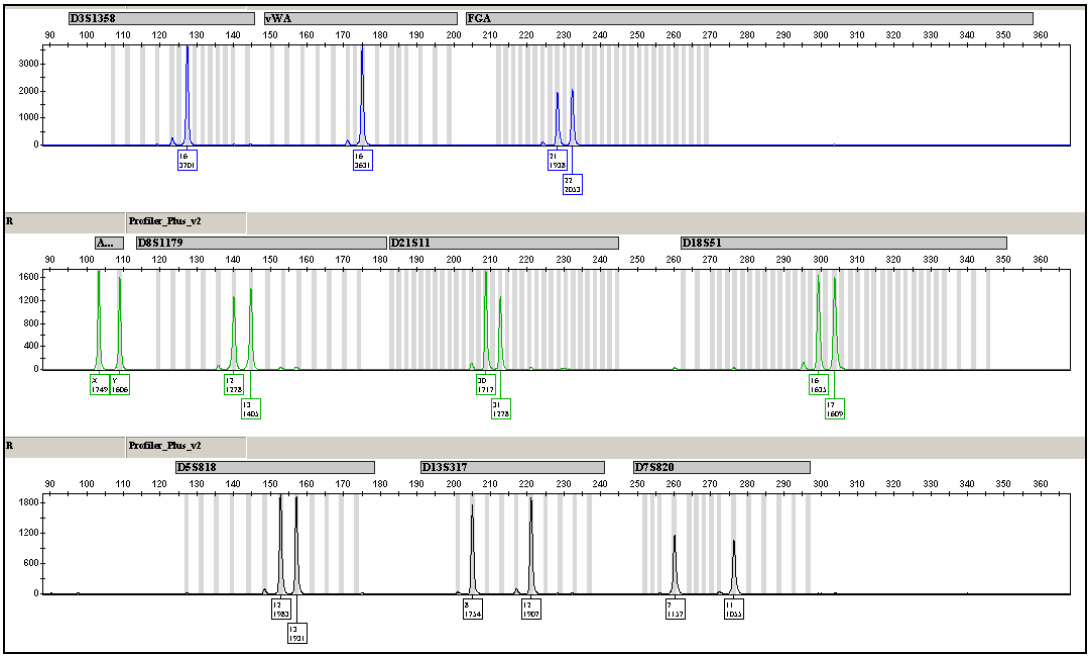


Figure 4.7: DNA profile of DNA extracted using the DNA IQ™ System in (a) the presence of 0.010 M caesium-133 and (b) the absence of caesium-133.

Similarly, the caesium nitrate salt has not produced any deleterious effects on the DNA extraction efficiency or DNA profile when extracted with ChargeSwitch® (Figure 4.8).

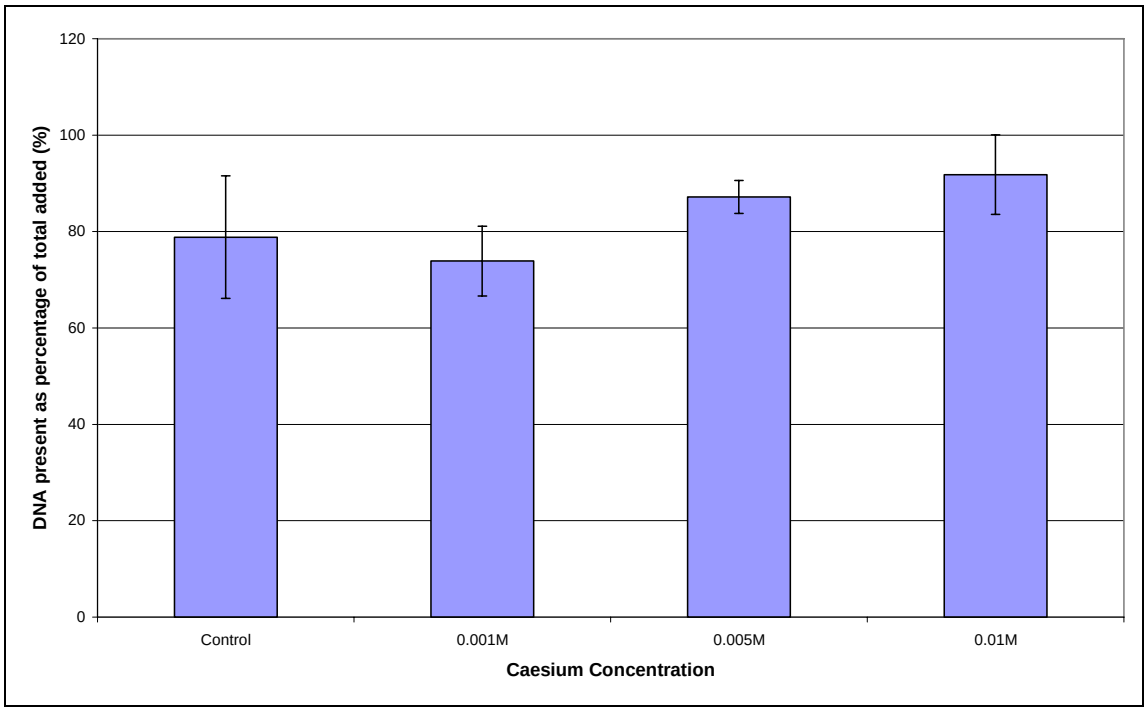


Figure 4.8: Percentage of DNA extracted using the ChargeSwitch® System in the presence of three concentrations of representative caesium-133 (n = 3)

4.4 Summary and conclusions

4.4.1 Comparison of DNA extraction efficiency and effects of contamination

Both the DNA IQ™ and ChargeSwitch® systems proved reliable and appropriate for the purification of DNA samples contaminated with caesium-133 (>99.95% removal for DNA IQ™; 99.99% for ChargeSwitch®). The final eluant contained approximately 0.06 µg of caesium-133 for DNA IQ™ and approximately 0.04 µg for ChargeSwitch®; the final concentrations did not appear dependent on the original concentration added, indicating that the extraction systems may be capable of removing a significantly higher caesium load. By comparison, the conventional Chelex® 100/Microcon® procedure

performed less efficiently at contaminant removal (> 98.8%), resulting in up to 3.0 µg of caesium remaining in some samples.

The results demonstrate that the presence of the contaminant did not have deleterious affects on the pH or ionic strength of the solid-phase extraction systems, and thus did not affect the efficiency of decontamination or DNA binding. The higher efficiency in caesium removal can be attributed to the action of the paramagnetic DNA-binding resin of the DNA IQ™ and ChargeSwitch® systems. Both systems provide a solid support to which DNA can reversibly bind and allow for washing and removal of contaminants.

4.4.2 Implications of efficiency on analyst exposure

The amount of contaminating caesium-133 that was remaining in the extraction eluates was extrapolated to reflect dose rates of caesium-137 (in µSv/h). The data demonstrated that both the DNA IQ™ and ChargeSwitch® protocols significantly reduced dose rates compared to the Chelex® 100 resin extraction with Microcon® filtration (< 0.01 µSv/h for DNA IQ™ and ChargeSwitch® versus up to 0.56 µSv/h for Chelex® 100). From this, it can be predicted that with the DNA IQ™ and ChargeSwitch® systems, numerous extracted samples could be handled per analyst before the derived dose rate limit of 0.5 µSv per work hour for a non-radiation worker is exceeded.

As the DNA IQ™ and ChargeSwitch® systems have been demonstrated as being effective for the removal and purification of DNA from contaminating caesium-133, their potential application for forensic DNA profiling following contamination with radioactive material have been realised (continued in Chapter 5).

4.4.3 Implications on DNA profiling

Contamination of the samples with representative caesium-133 showed no significant effects on the quantitation, amplification or profiling of DNA at the concentrations tested. Quantitation of the DNA samples extracted using DNA IQ™ demonstrated

approximately 60% recovery, a value that is consistent with both the DNA IQ™ patent and the control samples. By comparison, quantitation of the samples from ChargeSwitch® extraction demonstrated approximately 80% recovery (note: the ChargeSwitch® procedure had been previously optimised by the AFP).

The caesium-133 contaminant did not inhibit/influence the amplification process or affect the quality of the DNA profiles. The profiles produced from all contaminated samples, regardless of extraction method, were consistent with control (uncontaminated) samples, indicating no effects of the contaminant at the targeted loci/alleles. However, it should be cautioned that other radioactive contaminants may not behave this way.

In this regard, the presence of this representative contaminant did not have a deleterious effect on the DNA profile of the samples analysed.

4.4.4 Implications in the laboratory environment

As alluded to in the discussion of radiation dose rates, the laboratory would need to take into account the number of samples processed when determining overall dose rates for personnel. In addition, several other important considerations should be addressed by any laboratory planning to handle and analyse forensic biological samples that are contaminated with radioactive material. These include, but are not limited to, personal protective equipment, exposure monitoring, contamination monitoring, and waste disposal.

Chapter 5 will further explore the analysis of samples contaminated with radioactive caesium-137, in addition to conditions of the working environment.

4.5 Future directions

The findings presented here suggest that the solid-phase extraction protocols, the DNA IQ™ and ChargeSwitch® systems, are more efficient at removing a caesium

contaminant than the standard Chelex® 100 resin and Microcon® filtration method typically used for DNA extraction. That said, there are several items to consider for further investigation.

As previously mentioned, the ChargeSwitch® extraction protocol was optimised as part of the validation process by staff of the Biological Criminalistics laboratory, Australian Federal Police. While several parameters in the published DNA IQ™ protocol were modified in this study, with little effect observed, further research should be considered.

In these efforts, DNA recovery was not improved by changes to the length of mixing (elution time), length of incubation, binding time, or elution volume. However, Prinz and colleagues presented a paper on the challenges posed when processing samples that were compromised as a result of the September 11, 2001 attacks on the World Trade Centre in the USA (Prinz *et al.* 2002). The medical examiner utilised DNA IQ™ in the analysis of samples and showed that DNA recovery could be increased by tripling the amount of magnetic beads used in the protocol. Additionally, in the presence of bacterial contamination (silica bead extraction protocols such as DNA IQ™ and ChargeSwitch® do not differentiate between bacterial and human DNA), an increase in the amount of beads added would allow for greater overall recovery, limiting the loss of human DNA, and increasing the likelihood that the majority of DNA will be retrieved (Prinz *et al.* 2002).

The solid-phase extraction systems explored in this research could also be applied as an alternative to purification systems currently employed. Work by Cattaneo *et al.* (1995) demonstrated the need for contaminant removal from a DNA extract from post-mortem skeletal material by agarose gel electrophoresis. The process removed contaminants that were preventing the PCR amplification step (Cattaneo *et al.* 1995). As an alternative, DNA IQ™ or ChargeSwitch® could be used as an efficient alternative to gel purification in a single step within the extraction protocol or after the phenol/chloroform extraction (Arismendi *et al.* 2004; Ye *et al.* 2004). Ye *et al.* (2004) also describes the use of the DNA IQ™ system as a purification method following DNA extraction with

cetyltrimethylammonium bromide (CTAB) buffer and isoamyl alcohol-chloroform. The authors identified the DNA IQ™ system as equal to other silica-based methods, specifically the QIAquick™ system (Qiagen, USA), and recognised additional benefits such as higher DNA yield as centrifugation and rinsing was not required. The method was also reported as faster and easier to implement (Ye *et al.* 2004).

Furthermore, particularly in light of technological advances, recent applications of silica bead extraction methods to microfluidic devices have demonstrated the potential usefulness of solid-phase extraction for on-site or lab-on-a-chip applications (Baker *et al.* 2001; Bienvenue *et al.* 2006). Bienvenue *et al.* (2006) demonstrated the application of microscale solid-phase extraction to DNA extraction from sperm cells, while Baker *et al.* (2001) applied silica-based mitochondrial DNA extraction to hair shafts and teeth (these options could also be explored). Similar studies for radiological contamination such as those explored in this research could be applied to microfluidic devices to investigate their amenability for decontamination of biological samples.

These cited publications also highlight the notion that further research on the DNA IQ™ and ChargeSwitch® protocols could be pursued on numerous alternative sample matrices in addition to the blood and genomic standard matrices tested here. In addition, other methods could be comparatively explored for the removal of radioactive contamination and DNA isolation from forensic matrices, such as the QIAamp DNA Investigator Kit (Qiagen, USA) that utilises micro-spin columns for purification of forensic samples.

Apart from expanding beyond the reported extraction systems, there are additional options to explore within the systems tested in this series of experiments. While the elemental composition of DNA IQ™ resin was assayed (via acid digestion and analysis by ICP-MS and ICP-AES), there may be some benefit to testing components of the ChargeSwitch® and Chelex® 100 resin / Microcon® assembly. While this may not be of significance to ChargeSwitch®, as the remaining caesium-133 was low, this could formally rule out any leached caesium-133 contributing to the relatively higher amounts

observed after Chelex® 100 / Microcon® extraction. In addition, trace levels of caesium-133 that may be present in plasticware, glassware and reagents could be explored further for their contributions, if any, to the measured quantities of caesium.

Lastly, as the DNA IQ™ and ChargeSwitch® systems have proven effective for the extraction of DNA contaminated with non-radioactive caesium-133, further studies should include assessment of the suitability of these methods for other representative non-radioactive (or, if possible, radioactive) contaminants such as americium oxide. In addition, many radioactive materials are manufactured as metals and vitrified ceramics, therefore investigation of the impact of these materials on the solid-phase extraction procedures would be of interest. If extraction efforts can be investigated using representative non-radioactive forms, analyst exposure to radioactive materials could be greatly reduced in preparing an analytical response to a real-world/genuine CBRN incident involving radioactive contamination.

In this regard, Chapter 5 addresses the systematic analysis of samples contaminated with radioactive caesium-137, from sample collection to the disposal of analytical waste, in addition to further discussing implications of radioactivity for the analyst and the laboratory setting.

Chapter 5: Extraction and Profiling of Blood Samples Contaminated with Radioactive Caesium-137

5.1 Introduction

In Chapter 4, the capability of the DNA IQ™ and ChargeSwitch® systems to remove a representative caesium-133 salt contaminant in the presence and absence of DNA was demonstrated. These systems therefore have the potential to also remove the radioactive equivalent, caesium-137. While this initial research was essential for establishing protocols and determining removal efficiency within safe parameters, a more complete examination with a radioactive caesium-137 contaminant was necessary for measuring the efficacy of caesium removal and observing the effects, if any, of the gamma/beta irradiation on the silica bead substrate (e.g. chemical or morphological effects that may influence DNA binding efficiency).

Based on the preliminary data obtained from non-radioactive caesium-133, this chapter presents a further investigation of the DNA IQ™ and ChargeSwitch® extraction systems for their amenability to purification and extraction of DNA from radioactively contaminated samples. In these experiments, a low concentration of gamma-emitting caesium salt was added to samples of blood, which were then taken through each extraction procedure and subsequently profiled. This allowed for a quantitative measure and a comparison of extraction efficiency (for both removal of caesium-137 and recovery of DNA), in addition to observing the effects of low-level radioactivity on the success of DNA quantitation, amplification and profiling.

In addition, safe working practices are proposed based on the overall procedures explored in this section. These include considerations for sample collection and storage, proposed guidelines for maximum dose limits (for a non-radiation worker), estimation of the time, distance and shielding required for safe handling/management, and identification of potential for contamination of a working forensic laboratory.

5.1.1 Aims

Findings obtained in Chapter 4 indicated the potential effectiveness of the DNA IQ™ and ChargeSwitch® systems for the extraction of DNA from biological samples contaminated with radioactive material. Therefore, the objectives of this experimental series were to:

1) Identify issues for handling radiologically-contaminated biological samples within a forensic laboratory and propose related safety protocols.
2) Conduct DNA extraction of blood contaminated with caesium-137 using the DNA IQ™ and ChargeSwitch® systems under the proposed safety protocols.
3) Quantitate the removal of caesium-137 and the DNA extraction efficiency achieved for the DNA IQ™ and ChargeSwitch® systems.
4) Propose future directions for forensic institutions that may be planning to implement (or are currently charged with) the analysis of radioactive samples.

5.2 Collaboration of research agencies

The procedures involved in the extraction and profiling of radioactive samples required technical and logistical collaboration between the Biological Criminalistics laboratory of the Australian Federal Police and the National Security Research group of the Australian Nuclear Science and Technology Organisation (ANSTO).

The DNA IQ™ and ChargeSwitch® extractions were conducted at the Institute of Environmental Research facilities, ANSTO in Lucas Heights, NSW. By undertaking the extractions at this facility, the activity of the samples could be closely monitored and the samples handled within an environment equipped to protect the operator from radiation exposure and to manage radioactive contamination.

DNA amplification and profiling required specialised instrumentation that was not available at the Lucas Heights facility. Consequently, arrangements were made for the

use of equipment and instrumentation at the Biological Criminalistics laboratory of the Australian Federal Police in Canberra, ACT.

5.3 Preparation for experimentation

5.3.1 Occupational health and safety considerations

Before commencing experimentation, occupation health and safety aspects of this research were discussed with the Occupation Health and Safety manager of the Australian Federal Police and agency Radiation Safety Experts. Organisational approval was obtained from all the relevant managers at Forensic and Data Centres, Australian Federal Police.

The laboratory personnel within Forensic Operations at the Australian Federal Police were made aware of the proposed use of the laboratory for the analysis of low-activity radioactive samples. As a safeguard, the location and dates of experimentation were planned so that the majority of staff were not present at the time of analysis. In addition, assurances were made and agreed upon, that all materials brought into the laboratory would be disposed of either through the use of biohazard waste units if on a solid material (e.g. lab coat, face mask, absorbent mat) or via the dilute and disperse method (i.e. flushed down the sink). Reagents and/or equipment were replaced as required (e.g. if the item came into contact with the samples or was otherwise contaminated).

Staff members were encouraged to ask any questions and express any concerns with respect to the research. This included questions relating to the potential activity of the samples, the potential for contamination, consequences for the laboratory, and health risks posed by the samples to staff members working in the laboratory. These issues were addressed and accommodated where appropriate (e.g. sample activity was measured and the values made available for staff review).

5.3.2 Legislative requirements

Under Schedule 2 of the Australian Radiation Protection and Nuclear Safety Regulations (1999), caesium-137 is exempt from licensing requirements in circumstances where the total activity of the sample is below 10,000 Bq or when the activity concentration is below 10 Bq/g (ARPANS Regulations 1999). The amount of material held at the Biological Criminalistics laboratory for this research was significantly less than 10,000 Bq; the material was not considered "radioactive" under the regulations and was therefore exempt for the purposes of handling or licensing of the premises. Irrespective of this classification, the samples were handled with the same care and consideration as would occur if the activity of the samples was much higher in order to explore appropriate handling protocols for analysis.

5.4 Experimental procedures

5.4.1 Sample transport and storage

Appropriate clearance was obtained from ANSTO prior to transporting samples to the Biological Criminalistics laboratory. Monitoring of the sample activity was conducted subsequent to sample extraction in order to confirm minimal activity was present in the samples before transport.

As the measured activity was below accepted background levels (Section 5.5.3), additional shielding was not required for transport. Instead, samples were transported by vehicle in a container clearly marked with radioactive labels and contact information. Once samples had arrived at the Australian Federal Police facility, they were stored in the laboratory in the transport container. Access to this area of the laboratory was restricted, with warning labels posted on all access points.

5.4.2 Radioactive contaminant

A previously verified salt solution of the radioisotope caesium-137 (ANSTO caesium-137 solution #2) was utilised for this experiment. The caesium-137 solution (pH 4.5 – 5.0) had an activity of 1.65×10^5 Bq/mL. As previously mentioned in Chapter 1, caesium-137 is a gamma/beta emitter with a gamma emission energy of 662 keV and maximum beta emission energies of 514 keV and 1180 keV (NNDC 2009). These emission types represent a hazard for external and internal exposure as well as contamination.

5.4.3 Sample preparation and monitoring

The experiments were designed to permit an examination of precision while limiting radiation exposure to the analyst. To best satisfy these conditions, a test of each system was conducted in triplicate. For this, 10 µL of 1:1 diluted whole blood (1:1 blood:TE buffer) was combined with 40 µL of caesium-137 solution (ANSTO caesium-137 solution #2). These samples were then tested using both the DNA IQ™ and ChargeSwitch® systems. It should be noted that, for these experiments, the relatively large amount of blood added was to ensure that sufficient DNA would be available to provide a full DNA profile.

The gamma emission of final extracts was measured using a P-type High Purity Germanium (HPGe) coaxial well detector at 662 keV and the remaining activity and dose rates of each sample was determined.

5.4.4 Protective equipment, exposure monitoring and radiation detection

DNA extraction using the DNA IQ™ System and ChargeSwitch® took place at the Institute for Environmental Research facility at the ANSTO. The experiment was conducted within a fume hood in a designated “blue” level laboratory (low level activity/contamination). As the extraction procedures required manual handling, lead

brick shielding was constructed and the experimental procedures were conducted behind the shielding at all times. Disposable nitrile gloves, protective glasses and a laboratory coat dedicated for use in a radiation laboratory were used during the experiments.

For the duration of the testing, an active portable Geiger-counter and personal electronic real-time dosimeter (for a total whole body dose of 2 μSv) were employed. In addition, the three recommended safety procedures of time, distance and shielding were implemented to reduce total dose. For example, during any period of time not requiring the analyst to be in close proximity to the sample, such as during incubation phases, the distance from the samples was increased to reduce the dose. This also reduced the overall time spent with the samples.

DNA processing (quantitation, amplification and capillary electrophoresis) of the decontaminated samples was conducted in the research laboratories of the Biological Criminalistics team at the Australian Federal Police. Prior to experimentation, the laboratory and equipment were screened with the Exploranium GR-135 plus Identifier portable radiation detector to determine a baseline gamma reading for comparison purposes. Gamma emissions were also monitored during the experiment and after analysis to ensure that contamination did not occur. All individuals present in the laboratory were issued with electronic real-time radiation dosimeters.

5.4.5 Decontamination and spill procedures

Decontamination and spill procedures were established for the Biological Criminalistics laboratory of the Australian Federal Police. All working surface areas (e.g. laminar flow hoods, bench tops) were covered with plastic-lined absorbent mats to contain accidental spills of contaminated material. In addition, all unnecessary equipment and consumables were removed from the immediate working area.

There were a number of procedures put in place for decontamination in the unlikely event of a spill (these were set according to regulations set out in the Australian

Radiation Protection and Nuclear Safety Regulations 1999). If a spill occurred on an absorbent mat, the absorbent mat was to be folded and placed in a hazardous waste bag and disposed of with remaining samples upon completion of the analyses. If sample was spilled on a solid surface, the spill was to be soaked up with absorbent paper and disposed of in the hazardous waste bag. The surface was then to be washed thoroughly with water and the area scanned with the Exploranium GR-135 survey monitor.

As previously discussed in Chapters 1 and 4, caesium-137 can cause damage and disruption of cellular function. For these experiments, the likely routes of exposure included external irradiation and external contamination; internal contamination may also be possible (e.g. via entrance through a wound, or by inhalation or ingestion), although considered unlikely under the circumstances. Protection from external and internal contamination included the use of disposable gloves, laboratory coat, safety glasses, and a face mask. The risk was assessed as minimal.

In the event of any accidental contact of contaminated material with the skin, eye or other areas, copious amounts of water were to be used to rinse the affected area(s), utilising the available eye wash and shower if necessary.

5.4.6 Clean-up and decontamination of work areas

At the facilities of the Australian Nuclear Science and Technology Organisation:
Following the completion of the DNA extraction experiments, all samples, reagents, equipment and personnel were screened for radionuclide contamination with a Geiger-Müller counter before exiting the laboratory. Any contaminated equipment was rinsed thoroughly with water, dried and re-screened until readings returned to background levels. Contaminated waste, such as pipette tips, gloves, and microcentrifuge tubes, were discarded in a radioactive waste container. This container remained within the laboratory for disposal via ANSTO standard operating procedures.

At the facilities of the Australian Federal Police:
All materials involved in the analysis (including samples) were discarded into biohazard waste containers following completion of the DNA processing.

5.4.7 Extraction procedures

Extraction procedures were carried out as presented in Sections 4.2.4.2 and 4.2.4.3 for DNA IQ™ and ChargeSwitch®, respectively.

5.4.8 Caesium-137 detection

Immediately following DNA extraction, each tube of extract and resin was tested for remaining caesium-137 contamination. Gamma emission was measured on a P-type HPGe well detector set to detect peaks at 662 keV. The activity of each sample and resin was recorded in Table 5.1.

5.4.9 Quantitation, amplification and DNA profiling

The extracts from the DNA IQ™ System and ChargeSwitch® were processed using DNA quantitation, PCR amplification (25 µL reaction), and DNA profiling with capillary electrophoresis as described in Sections 2.3.4 to 2.3.8.

5.5 Results and discussion

The results obtained from these experiments demonstrate the efficiency of the DNA IQ™ and ChargeSwitch® systems to remove a radioactive caesium-137 salt contaminant and to efficiently extract DNA in the presence of that contaminant. These findings allowed for a comparison of radioactive versus non-radioactive caesium removal and profiling success (i.e. from Chapter 4), an estimation of non-radiation worker exposure rates (e.g. a forensic biologist), and a basis upon which to propose

standard operating procedures for the handling and analysis of samples suspected of being contaminated with radioactive caesium.

5.5.1 Activity estimates for sample transport

The initial activity of the caesium-137 standard was 165000 Bq/mL, producing an initial measured activity of 6590 Bq for each sample (as each contained 40 µL of standard).

Following the extraction procedures, the "worst-case" scenario of maximum possible activity was estimated prior to transportation to the Biological Criminalistics laboratory. This was based upon the highest observed activity following extraction, which was approximately 2 Bq for a ChargeSwitch® replicate (see Table 5.1). Based on this value, the maximum activity of the samples was estimated at 16 Bq for 8 samples; in reality, this was likely to be much less as the average measured activity was below 1 Bq for the samples following extraction.

It should also be noted, particularly in the context of occupational health and safety and waste disposal, that the maximum activity of the extraction resin was estimated to be 192 Bq following extraction, over 10-fold higher than the samples (estimate was based on the highest activity sample i.e. 24 Bq × 8 samples, which gives 192 Bq). This resin remained at the ANSTO facility for storage and/or disposal. Therefore, the activity level of the resin post-extraction has implications for its safe disposal in standard operating procedures.

5.5.2 Efficiency of radioactive contaminant removal

One objective of this research was to investigate the efficacy of the DNA IQ^{TM} and ChargeSwitch® systems in removing radioactive caesium-137 contaminant (as a decontamination procedure for downstream sample processing). As previously discussed, caesium-137 nitrate salt solution was added to each sample and the spiked samples taken through each extraction procedure. The gamma emission of the final

extract was measured using a P-type HPGe coaxial detector (detection at 662 keV) and the remaining activity of each sample determined using manual calculations. The activity of each sample was determined by multiplying the number of counts detected by the number of Becquerels (Bq) per count. For example, for each count detected there is 0.24 Bq. For DNA IQ™ replicate 1, six counts were detected. Therefore, the sample contained 1.4 Bq of radioactivity. Results from the analysis are summarised in Table 5.1.

Table 5.1: Effectiveness of caesium-137 removal using the DNA IQ™ and ChargeSwitch® Systems

	Sample Name	Activity of sample before extraction (Bq)	Activity of Resin after extraction (Bq)	Activity of sample after extraction (Bq)	DNA Profiling Success (Y/N)
DNA IQ™ Samples	DNA Control	0	0	0	Y
	Cs Control	6.6×10^3	< 0.24[a]	< 0.24[a]	N/A[b]
	Replicate 1	6.6×10^3	< 0.24[a]	1.4	Y
	Replicate 2	6.6×10^3	1.4	0.24	Y
	Replicate 3	6.6×10^3	1.4	< 0.24[a]	Y
ChargeSwitch® Samples	DNA Control	0	0	0	Y
	Cs Control	6.6×10^3	7.9	1.2	N/A[b]
	Replicate 1	6.6×10^3	7.9	2.2	Y
	Replicate 2	6.6×10^3	7.0	0.98	Y
	Replicate 3	6.6×10^3	24	< 0.24[a]	Y

[a] Zero counts recorded in 120 second scan; [b] DNA was not added to the caesium control samples.

The results presented in Table 5.1 demonstrate that both the DNA IQ™ and ChargeSwitch® systems are able to effectively remove the bulk of the contaminating radioactive caesium-137, to leave a significantly low activity level. In all samples, the observed activity (in Bq) was reduced at least 3000-fold to levels approaching zero.

Moreover, considering the initial starting activity of 6.6×10^3 Bq, a successful DNA profile was obtained for all contaminated samples in addition to the DNA controls. This will be further discussed in Section 5.5.5.

Table 5.1 also demonstrates the activity of the resin after extraction, indicating the retention of caesium for each system. From these data, the ChargeSwitch® resin appears to be retaining more caesium than the DNA IQ™ resin. This could be due to the apparently larger amount of resin in the ChargeSwitch® extraction procedure or possibly due to the longer incubation time involved in the ChargeSwitch® procedure (20 minutes as compared to 5 minutes) allowing for a greater opportunity for caesium ions to bind to the resin. These results also support observations from Sections 4.3.3.1 and 4.3.3.2 which demonstrate a larger portion of the caesium salt remain on the ChargeSwitch® resin than the DNA IQ™ resin.

5.5.3 Discussion of dose rates

Dose rates for analyst exposure were calculated from the measured activities in Table 5.1 (Table 5.2). Dose rates were determined using an online calculator for Radiation Safety professionals (McGinnis 2008). Dose rates determined by the online calculator are comparable to manual calculations independently conducted.

Considering that permissible dose levels for a non-radiation worker equate to approximately 0.5 μSv/working hour[5], the calculated dose rates indicated in Table 5.2 are significantly lower (250-fold lower per sample pre-extraction, approximately 1-million-fold lower post-extraction).

[5] The 0.5 μSv/work hour is derived from an acceptable limit of 1 mSv/year = 1000 μSv/year = 20 μSv/week [50 work weeks/year] = 4 μSv/work day [5 days/week] = 0.5 μSv/working hour [8 hours/day]

Table 5.2: Estimated dose rates following extraction with the DNA IQ™ and ChargeSwitch® Systems (50 cm working distance)

	Sample Name	Dose rate[a] of sample before extraction (μSv/h)	Dose rate[a] of sample after extraction (μSv/h)
DNA IQ™ Samples	DNA Control	0	0
	Cs Control	0.0020	$< 7.3 \times 10^{-8}$
	Replicate 1	0.0020	2.2×10^{-7}
	Replicate 2	0.0020	7.3×10^{-8}
	Replicate 3	0.0020	$< 7.3 \times 10^{-8}$
ChargeSwitch® Samples	DNA Control	0	0
	Cs Control	0.0020	3.6×10^{-7}
	Replicate 1	0.0020	6.6×10^{-7}
	Replicate 2	0.0020	2.9×10^{-7}
	Replicate 3	0.0020	$< 7.3 \times 10^{-8}$

[a] Dose rates calculated based on a 50 cm working distance

These levels may provide guidance when planning for the volume of samples and time allowed per analyst in the forensic biology laboratory. Furthermore, time spent preparing the samples before extraction needs to be considered, i.e. 2 hours preparation time will result in an integrated dose of 0.0040 μSv. Preparation time will include searching of exhibits and collection of samples, sample incubation periods and possibly centrifugation depending on the sample and extraction procedure employed. Furthermore, these values may be further adjusted if certified radiation workers are charged with the extraction of such samples; the current standards for radiation workers permit an exposure limit of 20 mSv/yr (ICRP 1991; ARPANS Regulations 1999; ICRP 2007).

5.5.4 DNA extraction efficiency – DNA quantitation

Following extraction of the contaminated blood samples, it was demonstrated that the DNA IQ™ and ChargeSwitch® systems gave comparable overall DNA yield as a

percentage of control values (99.0% [DNA IQ™], 96.7% [ChargeSwitch®]; Figure 5.1). From the findings, however, it can be seen that ChargeSwitch® demonstrated a higher degree of repeatability (±19.3% [DNA IQ™], ±2.7% [ChargeSwitch®]; Figure 5.1). It should be noted, however, that small sample size and possible errors in pipetting may have contributed to this variation.

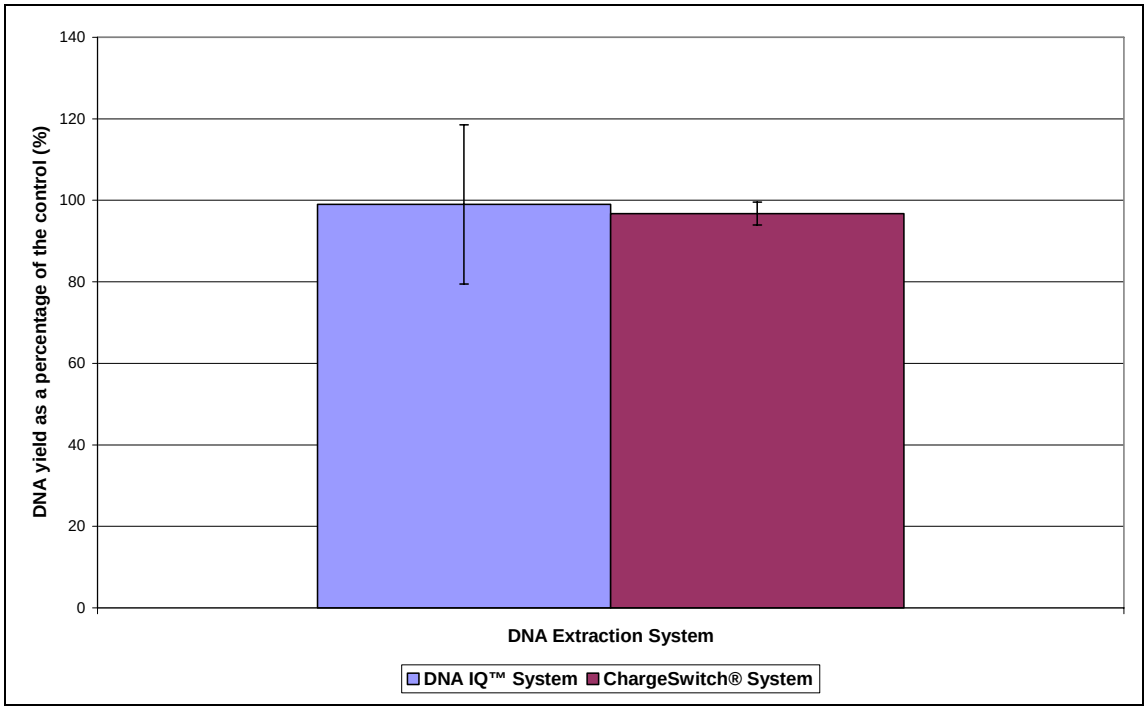


Figure 5.1: Quantitative comparison of DNA extraction yield using the DNA IQ™ and ChargeSwitch® systems in the presence of radioactive caesium-137 as a percentage of the control

Figure 5.1 also demonstrated a recovery greater than 100% for some samples extracted with the DNA IQ™ system. The Rotor-Gene™ real-time PCR system is a very sensitive method for quantitiation that utilises small volumes of DNA extract (2.5 µL) for the analysis. Slight changes in volume can therefore greatly affect the quantitation results. In addition, the DNA IQ™ system is recovering well below the expected capacity of the resin (see below and Figure 5.2) and therefore a DNA yield in excess of the control value is achievable.

Figure 5.2 demonstrates that the overall DNA concentration and extraction repeatability for ChargeSwitch® is better than that of the DNA IQ™ system (0.42 ± 0.08 ng/µL [DNA IQ™], 1.03 ± 0.03 ng/µL [ChargeSwitch®]). ChargeSwitch® is achieving its nominated potential for a maximum DNA extraction efficiency of 100 ng DNA (1 ng/µL in 100 µL); the DNA IQ™ system also has a maximum extraction efficiency of 100 ng DNA but is performing over 2-fold lower than this capacity. It should be noted that the ChargeSwitch® extraction procedure employed in these experiments was previously optimised by staff of the Biological Criminalistics laboratory of the Australian Federal Police; no changes to the optimised procedure were made for this study. However, as the treated samples performed to a similar capacity as the DNA controls (i.e. those with the same quantity of DNA but without the caesium contaminant), any effects of the contaminant on DNA binding and recovery can be reasonably excluded.

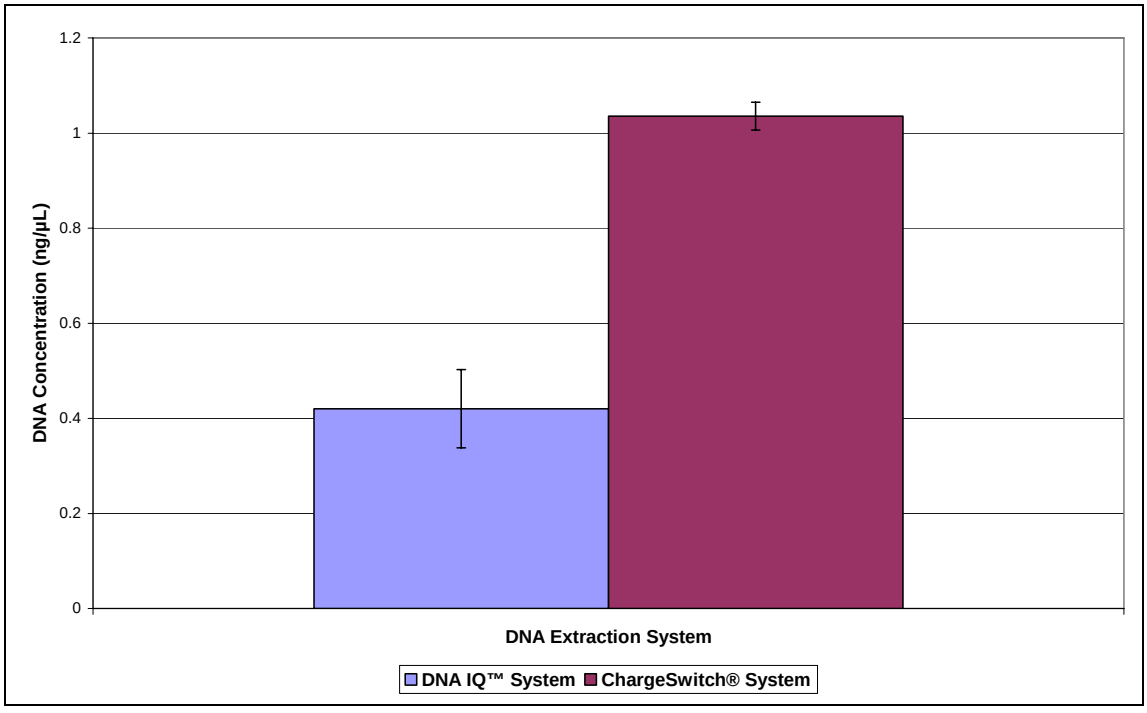


Figure 5.2: Quantitative comparison of DNA concentration (in ng/µL of extract) using the DNA IQ™ and ChargeSwitch® systems in the presence of radioactive caesium-137

While optimisation attempts for the DNA IQ™ System utilising genomic DNA did not reveal direct improvements for DNA extraction efficiency, further testing with biological matrices such as blood is recommended as it may have been beneficial to the overall extraction efficiency. For example, increasing the incubation time of the blood sample prior to addition of the beads, in a similar manner to the ChargeSwitch® protocol (Section 4.2.4.3) , may allow for a more complete release of DNA from the cells resulting in increased yield and repeatability.

In addition, results from Chapter 4 (Section 4.3.2) have shown that the DNA IQ™ system is more efficient at extracting lower quantities of DNA (< 50 ng). This is clearly demonstrated in results Figures 4.2 – 4.4 where the system was able to extract approximately 90% of the 10 ng of DNA added as compared to approximately 60% for 100 ng DNA. The reasons for the decrease in binding efficiency as outlined previously e.g. steric hindrance are applicable in this instance as a larger quantity of blood was added to ensure a profile was obtained after the extraction.

Despite the discrepancies observed between the DNA IQ™ and ChargeSwitch® extraction systems, it has been demonstrated that the presence of caesium-137 and the emission of ionising radiation at the levels tested did not appear to affect the capability of either system to obtain a suitable sample of DNA for profiling purposes. The effects of the contaminant will be discussed further in the following section.

5.5.5 Success of DNA profiling

The findings from these studies demonstrate that, following capillary electrophoresis, no significant differences were observed between the samples contaminated with caesium-137 and extracted using either the DNA IQ™ or ChargeSwitch® systems (Figure 5.3). From Figure 5.3, it can be seen that peak heights were obtained from both extraction systems for all targeted loci, and at levels in accordance with those obtained for the DNA controls.

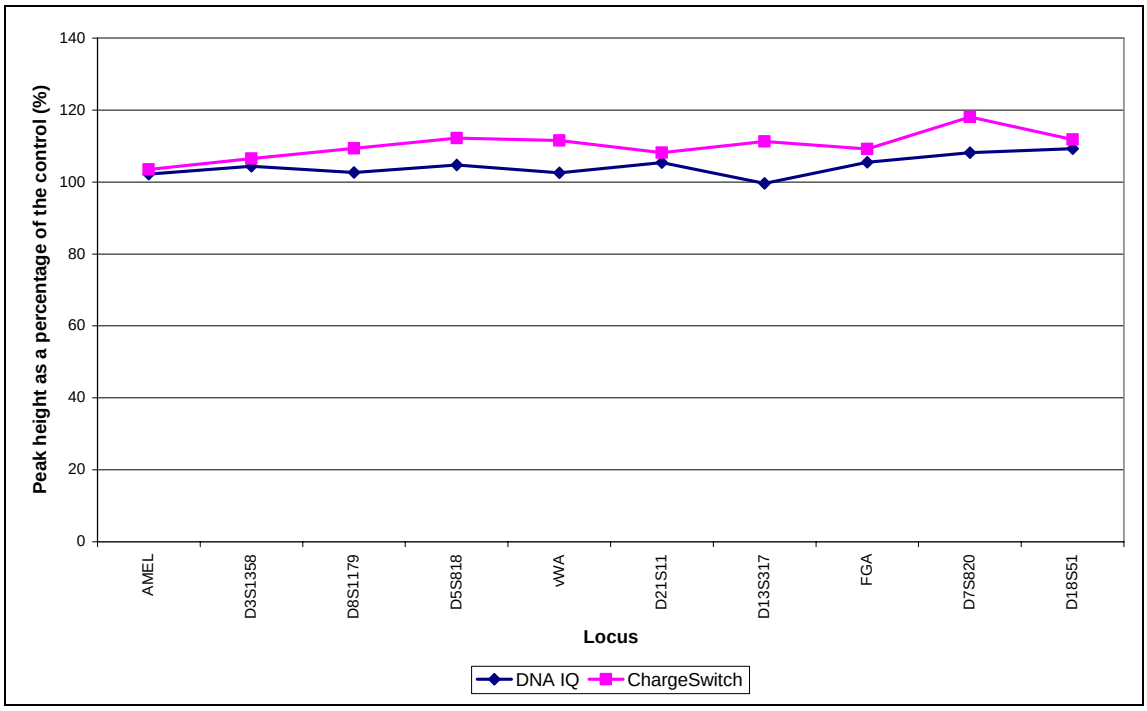


Figure 5.3: Quantitative comparison of locus peak heights using the DNA IQ™ and ChargeSwitch® systems in the presence of radioactive caesium-137 (expressed as % of the control)

As demonstrated in Figure 5.3, both the DNA IQ™ and ChargeSwitch® systems produced peak heights consistent with their respective controls. The relatively superior extraction efficiency observed with the ChargeSwitch® system (Section 5.5.4) has not significantly impacted on the overall DNA profile produced, as one would expect if DNA degradation had occurred. If lower amounts of starting DNA were used, such as those typical for trace DNA analysis, poorer extraction efficiency may have a significant impact on the production of a full DNA profile, with allele/locus dropout.

5.5.6 Statement on the extraction of radioactive versus non-radioactive caesium samples

The previous chapters demonstrated the capability of the DNA IQ™ and ChargeSwitch® systems to remove a non-radioactive caesium-133 salt contaminant in the presence and absence of DNA. It was shown that, in either system, the caesium-133

contaminant did not affect the amount of DNA recovered, nor did it affect the suitability of the purified DNA for profiling using capillary electrophoresis.

In these experiments, radioactive caesium-137 salt was used to contaminate blood samples intended for extraction and DNA processing. From the findings at the studied activity level, it has been demonstrated that, regardless of the contaminant being radioactive or non-radioactive, there were no significant changes observed in the DNA profiling results compared to those from the control samples.

This is a particularly important consideration in that, should caesium be considered a representative example, these and future extraction methodologies may be investigated and/or validated using non-radioactive counterparts. This would greatly reduce analyst exposure to radioactive materials in preparing an analytical response to a real-world radiological incident (e.g. in an investigation of a terrorist act involving an RDD or other radiological contamination event).

5.6 Conclusions

The results of these experiments have demonstrated that both the DNA IQ™ and ChargeSwitch® systems are able to effectively remove contamination by radioactive caesium-137 at the levels tested. In all samples, the observed activity (in Bq) was reduced at least 3000-fold to levels approaching zero. This activity was well below that considered permissible for non-radiation workers (250-fold lower per sample pre-extraction, approximately 1-million-fold lower post-extraction), which may provide guidance when planning for the volume of samples processed and the time required per analyst in an operational forensic biology laboratory.

It has also been demonstrated that the DNA IQ™ and ChargeSwitch® systems give comparable overall DNA yield as a percentage of control values (99.0% [DNA IQ™], 96.7% [ChargeSwitch®]; Figure 5.1), with ChargeSwitch® demonstrating more favourable repeatability and a maximum extraction efficiency of 100 ng DNA

(extraction capacity more than 2-fold higher than that achieved with DNA IQ™). Despite the discrepancies observed between the DNA IQ™ and ChargeSwitch® extraction systems, it has been demonstrated that the presence of caesium-137 did not affect the capability of either protocol to obtain a suitable sample of DNA for profiling. Additionally, it has been demonstrated that the starting pH of 4.5–5.0 for the contaminant solution did not adversely affect either DNA IQ™ or ChargeSwitch® (as previously stated in Chapter 2, the ChargeSwitch® system requires a pH of 4.0 for DNA binding). The poorer performance of the DNA IQ™ system at capturing 100 ng of DNA was not unexpected as Promega's Small Sample Casework Protocol states that DNA IQ™ is designed to purify small quantities of DNA and becomes more efficient with samples containing less than 10 ng of DNA (Promega 2002b).

The findings from these studies demonstrate that, following capillary electrophoresis, no significant differences were observed in the results achieved using the two different extraction systems. Suitable peak heights were obtained in both systems for all loci, and at levels in accordance with those obtained for the DNA controls.

The DNA IQ™ and ChargeSwitch® systems have therefore proven to be robust methodologies, not only for the efficient extraction of DNA from blood in the presence of the radioactive caesium-137 contaminant, but also as a decontamination process for subsequent downstream processing.

5.7 Recommendations for laboratory standard operating procedures

First and foremost, it is important that all handling, transport and storage of radioactive materials comply with State and Federal regulations (e.g. Australian Radiation Protection and Nuclear Safety Regulations 1999). The operating procedure listed below was considered both appropriate and adequate in protecting against exposure to (and contamination by) caesium-137 at the levels being studied. This information may serve as a guide for interested institutions; however, it is still recommended that other sources of information are reviewed in preparing a response strategy (e.g. radiation and nuclear

safety legislation may provide specific guidance for particular agents, as well as advice on handling or related activities under special circumstances). In addition, analysts that may be required to process biological evidence contaminated with radioactive substances and manage radiation exposure levels should undertake relevant radiation training to complement the procedures discussed below.

In a laboratory response to a radiological incident, as a safeguard, the location and time of analysis should be planned so as to avoid subjecting uninvolved staff to unnecessary exposure and potential contamination. All testing in the laboratory should be conducted within a hood or alternative closed containment systems designated to handle appropriate levels of radioactivity. Shielding should be utilised (e.g. lead bricks), with analytical procedures taking place behind the shielding at all times.

As discussed earlier, Personal Protective Equipment (PPE) should consist of chemical-resistant gloves, protective glasses (and/or a face shield), and a laboratory coat dedicated for use in a radiation laboratory. In addition, procedures should be in place for the management of accidental contamination of the skin, eye or other areas with radioactive materials with protocols for treatment or remedial actions such as rinsing of the affected area(s) with copious amounts of water. Analyst time, distance and shielding should be controlled to reduce total dose, and sample activity should be monitored to adjust these parameters accordingly over the course of testing (in addition to monitoring area contamination). Portable Geiger-Müller counters and personal real-time dosimeters should be employed over the duration of the analyses.

Decontamination and spill procedures should be established. In these experiments, working surface areas (e.g. laminar flow hoods, bench tops) were covered with plastic-lined absorbent mats to contain spills and procedures were put in place for clean-up, decontamination of surfaces, disposal and confirmation of decontamination in the event of an incident.

Care should be taken to avoid contamination of both human traffic areas and the immediate environment. The handling of considerable amounts of waste should be coordinated with agencies certified to safely remove and dispose of radioactive materials.

5.8 Future directions

The DNA IQ™ and ChargeSwitch® extraction systems have proven to be effective for both sample decontamination and the efficient extraction of DNA from blood in the presence of the radioactive caesium-137 contaminant. This research investigated several aspects of dealing with radioactive contaminants in blood, including handling protocols, and documented preliminary results on the removal of caesium-137 contaminant and subsequent DNA profiling. There was also the intention of identifying key issues for research and proposing future directions for forensic institutions involved in the investigation and analysis of forensic samples contaminated with radioactive material.

There were several aspects identified in this research that merit further study. These include procedural components (e.g. methodologies, contaminants, matrices) as well as potential policy considerations. Of particular interest is to confirm the point at which the caesium-137 is removed during the extraction process. While conducting these experiments, there was an effort to adhere to the ALARA principle (As Low As Reasonably Achievable) to ensure minimal radiation exposure to the analyst. As a result, and as per ANSTO's protocols, the highly contaminated solutions were discarded as soon as possible. It would, however, be of benefit to confirm the pattern observed with the caesium-133 experiments, which demonstrated that the majority of the contaminant is removed during the lysis step (Chapter 4).

This study involved the analysis of small blood volumes contaminated with radioactive caesium-137 of known activity. One critical aspect not previously discussed involves the searching and/or collection techniques used to acquire forensic specimens for analysis from items of evidence. Thorough screening of each evidentiary item is necessary for

documentation and sampling of appropriate types of biological evidence for forensic analysis. This may require varying lengths of time and proximity to the contaminated material. Such exposure must be considered when executing the examination, taking special care to limit the dose received by the analyst to within permissible levels for non-radiation workers. Policies may dictate strategies such as the allocation of duties such as searching, extraction, and profiling to individual analysts with a defined maximum radiation exposure or, alternatively, as the level of radioactivity will decrease as the samples are processed, it may be more beneficial for a single analyst to take samples through from searching to completion, and where appropriate limit exposure to one analyst. A further discussion on search and collection techniques is presented in Chapter 6 and includes consideration of a multi-disciplinary approach for the order of searching/collection/analysis of forensic evidence contaminated with radioactive material.

The extraction efficiency of the DNA IQ™ and ChargeSwitch® systems utilising ICP-MS for the quantitation of caesium from various matrices (blood, Hep-2 cells, saliva, and genomic standard) is discussed in Chapter 4. The experiments detailed in this chapter, involving the radioactive caesium-137 contaminant, were based on the parameter of activity (i.e. the volume of standard added to each sample was equivalent to an initial activity of 6590 Bq). The ICP-MS instrumentation was not used to analyse samples in these studies due to sample volume constraints; however, future studies could utilise the instrumentation to determine the clear association of caesium-137 concentration with activity.

In addition, levels of activity should be investigated that are in excess of the studied 6590 Bq. Given that the extraction methods were demonstrated to reduce the sample activity by approximately 3000-fold, and previous experiments involving various concentrations of non-radioactive caesium are able to be removed, it may indicate that these systems are able to achieve a higher capacity of caesium-137 removal (see chapter 4). This would depend on the correlation of concentration to activity as

previously mentioned, and would need to be restricted to practical activity levels that do not endanger the analyst.

It was also observed that ChargeSwitch® provided better overall DNA yield and repeatability than that of the DNA IQ™ system, with the ChargeSwitch® system achieving its nominated potential for a maximum DNA extraction efficiency of 100 ng DNA (1 ng/µL in 100 µL). The DNA IQ™ system also has a maximum extraction efficiency of 100 ng DNA but performed at less than half of this capacity. Given that the negative controls performed to a similar capacity excludes any effects of the contaminant on DNA binding and recovery. While the DNA IQ™ extraction procedure has been previously optimised using genomic DNA, further adjustments may be required to improve on this efficiency utilising blood and other test matrices. Particular attention should also be paid to assessing the extraction efficiency for low levels of DNA.

The DNA IQ™ and ChargeSwitch® systems have proven to be effective for the extraction of DNA contaminated with radioactive caesium-137, a gamma/beta emitter that may possibly be used in illegal or subversive activities. Further studies should include assessment of the suitability of these methods for other radioactive contaminants such as strontium, iodine, iridium, americium and uranium, particularly uranium oxides. The investigation of a negatively charged ion, such as iodine, will further establish if there is any interference in the DNA binding for either method. As previously mentioned, such efforts may be investigated using non-radioactive forms, as the extraction behaviour of caesium-133 has been demonstrated to be appropriately representative of caesium-137. This approach could greatly reduce analyst exposure to radioactive materials in preparing for an analytical response to a real-world incident involving radioactive contamination.

Furthermore, as this study focused on the analysis of blood, further validation studies on radioactive contamination should be carried out with other matrices using either the DNA IQ™ or ChargeSwitch® extraction systems. These matrices may include any free-

flowing dilute liquid that will not affect the binding capacity of the beads as well as biological samples collected on swabs, adhesive tape, and other collection apparatus that may affect the efficiency of the DNA extraction process. Such amenable matrices may include saliva as well as tissue homogenates (such as bone, muscle and other tissues) that have been contaminated with radioactive materials. Care must be taken with other matrices, as critical pre-treatment may be required to maximise DNA recovery. Additionally, the ChargeSwitch® system does not have standard operating procedures that have been validated for tissue and bone samples, hence the available protocols may require significant revision in terms of reagent volumes and optimisation of methodology.

Chapter 6: General Discussion and Future Directions

6.1 Introduction

The principal aims of this research were to investigate the impact that an unconventional weapon incorporating radioactive material may have on the analytical processes and the interpretation of forensic DNA evidence. Experiments were designed to examine the impact of ionising radiation, specifically gamma and alpha radiation, on the DNA profiles of forensically-relevant biological matrices.

The effects of electromagnetic gamma radiation was explored and characterised with regard to blood, saliva, and bone specimens, as well as a genomic DNA control. For comparison, the effects of alpha particle radiation were also investigated for blood and saliva, in addition to a human epithelial cell line, HEp-2. Both experimental series sought to obtain an insight into the stability of the DNA sample post-irradiation, in addition to addressing concerns regarding sample integrity and dose thresholds for DNA degradation.

This research has also examined issues of sample contamination and the impact that radioactive material will have on the forensic analyst and the laboratory environment. Current and novel DNA extraction methodologies were investigated for their effectiveness at decontamination of non-radioactive caesium-133 salt, in addition to characterising potential interference with extraction efficacy. Confirmatory studies were then conducted using the corresponding radioactive caesium-137 species.

It was desirable to carry out original research that could satisfy the present needs of the forensic community. Therefore, this research focused primarily on applications that were both feasible and practical for a working forensic laboratory. By experimental design, the opportunity to contribute to the existing literature pool has been realised through the presentation of these findings.

The following sections summarise the conclusions from these experiments and highlight directions for future research.

6.2 Effects of gamma radiation on DNA profiling

The research presented in Chapter 2 sought to address several objectives, including investigation of the effects of γ-radiation from a cobalt-60 source on DNA from a range of biological matrices (blood, saliva, bone and commercially-available genomic male DNA), investigation of the potential impact of the time-to-analysis on the qualitative and quantitative aspects of DNA profiling, and to establish limits of exposure for successful profiling of gamma-irradiated (cobalt-60) biological samples. These were in addition to a fourth objective in that the Alonso *et al.* (2001) organic extraction method was identified as the most robust and reliable technique, of those examined, for the extraction of DNA from bone samples.

The pattern of profiling results obtained across matrices, examined as average loci peak height, demonstrated a progressive loss of the higher molecular weight loci as the gamma radiation dose increased. The smallest target fragment, amelogenin (109 bps), was successfully analysed after exposures of up to 100,000 Gy for all matrices investigated, while one of the largest target fragments, D18S51 (264-344 bps), was typically absent at both the 50,000 and 100,000 Gy doses (loss of signal was also observed at the D13S317, FGA and D7S820 loci). This observation reflects the typical pattern of degraded DNA, where the longer fragments presented a greater opportunity for interaction with ionising radiation than the shorter fragments. It is proposed that degradation of the DNA molecule in these cases (as indicated by the failure of PCR amplification) is likely due to fragmentation of the DNA strand, in addition to inter-strand cross-linking, deamination and dimer formation. This view is also supported by the work of Hutchinson (1985), Lindahl (1993) and Irwin *et al.* (2007).

Furthermore, the findings from this research have demonstrated that gamma irradiated biological matrices are particularly robust for DNA analysis using commercially available STR systems, such as Amp*Fl*STR® Profiler Plus®. Overall, there appears to be little observable degradation up to an exposure of 5,000 Gy for all samples tested, and it is possible to obtain a full DNA profile at doses at least up to 10,000 Gy. This

suggests that there is a threshold dose of between 5,000 and 10,000 Gy for degradation in the DNA profile to be observed. In addition, the effects of gamma radiation on DNA appear to be consistent with other degradative processes (e.g. temperature, bacterial contamination, fire and moisture) and therefore current standard operating procedures used for the interpretation of degraded DNA can be applied to DNA that has been exposed to ionising radiation. With regards to time-to-analysis (i.e. delay between exposure and the commencement of sample processing for DNA analysis), general trends in the data suggest a marginal reduction in response for the samples over time. Therefore, if possible, steps should be taken to process samples within this timeframe.

With this in mind, and taking into consideration analyst exposure, high dose thresholds for all matrices suggest that potential adverse effects on DNA evidence would be unlikely to direct the pace of such an investigation. Instead, operational decisions regarding collection of the evidence should be based largely on potential exposure to the personnel present at the scene.

From the insights gained through this experimental series, additional studies may be of interest to further explore the impact of gamma radiation on DNA evidence. Future research may include investigation of alternative sources of gamma radiation, such as the gamma/beta-emitter caesium-137, which has a single gamma ray energy of 662 KeV, to investigate the potential impact of energy levels on threshold doses for degradative effects. In addition, responses from trace levels of DNA should be explored (as the starting amount of DNA template was kept consistent in this research to ensure that a full profile could be obtained), including DNA on clothing and other substrates on which biological materials are typically found.

Also, in this study, significant allelic/locus dropout was not observed until doses reached 50,000 Gy. Upon further review, there may be effective alternative methods of analysis that can retrieve information from degraded samples. One example is the use of miniSTR primers, which amplify a shorter segment of the DNA locus by binding as close to the repeat region as possible. These primers can potentially recover information

from an additional two or three loci depending on the template size of the remaining target DNA molecule. MiniSTR primer sets have already demonstrated efficacy with current systems (e.g. Profiler Plus®, Identifiler® or PowerPlex16®) and have been successfully applied to the analysis of a number of naturally and artificially degraded DNA samples (Butler *et al.* 2003; Chung *et al.* 2004; Coble and Butler 2005; Opel *et al.* 2006; Meissner *et al.* 2007; Opel *et al.* 2007; Hill *et al.* 2008; Mulero *et al.* 2008). Further options worthy of exploration include mitochondrial DNA analysis, YSTR analysis, Single Nucleotide Polymorphisms (SNPs), and Low Copy Number (LCN) STR typing. Swango *et al.* (2006; 2007) have also presented research into multiplex qPCR for the assessment of DNA degradation in forensic samples, with target loci (TH01 and CSF1PO) that may give a more accurate representation of the true quantity of useful DNA in the sample and the evidentiary quality of the sample.

From the results of the cobalt-60 gamma irradiations, it has been demonstrated that DNA is very robust. A full DNA profile can be obtained from blood, saliva and bone up to 10,000 Gy and over at least a 4-week period from exposure to extraction.

6.3 Effects of alpha radiation on DNA profiling

The experiments performed in Chapter 3 were designed to include investigation of the effects of alpha particles from a particle accelerator on a range of biological matrices (blood, saliva, human epithelial cells [HEp-2]; particle energy of 5.5 MeV), demonstration of the potential impact of time-to-analysis on the qualitative and quantitative aspects of a DNA profile, to establish limits of exposure for the analysis of alpha particle-irradiated samples, and to compare the general effects of gamma and alpha radiation on DNA profiling. Doses were designed to simulate a 3.7×10^{11} Bq (10 Ci) americium-241 source in direct contact with the sample.

From the observed data, there is a general reduction in profiling success with increasing dose of alpha particles (energy 5.5 MeV). DNA profile degradation was observed at doses at and above 66,000 Gy across all matrices, and a characteristic pattern was

demonstrated with allelic dropout first occurring at the higher molecular weight loci. With regards to effects due to time-to-analysis, the pattern, if any, was difficult to discern. Although general trends over the three time periods suggest a reduction in response for the samples, which was demonstrated in the HEp-2 cells and the blood.

Based on the selected source parameters, the doses examined were exceptionally high; however there was a significant resilience demonstrated by the DNA in comparison to that observed with the gamma irradiation. The most significant factor influencing this effect is likely to be the limited penetrability of the alpha particle (approximately 0.04 mm in tissue) and the respective compositions of the tested matrices, the latter being demonstrated in the variable effects seen across matrices in these experiments. In a real-world scenario, there should not be any detrimental effects on DNA evidence from alpha radiation unless the sample is in direct contact with the radioactive material. Further in support of this reasoning, bone was not analysed as the structure of bone allows for adequate protection from the limited penetration of alpha particles. In addition, sample collection procedures for human identification usually target specimens from deep within the matrix (e.g. bone marrow or muscle).

The STAR particle accelerator was used to simulate an americium-241 source in this research, with the instrument and rigid sample requirements imposing limitations on the experimental design (e.g. all samples required mounting on a conductive surface, completely dry, and as flat as possible in a single layer of cells). To further explore the findings presented here, future research could include the use of the americium-241 radioactive source itself. By using such a source, experimental limitations could be addressed, such as alternative sample dilutions of biological materials, alternative substrates, additional biological matrices, and effects from other dose activities. Further studies using alternative alpha sources, such as plutonium-238, would also allow for a more thorough characterisation of exposure effects.

Lastly, and similar to the findings from the gamma irradiation experiments, further studies to more closely examine the DNA degradation may be extended to alpha

radiation experiments, such as the use of alternative miniSTR primers. Should it be deemed necessary, additional studies might also consider exploring greater time-to-analysis periods to demonstrate more defined effects (e.g. delays from three to six months from irradiation).

Also similar to the findings for the gamma-irradiated samples, this study has shown that conventional methodologies used in the interpretation of degraded DNA can also be applied to the interpretation of DNA irradiated with alpha particles. From an operational perspective, access to an affected scene will not be impeded by concerns regarding external exposure to alpha radiation (in contrast to gamma irradiation). Irrespective of the limited penetrability of alpha particles, protective equipment must still be implemented to safeguard from internal exposure to the contaminant material, where uptake of the radioactive material into the body can cause significant and irrecoverable damage to tissues and organs. This would include the use of a filtered breathing apparatus to prevent inhalation and ingestion, and protective clothing to prevent uptake by wounds and absorption though the skin.

6.4 Extraction and decontamination of DNA

The DNA extraction step is critical for the successful processing of biological evidence. With clever design, this step is capable of both effective decontamination of the sample and recovery of purified DNA for downstream profiling. The research presented in Chapter 4 therefore aimed to explore existing and novel methodologies, to include investigation of the usefulness of the DNA IQ™ and ChargeSwitch® extraction systems for the removal of contaminants from biological samples, comparison of these novel systems against the conventional Chelex^{100} resin extraction procedure, quantification of the efficacy of the extraction systems for the removal of a contaminating caesium-133 salt, investigation of the quantity of recovered DNA from each extraction system, and examination of the quality of the subsequent DNA profiles.

Both the DNA IQ™ and ChargeSwitch® solid-phase extraction systems proved reliable and suitable for the purification of DNA samples contaminated with the representative non-radioactive caesium-133 (>99.95% removal for DNA IQ™; 99.99% for ChargeSwitch®). The final eluant contained 0.06 µg of caesium-133 for DNA IQ™ and 0.04 µg for ChargeSwitch®, in comparison with the conventional Chelex^{100}/Microcon®, which resulted in up to 3.0 µg of caesium remaining in some samples (> 98.8% removal). The higher efficiency in caesium removal can be attributed to the paramagnetic DNA-binding resin of the DNA IQ™ and ChargeSwitch® systems, which provides a solid support to which DNA can selectively (and reversibly) bind to permit washing and removal of contaminants. Furthermore, the results have demonstrated that the presence of the caesium-133 contaminant did not negatively affect the pH or ionic strength of the solid-phase extraction systems, which could otherwise potentially influence the efficiency of decontamination and/or DNA binding.

From the extraction efficiency data, the amount of remaining caesium-133 in the extraction eluants was extrapolated to reflect dose rates of radioactive caesium-137 (in µSv/h). The calculated data demonstrated that both the DNA IQ™ and ChargeSwitch® protocols significantly reduced dose rates compared to the Chelex^{100}/Microcon® extraction (< 0.01 µSv/h for DNA IQ™ and ChargeSwitch®; up to 0.56 µSv/h for Chelex^{100}). From this, it can be estimated that numerous extracted samples from the DNA IQ™ or ChargeSwitch® systems could be handled before the dose rate limit of 0.5 µSv per work hour for a non-radiation worker is exceeded. However, the laboratory must still account for the number of samples to be processed (and time required) to ensure that task allocation is in accordance with legislated total integrated dose for personnel. Workplace risks may be further mitigated through the use of appropriate personal protective equipment, exposure monitoring, contamination monitoring, and waste disposal.

From the study of decontamination efficacy, the effects of the contaminant itself on the DNA recovery process were also investigated. The findings demonstrated that contamination of the samples with caesium-133 did not result in any significant effects

on the quantitation, amplification or profiling of DNA at the concentrations tested (0.010 M, 0.005 M and 0.001 M). Quantitation of the DNA samples extracted using DNA IQ™ demonstrated approximately 60% recovery, a value that is consistent with both the DNA IQ™ patent and the results from the control samples. By comparison, quantitation of the samples from ChargeSwitch® extraction demonstrated approximately 80% recovery. In addition, the caesium-133 contaminant did not affect the amplification process or affect the quality of the DNA profiles. Results from all the contaminated samples were consistent with those from the control samples, with no significant effect of the contaminant at the targeted loci/alleles. However, it should be reiterated that other radioactive contaminants may not behave in this manner.

The findings from this research suggest that the solid-phase extraction protocols inherent with the DNA IQ™ and ChargeSwitch® systems are efficient at removing the caesium contaminant. Given the scope of these experiments, there are several issues to consider for future investigation. For example, several parameters in the DNA IQ™ manufacturer's protocol were modified in this study, with little observable effect. In these experiments, DNA recovery did not appear to be improved by manipulation of elution time, the length of incubation, binding time, or elution volume. However, Prinz and colleagues (Prinz *et al.* 2002) demonstrated that that DNA recovery could be increased by tripling the amount of magnetic beads used in the protocol. An increase in the amount of beads employed was also demonstrated to improve recovery in the presence of bacterial contamination (Prinz *et al.* 2002).

The solid-phase extraction systems explored in this research could also be applied as an alternative to current DNA purification systems (e.g. gel purification), such as in the removal of contaminants that are known to prevent PCR amplification. In addition, further research on the DNA IQ™ and ChargeSwitch® protocols could be pursued on sample matrices other than the blood and genomic standard matrices tested here.

Lastly, the non-radioactive caesium-133 served as a useful surrogate for radioactive caesium-137, with the advantage of being able to test both in this research. Further

studies should include assessment of the suitability of these methods for other radioactive contaminants such as americium oxide (or, for safety purposes, non-radioactive representatives). In addition, as many radioactive materials are manufactured as metals and vitrified ceramics, investigation of these varieties and their impact on extraction procedures would be of interest.

6.5 Analysis of blood samples contaminated with caesium-137

The DNA IQ™ and ChargeSwitch® systems were demonstrated in the research described in Chapter 4 as being effective for the removal and purification of DNA from contaminating caesium-133. From this, the success of these applications for DNA profiling following a true radiological contamination was hypothesised.

This experimental series was designed to confirm this hypothesis through the analysis of samples contaminated with caesium-137. It was designed, in effect, to implement insights gained through the culmination of the research described in Chapters 2 through 4, and provide a novel demonstration that approximates a real-world contamination scenario. In addition, to achieving a true representation of effects, safe working practices were further explored based on the procedures employed in the experiments. Considerations included issues related to sample collection and storage, proposed guidelines for maximum dose, estimation of the time, distance and shielding required for safe handling, and identification of potential for contamination of the laboratory itself.

Therefore, the objectives of this experimental series were to identify issues for the management of radiologically-contaminated biological samples within a forensic laboratory (and to propose related safety protocols), conduct DNA extraction of blood contaminated with caesium-137 under the proposed safety protocols (including the quantification of decontamination and DNA extraction efficiency), and propose future directions for forensic institutions that may be planning to implement the analysis of radioactive samples.

The results of these experiments demonstrated that both the DNA IQ™ and ChargeSwitch® systems were able to effectively remove contamination by radioactive caesium-137 at the levels tested (6.6×10^3 Bq). The activity (in Bq) was reduced to levels approaching zero (<0.24 to 2.2 Bq), and dose rates derived from these activities proved to be negligible ($<6.6 \times 10^{-7}$ μSv/h). As discussed in Chapter 4, this efficiency data can be used to provide guidance when planning for the volume of samples processed and the maximum exposure time per laboratory analyst.

It was also demonstrated that the DNA IQ™ and ChargeSwitch® systems exhibited comparable overall DNA yield as a percentage of control values (99.0% for DNA IQ™; 96.7% for ChargeSwitch®). ChargeSwitch® also demonstrated more favourable repeatability and a maximum extraction efficiency of 100 ng DNA, an extraction capacity more than 2-fold higher than that of DNA IQ™. Despite any discrepancies between the DNA IQ™ and ChargeSwitch® extraction systems, it was concluded that the presence of caesium-137 did not affect the capability of either protocol to obtain a sample of DNA suitable for profiling. From the DNA profiles generated, suitable peak heights were obtained in both systems for all loci, at levels equivalent to those obtained for the DNA controls. The DNA IQ™ and ChargeSwitch® systems were therefore both proven to be practical for the efficient extraction of DNA from blood in the presence of a radioactive caesium-137 contaminant and as a decontamination process for subsequent downstream processing.

In examining safe work practices, it was important that all handling, transport and storage of radioactive materials comply with State and Federal regulations (e.g. Australian Radiation Protection and Nuclear Safety Regulations 1999). In this research, an operational procedure was devised that was appropriate and sufficient in protecting against exposure to and contamination by caesium-137 at the levels studied.

In a laboratory response to a radiological incident, the location and time of analysis should be carefully planned to avoid subjecting uninvolved staff to unnecessary exposure or contamination. All testing in the laboratory should be conducted within a

hood or alternative closed containment systems designated to handle appropriate levels of radioactivity. Shielding should be utilised (e.g. lead bricks), and Personal Protective Equipment (PPE) should consist of chemical-resistant gloves, protective glasses (and/or face shield), and a laboratory coat dedicated for use in a radiation laboratory (note: procedures should be in place to respond to accidental contamination of the skin, eye or other areas). Analyst time, distance and shielding should be controlled to reduce total dose, and sample activity should be monitored over the course of the analyses, including the use of portable Geiger-Müller counters and personal real-time dosimeters, where available.

In addition, care should be taken to avoid contamination of the working environment, and the handling of considerable amounts of analytical waste should be coordinated with agencies certified to remove and dispose of radioactive materials. Decontamination and spill procedures must also be established in the laboratory.

With the favourable results from these preliminary studies with caesium-137, this research has investigated several issues in dealing with radioactive contaminants in blood, including handling protocols and characterising the removal of the contaminant and potential effects on downstream DNA profiling. From these findings, several additional aspects were identified that merit further study. These include procedural components (e.g. other analytical strategies, contaminants, and alternative matrices), and even considerations for related policy formulation, particularly in guiding searching and collection techniques used to acquire forensic specimens for analysis.

Thorough screening of each evidential item is necessary for documentation and sampling of biological evidence for forensic analysis, which may require varying lengths of time and proximity to the contaminated material. This exposure must be considered when performing the examination, in order to minimise the dose received by the analyst to within permissible levels. Institutions must decide on practicality whether procedural policy dictates that individual analysts be allocated separate duties with a defined maximum radiation exposure (e.g. individual tasks related to searching,

extraction, and profiling) or, alternatively, if it is more beneficial for a single analyst to be charged with all stages of the analysis (thus limiting exposure to one analyst). In addition, examination of the impact that contaminating radioactive materials may have on presumptive and confirmatory screening procedures employed in forensic DNA analysis would be beneficial. This would allow a greater understanding of the overall impact that radioactive materials may have on current operating procedures and the subsequent results and conclusions from these analyses. In furtherance to this, other forensic disciplines, such as fingerprint, document examination and fibre analysis, should investigate possible implications of contamination with radioactive materials and subsequent analyst exposure. ANSTO is currently undertaking valuable research into the impact of radiation on forensic evidence relevant to various forensic disciplines.

From an analytical perspective, the experiments detailed in Chapter 5 were based on the parameter of radiation activity. As ICP-MS instrumentation was used to characterise caesium-133 content in contaminated samples (Chapter 4), future studies could utilise the available instrumentation to determine a clear association between caesium-137 concentration and radiation activity. In addition, levels of activity should be investigated that are in excess of that targeted for this research (6.6×10^3 Bq). Given the demonstrated efficiency of the extraction methods, it is reasonable to believe that these systems are capable of removing a higher amount of contaminant. This would, however, depend on the correlation of concentration with activity, and would need to be restricted to practical levels that do not compromise the safety of the analyst.

It was also observed that ChargeSwitch® provided better overall DNA yield and repeatability compared to the DNA IQ™ system. In addition, the ChargeSwitch® system achieved its nominated potential for a maximum extraction efficiency of 100 ng DNA (1 ng/µL in 100 µL). This is similarly stated for the DNA IQ™ system; however, the demonstrated performance was less than half of this capacity for this system. Therefore, further adjustments should be explored to improve on this efficiency, utilising blood and other matrices. Further studies could include assessment of the suitability of these methods for other radioactive contaminants such as strontium, iodine,

iridium, americium and uranium (and oxides), and the investigation of a negatively charged ion such as iodine would further establish if there are contaminants that can interfere with the DNA binding for either method. Such efforts may be investigated using non-radioactive forms of each contaminant to reduce analyst exposure to radioactive materials. This is a notion supported by the findings of this study, demonstrating that the extraction behaviour of caesium-133 is appropriately representative of that of radioactive caesium-137.

Lastly, as this study focused on the analysis of blood, further validation studies on radioactive contamination should be carried out with other common matrices using these (and other) extraction systems. Amenable matrices may include saliva, tissue homogenates (such as bone, muscle and other tissues), and other matrices that will not affect the binding capacity of the beads and/or efficiency of the DNA extraction process. Care must be taken with other matrices, as critical pre-treatment of samples prior to extraction may be required to maximise DNA recovery.

6.6 Searching and collection of biological evidence contaminated with radioactive materials – considerations and issues

Chapter 5 addressed a number of core issues relating to operating procedures when purifying and extracting DNA from blood contaminated with caesium-137 nitrate solution. However, further issues relating to searching, collection and analytical techniques for biological evidence contaminated with radioactive materials need to be addressed, within the context of forensic DNA analysis.

6.6.1 Prior to evidence collection

Before handling or analysis commences, radiation type and dose rate must be established to provide safety information to the analyst, to evaluate the potential impact on the evidence, and to record in official examination documents (case files). For example, dose rates exceeding safe working levels will preclude analysis from taking

place, in addition to identifying whether samples have received an excessive radiation exposure that may compromise evidential value. From Chapters 2 and 3, threshold values for gamma and alpha radiation have been identified above which significant effects are observed on the profiling success of a DNA sample. These values can be utilised for guidance purposes. However, this research has also demonstrated that the DNA molecule is extremely robust for both radiation types and can sustain a significant dose, well in excess of safe levels of exposure for humans prior to damage occurring.

6.6.2 Multi-disciplinary approach

During larger investigations, it is often the case that multiple disciplines will examine an item concurrently, not only to process the evidence quickly, but also to ensure that evidence of interest to one discipline is not compromised by the examinations conducted by another. There are several advantages to a multi-disciplinary approach when processing evidence contaminated with radioactive materials, including limiting the number of individuals affected, reducing the burden on each analyst to return to the exhibit and collect samples individually, and limiting the handling of the evidence to a single occurrence, which is especially important if a glove box is required each time the item is removed for examination.

In addition, while the impact of time-to-analysis in this research did not clearly correlate with observed degradative effects, concurrent collection and examination of the exhibit will reduce the length of time until analysis.

6.6.3 Examination area

To limit the potential spread of the material into the work environment, the examination of a contaminated exhibit can be conducted in a radiation glove-box. In addition, use of a bench protector with lead acrylic shielding would allow for visualisation of the sample and protection from low level gamma emitting sources. A recent presentation given at the 19th ANZFSS International Forensic Science Symposium discussed the use of a

collapsible and disposable glove-box, which would maintain the integrity of the evidence by preventing cross-contamination from other biological materials as well as contain the spread of radioactive material during examination (Garrett 2008).

6.6.4 Sampling procedures

As discussed in Chapter 1, forensic biologists typically utilise five types of sample collection procedures: hand picking, cutting, tape lifting, swabbing, and scraping. Certain procedures, particularly tape lifting and swabbing, present an increased likelihood for the accumulation or concentration of radioactive materials into a single sample. Each sample should be measured for dose, prior to extraction and purification, to determine if the radioactive material has concentrated in the sample, and apply these measurements to determine the total number of samples that can be processed safely.

6.6.5 Examination time

The time-frame for an individual to examine an exhibit can range from around 15 minutes for items such as a swab or a cigarette butt, to 2 or 3 days for larger, more complicated items such as clothing or bed sheets. To reduce the time taken for examination, and to provide a safer working environment, a team of analysts, including a scribe/director of examination (to document and direct the examination), a person to search (to conduct the examination), and a photographer (to photographically document the examination) could be utilised. In addition, the following safety measures could also be implemented to provide a safer working environment: the presence of a trained radiation worker to provide relevant information to the analysts during examination; specific and mandatory radiation awareness training for analysts undertaking the searching of exhibits contaminated with radioactive materials; and, participation in mock scenarios to test the relevant operating procedures and protocols.

6.6.6 Storage of evidence

Consideration must also be given to the storage and future analysis of contaminated evidence. Exhibits are typically stored with exhibit management personnel before and after examination and until court proceedings are complete. In addition, exhibits are occasionally presented and/or tabled in the court room. In the case of exhibits contaminated with radioactive materials, the cumulative dose rate of these items must be monitored so that it does not exceed safe working conditions for relevant personnel and cause continued radiation damage to itself and other exhibits. This may be achieved by storing the items in specialised radiation shielding containers. Alternatively, all exhibits of this nature may need to be analysed in one phase and then destroyed.

In addition, DNA extracts of samples are typically stored indefinitely for repeat or future analysis. While radiation exposure from contaminating materials is reduced by extraction with DNA IQ™ or ChargeSwitch® to levels well below background, these samples may still require separate storage. This may be necessary as fears surrounding radiation, even at these levels, can cause concern for some individuals.

6.7 Final comments

It is believed that this research is innovative and has contributed to an improved understanding of the effects of ionising radiation on forensic DNA evidence. The findings presented in this thesis have revealed the promise of select extraction systems in meeting the practical needs of forensic laboratories faced with sample analysis for a radiological incident, in addition to providing valuable insights into associated operational procedures. The importance of both analyst safety and evidentiary integrity has remained paramount throughout these efforts, at no sacrifice of either.

This study also demonstrated that a dedicated forensic laboratory can process radiologically-contaminated samples provided the radiation levels have been sufficiently reduced. If the sample activities prior to extraction exceeded legislated levels, analysis

in these laboratories would not be possible or appropriate. Therefore, a separate facility that can handle these contaminated samples or a purpose-built laboratory should be considered.

This work has endeavoured to contribute stimulating new information to the fields of forensic biology and radiation science. In the event of a radiological incident, the forensic community can turn to this research as a valuable model for analytical response with respect to DNA evidence recovery.

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Appendix

Table A1: Average peak heights and standard deviation for genomic DNA at 1-day post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1177	172	1267	55	1742	120	1552	41	1345	229	762	203	684	71	658	71	369	27	642	60
50	1113	400	1376	514	1464	199	1292	190	1315	435	748	268	794	266	794	401	370	155	766	326
100	973	23	1431	237	1289	161	1125	286	1274	172	804	104	665	168	817	60	431	99	705	75
500	1206	513	1594	823	1593	412	1376	572	1772	608	828	468	902	393	813	473	396	250	778	413
1000	859	485	1010	877	1200	482	874	823	1058	530	514	452	510	442	507	439	253	224	534	508
5000	687	268	776	187	871	139	704	197	834	139	367	70	399	80	321	79	158	21	303	72
10000	605	251	703	315	1012	308	906	153	676	126	340	113	326	85	231	38	131	14	238	37
50000	498	71	633	187	444	45	377	82	371	46	217	93	134	44	124	20	56	55	88	28
100000	274	83	289	18	226	72	170	91	132	50	17	30	40	35	0	0	0	0	0	0

Table A2: Average peak heights and standard deviation for genomic DNA at 1-week post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1706	491	2316	730	2422	792	2565	865	2614	932	1659	795	1496	387	1474	601	885	440	1462	643
50	2060	243	2958	262	2345	48	2353	76	2949	443	1916	36	1788	82	1870	294	946	214	1555	229
100	1425	714	1892	1162	1873	117	1490	468	1682	652	1250	801	1057	694	1191	863	649	515	1094	730
500	1340	120	1560	343	2287	166	2260	199	2290	241	1064	160	1076	217	906	251	491	37	1066	73
1000	2057	1030	2640	1276	2177	1103	2246	1140	2552	1130	1619	959	1531	822	1584	968	867	537	1553	824
5000	1152	295	1333	554	1644	500	1018	181	1346	143	587	171	538	225	553	293	196	122	435	187
10000	732	294	807	401	979	547	525	280	689	314	206	187	254	153	180	194	60	57	163	146
50000	537	152	539	167	516	183	406	120	282	53	105	116	121	77	48	83	18	31	0	0
100000	190	29	201	53	148	76	43	38	109	43	0	0	22	38	0	0	0	0	0	0

Table A3: Average peak heights and standard deviation for genomic DNA at 4-weeks post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	862	174	1154	275	1019	366	1262	356	958	202	647	93	611	229	652	198	407	56	616	139
50	819	93	1041	310	1153	308	1036	382	991	119	605	176	529	121	656	281	340	194	527	189
100	1001	196	1319	244	1081	315	1421	779	1097	209	770	289	713	277	838	259	433	215	718	139
500	776	288	893	278	757	373	839	553	705	420	471	291	431	154	513	274	297	128	444	223
1000	745	218	900	499	1058	39	995	84	870	415	436	234	452	189	487	358	229	235	443	224
5000	912	529	1149	839	823	467	899	218	794	420	485	285	448	247	479	260	202	95	456	322
10000	531	136	615	115	382	38	387	102	388	43	239	66	220	82	274	20	107	36	213	73
50000	207	100	186	65	224	16	183	46	118	59	53	52	0	0	0	0	0	0	0	0
100000	125	110	123	64	123	63	80	90	24	42	0	0	0	0	0	0	0	0	0	0

Table A4: Average peak heights and standard deviation for blood DNA at 1-day post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	2145	404	2187	651	2030	252	2037	403	2459	539	1971	632	1589	406	1965	623	1182	309	1637	517
50	1803	555	1951	484	1471	253	1613	261	1906	447	1437	206	1387	326	1553	328	1000	177	1507	318
100	1831	87	1931	232	1856	17	1643	146	2201	182	1819	231	1497	186	1630	81	1161	125	1537	50
500	2360	345	2508	324	2129	488	2157	331	2383	502	1892	123	1696	203	2003	264	1212	121	1651	271
1000	1867	561	1775	531	1781	404	1689	413	2143	637	1595	502	1369	459	1443	493	1057	406	1470	402
5000	1640	316	1752	323	1396	257	1399	323	1912	475	1494	364	1348	269	1546	321	1069	268	1371	257
10000	1986	668	1872	882	1707	532	1770	511	2250	821	1799	678	1476	354	1550	712	1106	249	1265	620
50000	1430	482	1669	652	1175	397	978	269	1455	425	926	352	781	146	758	300	429	143	488	165
100000	1025	101	877	47	696	102	575	104	707	90	427	42	374	41	295	62	150	16	187	11

Table A5: Average peak heights and standard deviation for blood DNA at 1-week post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1658	389	1726	266	1450	326	1215	295	1903	303	1191	240	1196	124	1268	66	703	102	1150	51
50	1514	227	1744	406	1338	287	1247	148	1773	462	1098	310	1025	220	1222	361	717	209	1155	354
100	1768	21	1781	250	1428	168	1333	162	1751	235	1255	40	1170	11	1525	24	783	66	1271	108
500	1418	175	1494	474	1377	425	1208	420	1798	597	1080	444	1125	591	1238	435	693	316	1285	641
1000	1243	274	1189	280	1181	276	933	259	1526	336	888	177	830	233	941	341	592	114	1015	316
5000	1368	136	1472	318	1168	135	1223	178	1579	299	1019	116	1094	272	1148	134	676	72	1049	207
10000	1134	221	1118	333	923	173	867	173	1219	230	761	207	799	137	891	225	497	109	792	121
50000	969	18	861	33	746	124	551	37	899	170	392	115	401	52	402	63	200	30	262	13
100000	505	119	459	162	266	71	115	53	242	104	17	30	20	35	60	60	0	0	0	0

Table A6: Average peak heights and standard deviation for blood DNA at 4-weeks post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	2276	740	2173	976	1552	623	1564	506	2058	721	1562	498	1416	313	1766	688	969	150	1545	472
50	1591	38	1877	25	1361	228	1294	104	1614	72	1041	329	932	66	1349	74	712	115	1103	163
100	1796	73	1687	224	1519	79	1455	15	1797	218	1457	320	1265	28	1484	185	941	84	1432	169
500	1866	511	1882	333	1544	308	1415	463	1814	498	1234	309	1214	256	1470	325	903	301	1334	296
1000	1575	237	1900	361	1381	307	1262	155	1738	339	1143	200	1107	236	1337	212	750	73	1173	124
5000	1663	239	1595	180	1344	213	1287	261	1537	262	1150	150	980	215	1113	254	676	125	1010	121
10000	1749	278	1854	236	1284	213	1197	257	1704	393	1086	205	1009	192	1323	156	687	165	1061	236
50000	1261	290	1235	548	771	300	552	113	874	160	431	98	432	163	503	150	234	24	332	49
100000	545	105	461	36	302	78	199	28	314	120	43	74	100	51	102	36	0	0	0	0

Table A7: Average peak heights and standard deviation for saliva DNA at 1-day post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1751	305	1894	419	1034	175	735	140	966	22	445	119	549	137	772	215	286	49	517	207
50	1507	492	1411	613	883	304	685	160	770	359	431	215	437	189	604	260	206	93	380	137
100	1633	198	1721	127	1036	333	635	117	987	310	530	268	440	148	784	269	273	124	490	126
500	1424	212	1695	121	942	196	711	216	940	146	501	179	488	151	736	127	233	59	525	182
1000	1312	303	1487	312	779	249	610	255	803	313	417	146	343	102	656	181	212	95	428	81
5000	1588	162	1829	245	1124	188	812	105	925	154	521	106	531	28	747	123	240	5	508	119
10000	2026	846	2285	1280	1224	482	798	355	1364	917	624	452	587	363	1030	621	279	163	574	339
50000	1116	458	788	682	566	32	302	270	384	124	128	123	115	102	271	237	18	31	42	73
100000	974	125	1062	135	464	87	316	104	310	105	135	24	79	3	140	7	0	0	0	0

Table A8: Average peak heights and standard deviation for saliva DNA at 1-week post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1687	474	1735	454	954	328	694	322	890	213	534	301	467	195	837	381	281	169	538	204
50	1007	216	1215	134	627	40	402	41	541	41	252	59	269	28	443	69	136	26	225	68
100	1528	168	1688	270	901	201	718	139	865	178	334	178	392	155	635	137	189	27	459	92
500	1213	212	1394	556	764	252	514	105	682	214	387	151	364	79	575	107	206	44	362	90
1000	1436	370	1706	522	872	221	686	355	884	422	372	106	429	174	648	256	213	95	421	162
5000	1853	232	2161	183	1207	178	822	158	1210	164	516	119	508	83	853	25	268	76	526	78
10000	1369	247	1567	255	778	219	560	227	752	247	306	148	349	150	509	224	114	103	329	89
50000	877	273	826	238	673	532	369	199	347	133	132	28	136	19	176	42	0	0	0	0
100000	504	110	492	95	209	12	123	60	139	39	0	0	0	0	70	24	0	0	0	0

Table A9: Average peak heights and standard deviation for saliva DNA at 4-weeks post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1453	267	1607	95	704	79	530	109	776	197	311	68	498	133	807	194	246	123	533	140
50	1304	83	1445	359	988	186	698	216	670	109	329	54	365	70	493	85	159	14	344	92
100	1344	235	1316	318	636	134	492	112	583	132	217	48	307	92	508	103	168	14	312	82
500	1563	447	1819	672	957	388	650	178	855	299	349	165	432	159	694	313	192	60	531	289
1000	1582	497	1747	700	890	343	636	128	898	514	399	144	427	174	800	376	205	59	532	246
5000	1411	460	1537	523	909	244	555	223	786	315	420	140	393	85	627	206	158	24	412	189
10000	1489	162	1609	167	863	140	585	106	712	14	308	30	304	33	503	69	165	32	284	62
50000	1389	185	1402	110	595	15	373	44	523	52	187	46	160	29	296	18	0	0	139	9
100000	949	98	871	140	319	15	238	48	210	78	42	73	27	47	116	5	0	0	0	0

Table A10: Average peak heights and standard deviation for bone DNA at 1-day post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	3586	257	4235	260	3784	1136	4423	554	5252	489	4229	173	4750	301	4110	450	3093	554	4737	1099
50	3685	107	5551	155	3611	317	4450	366	5321	1817	4400	1824	4127	1267	3832	1765	2430	745	3860	2040
100	3515	514	4983	667	3001	817	3494	333	4687	1153	3334	898	3158	952	2774	952	1893	488	2918	895
500	4018	200	5028	346	3704	268	4244	1148	5336	974	4695	1398	4833	1370	4275	1145	3101	942	4136	836
1000	4899	673	6749	1187	5119	492	5304	155	6236	77	5366	131	4824	933	4933	77	3096	482	4570	734
5000	3560	198	4293	2	3772	308	4031	90	4425	6	3710	594	3401	127	2999	113	1893	263	2561	325
10000	3896	229	5909	214	3405	330	3109	348	3792	583	2774	1063	2566	960	2466	118	1416	720	2148	567
50000	2590	664	2760	576	1799	450	1477	284	1214	316	687	222	507	196	354	61	192	78	224	73
100000	1853	54	1332	34	814	145	569	81	309	98	139	55	160	8	0	0	0	0	0	0

Table A11: Average peak heights and standard deviation for bone DNA at 1-week post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	4595	179	6584	195	4456	15	4520	350	5743	66	4343	1088	3858	199	4553	1068	2730	166	4191	453
50	3984	1057	5922	496	3612	912	4230	286	4505	421	3516	72	3459	283	3395	287	2249	88	2927	100
100	4562	630	6672	836	4577	50	4082	587	5110	239	3596	819	3010	317	3390	332	2021	223	3016	443
500	4407	99	6661	724	4535	173	5054	185	4580	264	3677	156	3500	17	3454	376	2309	194	2744	138
1000	4965	246	7410	1019	4931	737	5145	598	5838	1113	4385	346	4280	378	4058	294	2531	234	3785	232
5000	5111	703	7393	1454	4219	614	4646	149	5515	435	4076	537	3596	469	3282	557	1890	111	2378	122
10000	2909	188	3997	1011	2480	776	2645	240	2717	211	1913	268	1767	197	1824	535	998	16	1344	200
50000	1760	175	2024	248	1056	286	874	175	497	621	428	141	317	2	223	45	64	91	60	85
100000	1193	272	1155	143	555	28	333	46	327	11	124	8	0	0	69	98	0	0	0	0

Table A12: Average peak heights and standard deviation for bone DNA at 4-weeks post-irradiation with gamma rays

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	3859	235	5472	372	4031	571	4639	545	5044	18	3819	144	4019	334	3844	12	2204	52	3260	101
50	2672	847	4460	1540	2242	385	2692	776	3258	841	2389	735	2244	890	2270	719	1401	259	2132	806
100	3623	670	5837	200	3469	735	3907	673	4372	670	3575	605	3212	926	3353	501	2126	329	2732	490
500	2361	646	3778	1186	2074	371	2965	677	3075	767	2217	580	2403	450	2472	825	1268	316	1794	521
1000	2769	196	4285	676	2939	178	2953	28	3540	701	2615	245	2311	498	2356	482	1396	18	2214	134
5000	2369	463	3679	795	2086	339	2218	537	2393	248	1651	86	1792	210	1710	192	996	192	1329	359
10000	2425	192	3817	104	2079	131	2064	235	2496	12	1771	111	1517	319	1521	211	726	1	1024	39
50000	2184	494	2701	849	1466	385	1255	272	965	316	624	335	494	233	325	115	171	71	219	126
100000	1278	247	1117	216	644	8	370	66	353	160	176	6	52	74	50	71	0	0	0	0

Table A13: Average peak heights and standard deviation for HEp-2 cells at 1-day post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	2362	433	2140	169	2134	316	2662	512	2246	307	1725	233	1584	375	1881	309	1324	165	2060	303
66000	2147	151	1874	255	1696	171	1936	215	1572	203	1141	102	808	54	1018	75	628	56	862	42
220000	1181	707	961	728	1092	396	847	593	652	400	382	336	243	219	337	315	143	128	162	155
440000	1201	229	726	106	647	39	496	85	359	107	213	18	90	103	128	41	0	0	56	53
1320000	638	19	274	30	287	39	218	22	182	19	112	26	85	44	106	30	20	34	137	23
3960000	206	39	229	57	196	61	225	42	166	47	130	53	72	64	122	58	37	65	134	18
13200000	262	68	224	119	277	131	282	105	204	75	168	60	142	73	182	105	137	105	190	54
26400000	33	57	20	34	28	48	37	64	0	0	0	0	0	0	25	43	0	0	23	40

Table A14: Average peak heights and standard deviation for HEp-2 cells at 1-week post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	2602	239	2473	282	2763	425	2946	648	2466	251	2189	379	1728	298	2070	500	1471	229	2289	495
66000	1981	339	1861	171	1729	202	1812	321	1549	210	1147	177	793	164	1036	190	621	125	971	169
220000	1729	92	1396	84	1332	231	1286	137	941	51	657	80	419	17	571	11	312	23	353	65
440000	1496	284	1114	257	974	176	828	119	567	105	376	94	205	39	261	38	79	31	129	36
1320000	865	94	402	24	347	33	225	41	125	45	29	50	0	0	23	40	0	0	19	33
3960000	102	76	33	58	54	93	44	77	20	35	18	32	0	0	24	42	0	0	25	43
13200000	38	34	0	0	18	31	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26400000	23	39	0	0	21	36	0	0	0	0	0	0	0	0	0	0	0	0	22	39

Table A15: Average peak heights and standard deviation for HEp-2 cells at 4-weeks post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	2169	204	2203	363	1961	118	2348	287	2182	203	1607	66	1396	151	1773	145	1190	47	1729	37
66000	1610	420	1501	408	1272	361	1472	329	1292	297	908	219	683	137	926	263	495	171	787	220
220000	1475	201	1295	191	1173	113	1252	37	944	186	605	82	405	86	547	121	307	34	379	52
440000	939	115	699	16	620	46	572	65	462	60	261	25	137	24	202	19	60	10	89	17
1320000	1123	902	498	387	438	353	388	360	234	229	191	199	66	114	86	148	53	91	124	86
3960000	217	84	188	99	224	107	216	108	173	101	178	41	79	80	149	65	18	32	141	43
13200000	75	71	53	92	30	51	83	72	48	83	35	30	0	0	55	48	0	0	58	53
26400000	41	36	0	0	0	0	19	32	0	0	0	0	0	0	0	0	0	0	34	30

Table A16: Average peak heights and standard deviation for blood (group 1) at 1-day post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1411	166	1560	312	1367	254	1535	71	1531	308	1122	123	1021	27	900	163	723	201	904	144
66000	998	345	961	429	829	159	774	215	889	283	519	90	590	167	446	86	338	134	342	151
220000	970	488	1017	335	712	298	621	201	645	296	303	122	376	159	267	176	107	100	133	133
440000	344	135	322	133	216	95	132	87	147	68	62	6	27	46	18	32	25	43	25	44
1320000	96	119	63	67	56	54	38	66	69	66	41	70	17	29	17	29	17	30	43	75
3960000	175	89	191	100	193	113	167	31	202	118	157	138	158	139	157	108	86	79	121	107
13200000	170	40	171	19	100	59	99	90	153	30	108	65	91	27	67	20	18	32	61	55
26400000	169	98	127	69	112	97	83	144	157	65	57	99	85	101	101	40	30	51	55	95

Table A17: Average peak heights and standard deviation for blood (group 1) at 1-week post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1406	455	1702	803	1211	381	1174	168	1485	534	1139	409	1067	503	1165	627	809	414	1190	670
66000	1115	152	1033	337	934	67	872	73	946	55	614	64	649	85	567	64	339	75	423	49
220000	900	245	888	335	667	247	606	211	671	205	366	122	337	115	359	135	209	122	304	157
440000	753	226	715	246	581	141	555	175	628	287	363	130	328	213	306	85	117	99	205	119
1320000	374	125	306	154	239	46	198	47	215	117	105	141	109	53	93	34	69	16	25	44
3960000	142	134	129	76	76	84	60	2	105	113	93	91	97	84	67	74	64	71	62	67
13200000	138	60	213	148	152	40	180	70	168	43	91	108	107	93	96	75	78	92	83	29
26400000	39	34	90	32	0	0	35	61	31	54	20	34	42	37	18	31	0	0	0	0

Table A18: Average peak heights and standard deviation for blood (group 1) at 4-weeks post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1660	263	1518	640	1367	265	1522	175	1672	587	1242	393	1273	330	1174	599	762	413	975	475
66000	1065	405	993	449	864	359	759	391	977	323	520	255	640	390	367	252	187	198	242	220
220000	1010	376	839	398	648	180	508	204	670	74	290	104	374	147	175	100	90	156	106	183
440000	686	344	530	332	436	315	240	211	627	387	145	137	186	177	78	135	0	0	0	0
1320000	17	29	36	32	18	31	0	0	19	33	0	0	0	0	0	0	0	0	0	0
3960000	22	38	52	47	28	48	0	0	61	63	0	0	0	0	0	0	0	0	0	0
13200000	17	30	74	19	59	61	17	29	47	40	0	0	0	0	0	0	0	0	0	0
26400000	120	107	92	81	85	73	20	35	91	79	0	0	38	66	0	0	0	0	0	0

Table A19: Average peak heights and standard deviation for blood (group 2) at 1-day post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1170	411	1138	337	1073	441	1243	405	911	374	1268	449	783	416	1180	363	1158	292	1086	393
66000	966	251	852	427	801	240	866	171	863	227	799	227	711	150	783	127	452	95	612	126
220000	983	172	900	107	851	69	779	82	705	33	601	35	471	59	556	21	301	37	357	50
440000	814	191	606	203	582	123	471	133	405	86	414	96	282	33	348	40	195	49	218	34
1320000	954	153	859	147	994	180	848	72	807	183	876	108	699	144	722	149	511	125	626	139
3960000	741	356	743	287	737	317	791	297	731	423	755	359	640	263	690	394	410	192	570	329
13200000	962	500	882	372	1027	499	955	524	923	522	921	494	763	427	835	526	521	331	688	449
26400000	481	140	447	118	478	129	463	81	428	88	406	109	331	70	359	91	216	85	300	54

Table A20: Average peak heights and standard deviation for blood (group 2) at 1-week post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1821	340	1623	405	1809	354	1816	247	1707	335	1813	175	1664	212	1920	141	1152	113	1663	182
66000	1453	46	1273	170	1177	85	1282	86	1248	88	1117	49	1000	37	996	49	583	6	791	59
220000	1398	174	1270	424	1080	212	1103	66	874	278	735	157	600	57	659	55	335	41	452	67
440000	1103	83	901	154	805	120	575	79	573	126	452	97	404	37	401	1	209	5	268	16
1320000	573	129	472	129	508	100	409	1	378	148	364	66	354	53	315	68	241	78	300	23
3960000	476	63	502	70	505	44	441	81	431	74	492	48	388	25	445	63	270	25	334	15
13200000	729	268	692	530	641	220	619	235	620	455	579	210	489	190	565	205	373	101	442	138
26400000	423	88	426	149	438	35	454	49	395	112	366	62	317	62	301	49	185	53	287	60

Table A21: Average peak heights and standard deviation for blood (group 2) at 4-weeks post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1325	359	1223	376	1149	368	1423	461	1333	400	1510	381	1345	370	1543	451	942	384	1274	480
66000	1324	245	1233	226	1058	217	1189	399	1173	273	1048	200	928	207	1058	339	566	106	747	212
220000	1006	95	928	35	790	42	741	58	688	60	604	31	515	27	544	26	300	16	371	49
440000	907	98	678	66	574	71	523	95	565	20	478	26	425	38	403	121	231	67	306	93
1320000	844	223	692	85	734	113	721	48	874	287	678	160	669	136	654	88	345	85	484	96
3960000	402	117	415	72	476	161	439	192	433	122	404	112	398	146	418	124	239	115	339	108
13200000	524	133	464	147	419	100	448	95	554	140	452	169	394	140	449	115	254	67	312	76
26400000	352	253	296	181	342	235	338	252	300	214	275	181	252	151	268	156	119	112	207	92

Table A22: Average peak heights and standard deviation for saliva at 1-day post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1225	508	618	252	1044	367	1483	478	1417	448	752	303	620	304	1054	352	252	128	511	269
66000	1049	334	631	90	902	319	861	270	846	157	413	103	179	87	468	101	56	57	148	14
220000	556	86	338	78	430	87	419	90	348	80	163	40	19	33	165	78	0	0	0	0
440000	496	63	260	19	318	11	195	14	158	7	0	0	0	0	0	0	0	0	0	0
1320000	190	53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3960000	27	47	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13200000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26400000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table A23: Average peak heights and standard deviation for saliva at 1-week post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1116	486	758	306	939	358	1052	588	944	549	519	420	492	671	655	718	184	234	452	667
66000	952	414	669	429	858	461	723	271	742	298	372	191	206	159	410	201	69	120	183	159
220000	691	74	428	73	523	57	430	61	368	33	163	20	30	53	149	73	0	0	36	62
440000	438	25	245	95	279	44	172	26	136	72	18	31	0	0	0	0	0	0	0	0
1320000	202	25	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3960000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13200000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26400000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Table A24: Average peak heights and standard deviation for saliva at 4-weeks post-irradiation with alpha particles

	Amelogenin		D3S1358		D8S1179		D5S818		vWA		D21S11		D13S317		FGA		D7S820		D18S51	
Dose (Gray)	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD	Peak Height (avg)	SD
Control	1058	136	690	63	965	139	1186	264	1215	204	631	123	501	97	819	145	243	43	494	104
66000	816	66	514	34	705	74	670	50	737	51	355	76	186	109	412	134	60	61	143	76
220000	677	53	461	45	531	67	463	69	413	54	206	29	44	77	170	76	0	0	40	35
440000	589	139	379	83	429	133	273	60	271	114	99	28	20	35	95	60	0	0	0	0
1320000	344	78	114	71	97	84	19	33	0	0	0	0	0	0	0	0	0	0	0	0
3960000	99	52	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
13200000	69	18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
26400000	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0