The Internal Validation and Casework Application of MiniSTR Systems

A thesis submitted in partial fulfilment of the requirements for the degree of Magister Scientiae in the Department of Biotechnology,

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Keywords

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South African Missing Persons Task Team (SAMPTT)

Argentine Forensic Anthropology Team (EAAF)

Torture

uMkhonto weSizwe (MK)

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Abstract

The Internal Validation and Casework Application of MiniSTR Systems

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The objective of the study was to conduct an internal validation on miniSTR systems and apply it to cases received from the South African Missing Persons Task Team (SAMPTT). This was prompted by the fact that miniSTR systems have been shown to out perform some of the commercial kits available in the time of the study and provide an alternative to mtDNA when analysing degraded DNA from skeletal remains and that the DNA extracted from skeletal remains received from the SAMPTT would be degraded due to the remains generally being fragmented or charred and buried for many years. The miniSTR loci chosen for validation comprised the Combined DNA Index System (CODIS) thirteen core loci and were arranged into four triplexes and one uniplex. The internal validation studies included the following: a reaction volume study, sensitivity study, cycle number study, reproducibility and precision studies, environmental and matrix studies, mixture studies and concordance studies. Miniplex 1 and 2 exhibited sensitivity down to 100pg of DNA in 10μL reactions at 38 cycles while Miniplex 3 and 4 exhibited sensitivity down to 100pg at 33 cycles. The average allele size standard deviation ranged from 0.045-0.158 bases and concordance was observed in 99% of all allele calls made. The casework received from the SAMPTT involved six bone specimens, four highly fragmented and charred 20 year old bone specimens, a 25 year old bone specimen and a 44 year old bone specimen. The miniSTR systems produced four full CODIS profiles and two partial CODIS profiles for the bone specimens. This enabled the identities of four of the six bone specimens to be resolved. To support the miniSTR results and resolve the other cases, mtDNA and Y-STR typing were utilised. The DNA analysis data augmented the non-DNA evidence supporting the hypothesis that the bone specimens were derived from ANC activists who were abducted, tortured and murdered by the South African Security Police during the period 1963-1987.

Declaration

I declare that ‘The internal validation and casework application of miniSTR systems’ is my own work, that it has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full Name: Eugene Lyle Kleyn

Signed:

Date: March 2008

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I would like to thank my research supervisors for their guidance throughout this project.

Thanks also go to family and friends who have supported me during this endeavour.

List of Abbreviations

DNA	Deoxyribonucleic Acid
STR	Short Tandem Repeat
CODIS	Combined DNA Index System
mtDNA	Mitochondrial DNA
Y-STR	Y-chromosome short tandem repeat
SNP	Single nucleotide polymorphism
SWGDAM	Scientific Working Group on DNA Analysis Methods
EDNAP	European DNA Profiling Group
ENFSI	European Network of Forensic Science Institutes
CHI	Center for Human identification
AFDIL	Armed Forces DNA Identification Lab
OAFME	Office of the Armed Forces Medical Examiner
CILHI	Central Identification Laboratory, Hawaii
ICMP	International Commission on Missing Persons
FSD	Forensic Science Department
TRC	Truth and Reconciliation Commission
SAMPTT	South African Missing Persons Task Team
EAAF	Equipo Argentino de Antropología Forense, Argentine Forensic Anthropology Team
MHL	Minimal haplotype loci
PCR	Polymerase Chain Reaction
$MgCl_2$	Magnesium Chloride
dNTPs	deoxi Nucleotide TriPhosphates
BSA	Bovine Serum Albumin
EDTA	Ethylene Diamine Tetra Acetic Di-Sodium Salt

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RFU	Relative Fluorescent Units
TEMED	N, N, N', N' Tetramethyl-EthyleneDiamine
AMPS	Ammonium Persulfate

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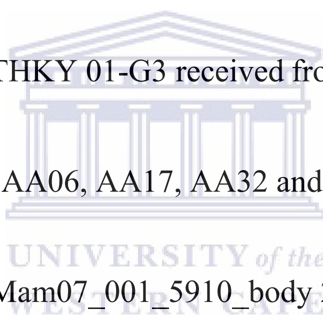
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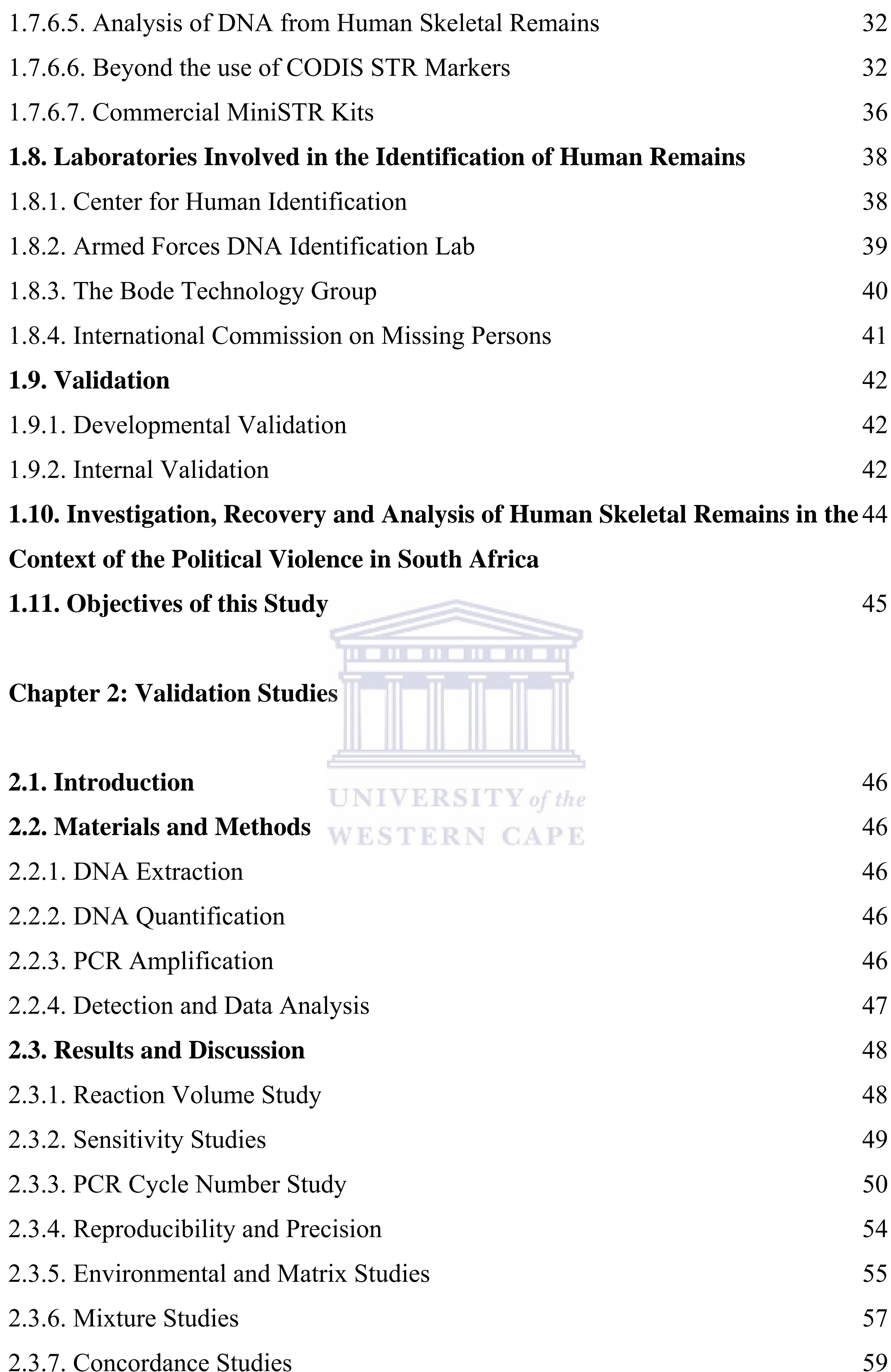

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Chapter 1: Literature Review

1.1. Introduction

The identification of highly degraded skeletal remains in mass disaster and missing person's cases can be extremely challenging. DNA obtained from these remains is often degraded to a size range of 50-200 base pairs. The use of commercial autosomal Short Tandem Repeat (STR) typing kits is not ideal as these systems generate amplicons with a size range of 100-450 base pairs (Krenke *et al*. 2002; Holt *et al*. 2002; Coble and Butler, 2005; Alonso *et al*. 2005). In situations like these mitochondrial DNA (mtDNA) analysis has traditionally been used because of its high copy number (Butler and Levin, 1998; Holland and Parsons, 1999; Coble and Butler, 2005). However, mtDNA analysis is both time-consuming and costly and its maternal inheritance and low power of discrimination make its use rather limited (Coble and Butler, 2005).

A system able to type degraded DNA with the high discrimination capacity of commercial autosomal STR typing kits would be ideal. This prompted the development of miniSTR typing systems, which generate products with reduced sizes (Butler *et al*. 2003). This was achieved by repositioning primers as close as possible to the STR target region (Yoshida *et al*. 1997; Ricci *et al*. 1999; Wiegand and Kleiber, 2001; Tsukada *et al*. 2002 and Butler *et al*. 2003). This reduction in product size allowed degraded DNA to be typed, as first observed in the analysis of skeletal remains from the Waco Branch Davidian fire (Whitaker *et al*. 1995) and subsequently in STR typing of human telogen hairs (Hellman *et al*. 2001); formalin fixed and mummified tissue (Wiegand and Kleiber, 2001); 17-26 year old bloodstains (Tsukada *et al*. 2002); human skeletal remains from the World Trade Center attack (Holland *et al*. 2003); enzymatically degraded DNA (Chung *et al*. 2004) and remains from mass graves of the Spanish Civil War (Martin *et al*. 2006). The conversion of the FBI Combined DNA Index System (CODIS) 13 STR markers to miniSTRs meant that a system had been developed which could type highly degraded DNA samples with the high discrimination capacity of commercial autosomal STR typing kits (Butler *et al*. 2003).

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This review will consider the issues and methodology involved in the analysis of skeletal remains. It will focus on mass disasters and missing persons, DNA degradation and preservation, DNA extraction methods, DNA quantification methods, DNA typing methods and validation.

1.2. Mass Disasters and Missing Persons

Missing persons definitions vary worldwide but generally refer to an individual whose whereabouts are unknown to relatives. In the United States of America alone there are one hundred thousand active missing persons cases with thousands disappearing under suspicious circumstances (Ritter, 2007). The ability to identify degraded skeletal remains is often important when addressing missing persons cases. The FBI's National Crime Information Center (NCIC) database registry consists of over 6000 unidentified skeletons and this is only 15% of the total being held in medical examiners evidence rooms across the United States (Ritter, 2007). All of these remains cannot be identified by conventional forensic methods and many labs are not capable of DNA analysis.

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Mass disasters generally fall into one of the following categories, accidental, natural or intentional (Budowle *et al*. 2005; Alonso *et al*. 2005). Accidental events include train, bus and aircraft crashes as well as ferry sinkings and fires. Intentional events include wars, the release of biological or chemical weapons, car, train and aircraft bombings (Budowle *et al*. 2005; Alonso *et al*. 2005). The war in the former Yugoslavia left behind hundreds of graves many of which were mass graves containing degraded remains. The World Trade Center attacks generated thousands of highly compromised skeletal remains (Holland *et al*. 2003).

1.3. DNA Degradation

1.3.1. Factors Leading to Degradation

DNA obtained from skeletal remains is often highly degraded. A number of factors lead to this degradation. The process is initiated by cellular nucleases and is followed by the release of the cells contents exposing it to microbial degradation (Hebsgaard *et al*. 2005). The degree to which these enzymatic processes affect DNA degradation depends on the environmental conditions. It can be slowed or stopped by freezing, rapid desiccation and high salt concentrations. However other much slower and long

term chemical factors such as hydrolysis and oxidation will continue to degrade the DNA even under ideal environmental conditions. These chemical factors result in the following types of damage; strand breaks; hydrolytic damage and oxidative damage (Paabo *et al*. 2004, Hebsgaard *et al*. 2005).

1.3.1.1. Enzymatic DNA Strand Cleavage

The process of autolysis occurs in dead or dying cells and results in the digestion and release of the cell contents due to the release of digestive enzymes from lysosomes (Paabo *et al*. 2004, Hebsgaard *et al*. 2005). This attracts bacteria and other microorganisms which initiate putrefaction. During this process endonucleases and exonucleases digest DNA into smaller fragments and sequentially remove nucleotides from strands. This results in reduction in strand size and overall DNA concentration.

Fragmentation of DNA by nucleases makes the use of commercial autosomal STR typing kits limited. This is due to the reduced length of available DNA fragments (50-200bp) while the kits amplify products with a size range of 100-450bp (Krenke *et al*. 2002; Holt *et al*. 2002; Paabo *et al*. 2004; Coble and Butler, 2005; Alonso *et al*. 2005 and Willerslev and Cooper, 2005).

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1.3.1.2. Hydrolytic Damage

The process of hydrolysis leads to deamination and the depurination and depyrimidination of bases. The products of the deamination are hypoxanthine (adenine), xanthine (guanine), uracil (cytosine) and thymine (5-methylcytosine) (Figure1-1 and 1-2). These modifications are referred to as miscoding lesions because they lead to alteration of sequences during PCR. The change from cytosine to uracil leads to the incorporation of adenine instead of guanine while the change from 5-methylcytosine to thymine results in the incorporation of adenine instead of guanine. The incorporation of these errors has been seen to occur at mtDNA polymorphic sites, which makes it even more of a concern because the errors could result in sequences that closely resemble expected evolutionary changes in humans and animals (Paabo *et al*. 2004 and Willerslev and Cooper, 2005).

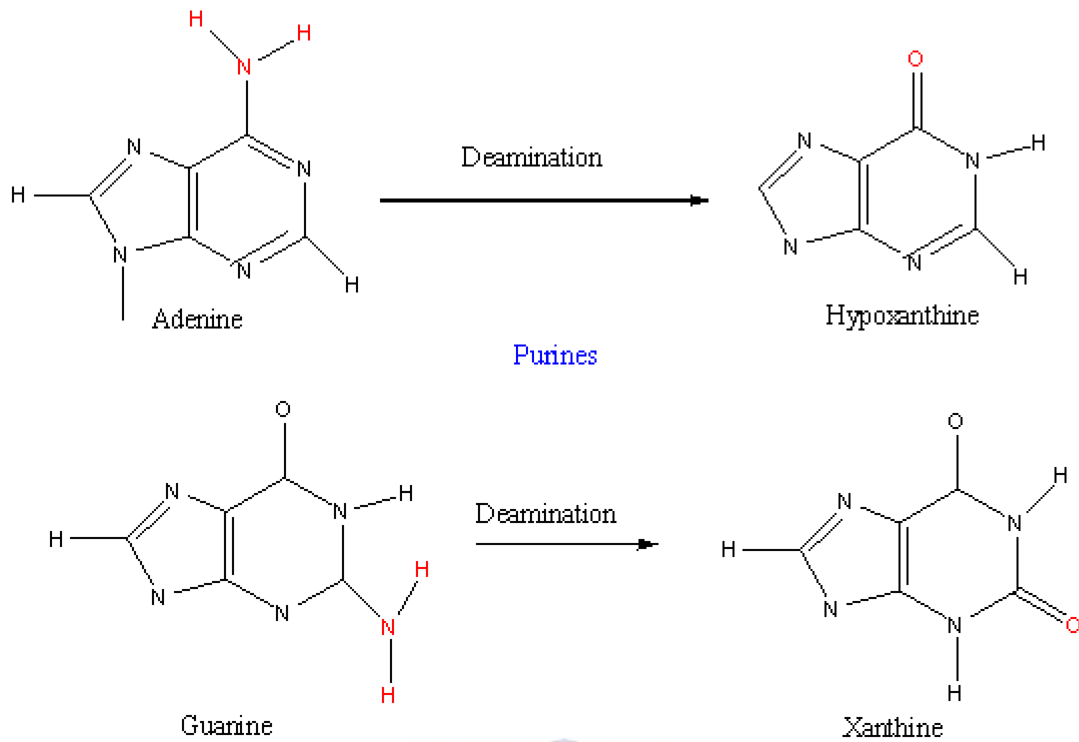

Figure1-1. Deamination of adenine and guanine. The deamination of adenine and guanine yields hypoxanthine and xanthine respectively (Adapted from www.ypatent.com/DNArepair.htm).

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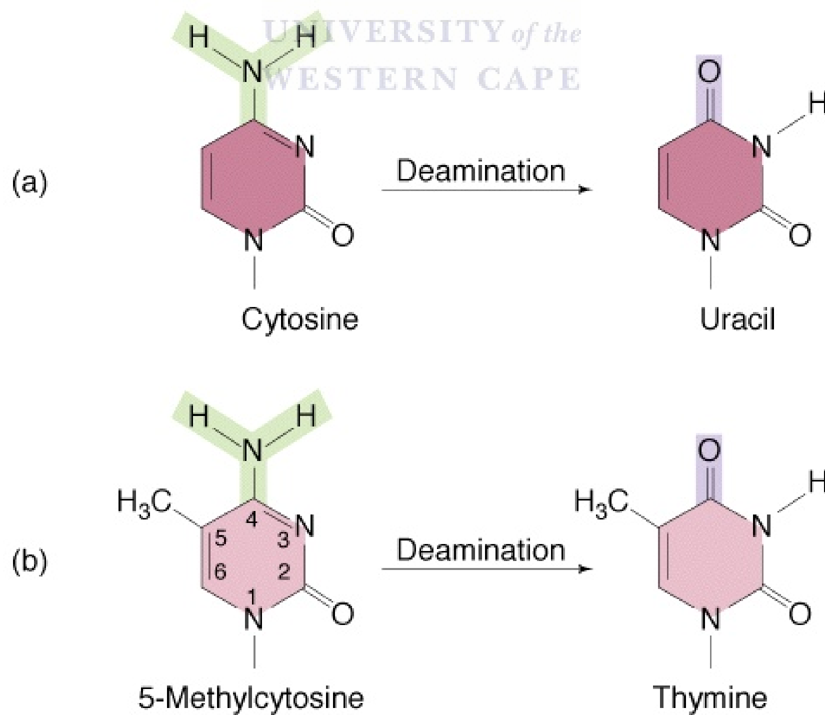

Figure1-2. Deamination of cytosine and 5-methylcytosine. The deamination of cytosine (a) and 5-methylcytosine (b) yields uracil and thymine respectively (www.bio.miami.edu/dana/250/25005_9.html).

Hydrolysis can also cause strand breaks via phosphodiester bond cleavage, depurination and depyrimidation of bases (Paabo *et al*. 2004; Hebsgaard *et al*. 2005 and Willerslev and Cooper, 2005). Direct cleavage of the phosphodiester bonds in the phosphate-sugar backbone by hydrolysis generates single stranded nicks. Depurination is the loss of purine bases (guanine and adenine) and depyrimidation is the loss of pyrimidine bases (cytosine and thymine). The hydrolytic cleavage of the glycosidic bond between the phosphate-sugar backbone and the bases result in depurination and depyrimidation (Sheppard *et al*. 2000). The loss of these bases result in apurinic or apyrimidinic (AP) sites. Figure 1-3 illustrates the steps involved in the process, hydrolytic cleavage of the glycosidic bond leads to the formation of an open chain aldehyde (compound 1), which is susceptible to β-elimination. Due to its susceptibility the 3` phosphoester bond is cleaved (compound 2). Cleavage of the 5` phosphoester bond occurs under alkaline conditions. The end result is DNA strand breaks, which causes fragmentation and reduction in overall DNA concentration.

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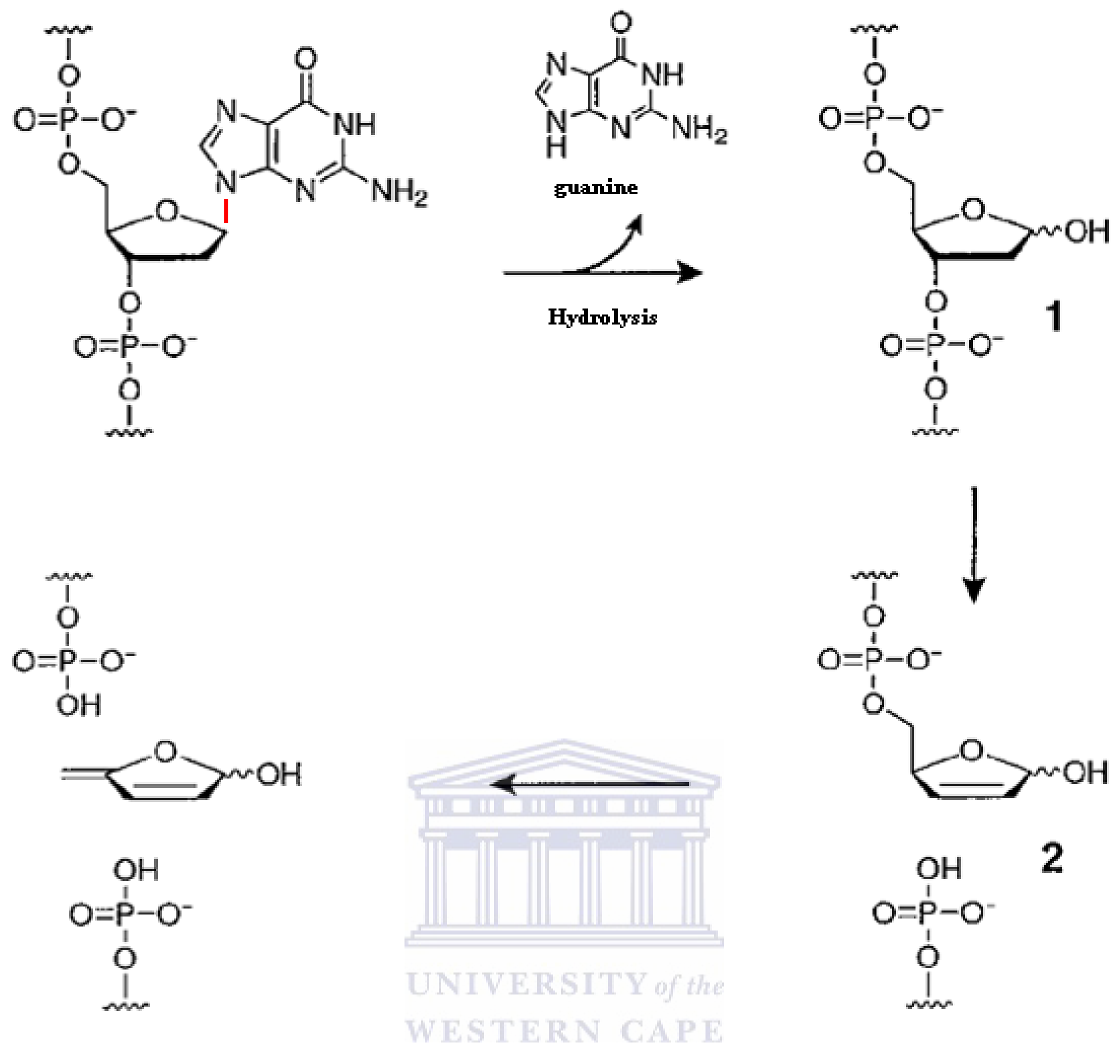

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Figure 1-3. DNA strand scission resulting from depurination and subsequent β-elimination at the apurinic (AP) site. Hydrolysis at the C1 position of the deoxyguanosine results in the release of guanine and the formation of an AP site (1). The open chain aldehyde is susceptible to β-elimination, which results in cleavage of the adjacent 3' phosphoester bond (2). This product in turn undergoes cleavage of the 5' phosphoester bond under alkaline conditions (Adapted from Sheppard *et al*. 2000).

1.3.1.3. Oxidative Damage

Reactive oxygen species such as superoxide ($O_2^{\cdot}$) and hydroxyl radicals ($OH^{\cdot}$), which are natural by-products of cellular respiration, can also cause DNA damage. They can cause structural mutations of bases as well as strand breaks. An example of mutagenic base damage caused by reactive oxygen species is 8-oxy-7, 8-dihydroguanine (Lindahl, 1996). It is a miscoding lesion since it facilitates base pairing with adenine rather than cytosine (Figure1-4).

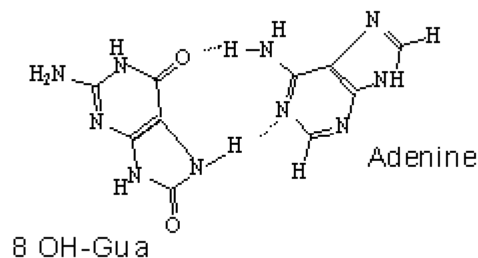
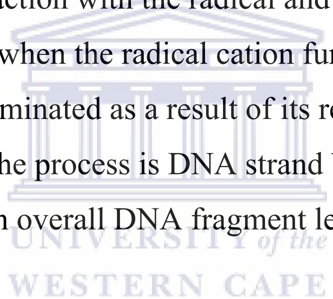


Figure 1-4. Base-pairing between adenine and the oxygen free-radical-induced lesion 8-oxy-7, 8-dihydroguanine (Adapted from Lindahl, 1996).

In addition, strand breaks are the most common damage caused by reactive oxygen species (von Sonntag, 1987). The sugar backbone is the primary target of reactive oxygen species. Figure 1-5 illustrates how reactive oxygen species cause DNA strand breaks. The C4 carbon of sugars are targeted by radical oxidants (1). The oxygen atom stabilises the radical due to its proximity (2). The C3 phosphate group is eliminated as a result of its reaction with the radical and a radical cation is formed (3). Another C4 radical is formed when the radical cation further reacts with water (4). The C5 phosphate group is eliminated as a result of its reaction with the new C4 radical (5). The end result of the process is DNA strand breaks, which causes fragmentation and reduction in overall DNA fragment length and concentration.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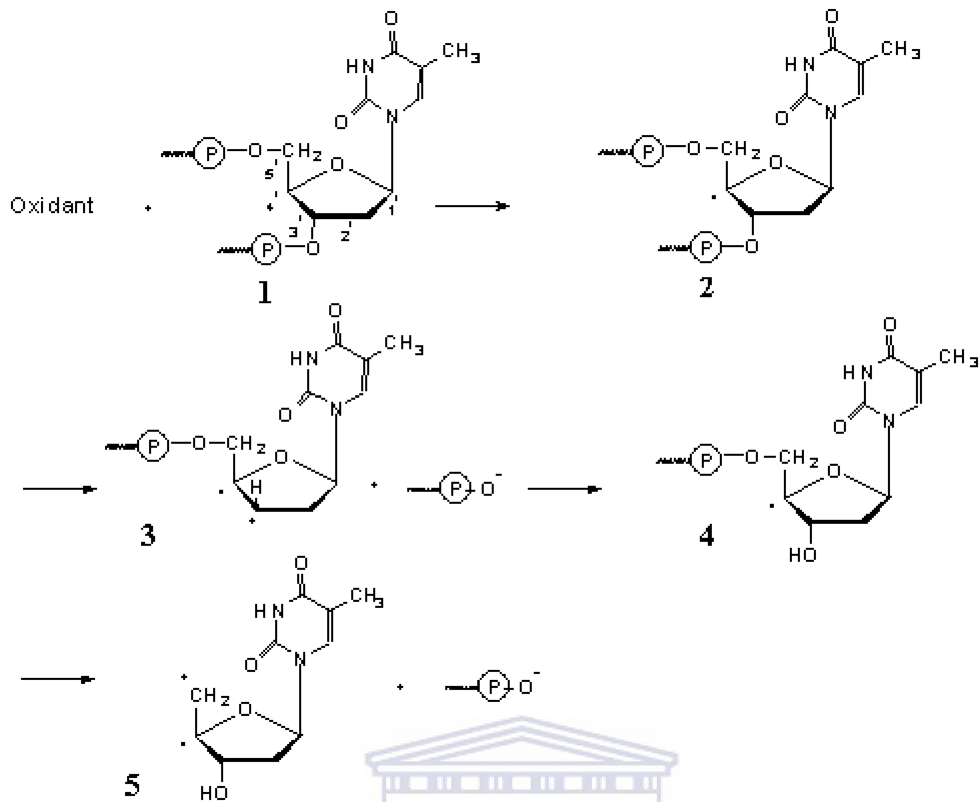

Figure 1-5. DNA strand breaks caused by reactive oxygen species.
The sugar backbone is the primary target of reactive oxygen species. The C4 carbon of the sugar is targeted by a radical oxidant (1). The oxygen atom stabilises the radical (2). The C3 phosphate group is eliminated as a result and a radical cation is formed (3). Another C4 radical is formed when the radical cation further reacts with water (4). The C5 phosphate group is eliminated as a result (5). (Adapted from von Sonntag, 1987)

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1.4. Contamination

In ancient DNA studies and forensic casework, contamination refers to the mixing of exogenous (i.e. not derived from the sample) DNA with endogenous DNA. This is of major concern in studies involving human skeletal remains because the levels of endogenous DNA are low and can easily be masked by exogenous sources of DNA. This could result in the exogenous (contaminant) DNA being preferentially amplified by the polymerase chain reaction (PCR).

1.4.1. Sources of Contamination

A number of potential sources of contamination are known. Every individual who handles the evidence is a potential source of contamination. This includes the excavators, anthropologists and laboratory personnel. In addition, those indirectly involved in the process like manufacturers of laboratory supplies are a potential

source of exogenous DNA (Hebsgaard *et al*. 2005 and Yang and Watt, 2005). Two of the major concerns are the washing stages of exhumed remains and contamination by PCR products in the DNA laboratory (Cooper, 1997; Willerslev and Cooper, 2005; Hebsgaard *et al*. 2005). Therefore both pre-laboratory and laboratory contamination controls and guidelines are critical to the success of a study.

1.4.2. Pre-Laboratory Contamination Controls

There have been cases where contamination could be traced back to individuals involved in the exhumation and anthropological analysis of samples (Sampietro *et al*. 2006). This illustrates the importance of general excavation controls and guidelines. Yang and Watt, (2005) suggest that the following contamination control guidelines be used by field archaeologists.

1. Samples for DNA analysis should not be cleaned as the dirt may protect against contaminants entering the sample.
2. Washing should be avoided as this could draw contaminant DNA into the sample and hydrolytic damage may occur.
3. The use of preservatives should be avoided as they may inhibit PCR reactions.
4. Further degradation can be limited by storing samples in a cool and dry environment.
5. Cross sample contamination can be prevented by storing modern reference and ancient samples separately.
6. All tools should be cleaned with at least a 10% (w/v) commercial bleach solution between each sample and whenever possible disposable tools should be used.
7. Disposable gloves and hair nets and depending on conditions protective suites should be worn at all times.
8. If a sample is not completely dry it could be stored in a paper bag but never in a plastic bag or tube as this would create ideal conditions for bacterial growth.

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Strict contamination controls of course only need to be in place for the collection of the bones or teeth that will be used for DNA analysis but excavators might not know which specimens will be sent for DNA analysis. A DNA analyst could help with this by giving advice on, which bones from the site would be best for analysis and if any

extra precautions should be taken. Notes should also be kept by field archaeologists recording the details of individuals involved in the exhumation. In addition, reference samples should be collected from all of workers.

1.4.3. Laboratory Contamination Controls

Criteria of authenticity have been developed to prevent and detect laboratory contamination. The criteria were developed for ancient DNA (aDNA) studies but can be useful when conducting forensic studies on human skeletal remains. The criteria include: (1) the use of a physically isolated pre-PCR work area or laboratory, (2) Negative controls: PCR and extraction blanks, (3) independent reproducibility, (4) cloning and sequencing, (5) decontamination of reagents and specimens, (6) DNA analysis of associated faunal remains, (7) appropriate molecular behaviour, (8) uracil-N-glycosylase (UNG) treatment and (9) quantification of starting templates. These criteria are briefly considered below.

1. Physically isolated pre-PCR work area or laboratory

The physical separation of pre and post-PCR laboratories will limit contamination by PCR products. The preparation of PCR reactions should be conducted in a dedicated pre-PCR laboratory and personnel should not be allowed to move from a post to pre-PCR environment on any one day (Yang and Watt, 2005 and Willerslev and Cooper, 2005). The further division of the pre-PCR laboratory into areas exclusively setup for bone preparation, DNA purification and PCR setup would also help to prevent contamination (Yang and Watt, 2005). Ideally each work area should be wiped down with at least 10% bleach and exposed to UV irradiation daily.

2. Negative controls: PCR and extraction blanks

A PCR blank control contains all PCR reagents but no DNA template, which allows contamination that occurs during the PCR setup to be detected. An extraction blank control contains all the extraction reagents but no skeletal sample material and is treated identically to the rest of the sample extractions. This allows any contamination that occurs during the DNA extraction process to be detected. It has been suggested that multiple PCR and extraction blank controls should be used per experiment (Poinar, 2003; Paabo *et al*. 2004 and Gilbert *et al*. 2005). This multiple blank control approach allows for the detection of sporadic and low-level contamination. However,

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PCR extraction blank controls only provide an indication of authenticity and do not take carrier-effects into account (Poinar, 2003; Paabo *et al*. 2004 and Willerslev and Cooper, 2005). Substances like sugars and microbial DNA that are found in certain extracts can act as “carriers” during PCR reactions thus allowing contaminant DNA of low concentration to be amplified. The low level contamination might be present in the PCR blank control as well but because it does not contain any “carriers” it will not be detected.

3. Independent reproducibility

Independent replication of results by an external laboratory is seen as the best way of demonstrating that internal laboratory contamination has been avoided (Willerslev and Cooper, 2005). This is because it’s unlikely that the same erroneous result would be generated independently in another laboratory. It has therefore been suggested that 10% of all key results of a study should be independently replicated.

4. Cloning and sequencing of PCR products

Cloning and sequencing can be used to assess contamination and DNA damage (Poinar, 2003 and Willerslev and Cooper, 2005). The detection of the same type of mtDNA sequence from several unrelated individuals or the presence of more than one mtDNA sequence type from one individual is a clear indication of contamination. The percentage and number of different mtDNA types present in a sample can be determined by cloning. Ancient endogenous sequences are prone to sequence errors due to the accumulation of DNA damage like miscoding lesions. Such errors can be detected by sequencing of several independently cloned fragments.

The most frequently occurring and the most damaged or modified sequence is considered to be the endogenous one (Poinar, 2003 and Sampietro *et al*. 2006). This is because damage and modification has been thought to occur over time and therefore the older (authentic) sequence should be more damaged or modified than the contaminant sequence.

5. Decontamination of reagents and specimens

All tools and reagents, even if labelled as sterile, are also potential sources of contamination and therefore must be decontaminated. Appropriate treatments can

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include exposure to high concentrations of bleach for 48hours, 2.5M Hydrochloric acid for 48 hours, UV irradiation or baking at 180°C for more than 12 hours (Willerslev and Cooper, 2005).

Specimens must also be decontaminated due to the problem of pre-laboratory contamination. Recent studies by Gilbert *et al*. 2006 and Sampietro *et al*. 2006 have indicated that skeletal remains are most susceptible to contamination during pre-laboratory handling. Therefore all specimens need to be decontaminated before DNA extraction and analysis. The commonly used procedures to decontaminate human skeletal remains include washing and physical removal of the outer surfaces, extraction of internal material, acid washing of surfaces, UV irradiation, ethanol immersion, bleach immersion and various combinations (Kemp and Smith, 2005).

6. DNA analysis of associated faunal remains

The preservation of DNA from associated remains can provide evidence for DNA survival and against contamination (Poinar, 2003 and Willerslev and Cooper, 2005). The amplification of DNA from associated faunal remains indicates whether the long-term environmental conditions of the particular exhumation site favoured DNA survival and if contamination occurred.

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7. Appropriate molecular behaviour

Due to degradation, the DNA from skeletal remains is generally fragmented to a size range of 50-200bp. Therefore PCR success should be inversely related to product size. If this is not the case then it’s likely that the particular sample has been contaminated with modern DNA (Paabo *et al*. 2004).

8. Uracil-N-glycosylase (UNG) treatment

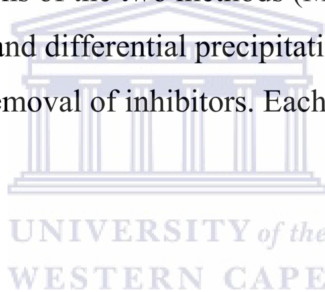
Previously amplified PCR products may contaminate subsequent PCR reactions. Carry over of PCR products can be prevented by substituting dTTP with dUTP in reactions. Treatment of subsequent reactions with Uracil-N-glycosylase facilitates the elimination of contaminating post PCR products.

9. Quantification of starting templates

An assessment of the copy number of the target DNA should be made. This is important because it may be impossible to avoid sporadic contamination when template levels are extremely low.

1.5. DNA Extraction Methods

The success of DNA analysis depends on the purity, quality and quantity of the DNA extracted. Therefore extraction methods that reduce the loss of DNA and reduce or remove inhibitors are desirable. There are four basic extraction categories with many variations: denaturation and dilution, organic extraction, differential precipitation, and differential affinity (Glenn, 1996). The methods of choice for ancient DNA extraction over the years have been variations of the phenol/chloroform method, which is an organic extraction method and the silica method, which is a differential affinity method, as well as combinations of the two methods (Mac Hugh *et al*. 2000). While the denaturation and dilution and differential precipitation methods generally result in loss of DNA and inefficient removal of inhibitors. Each of the methods will be briefly considered.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1.5.1. Chelex Extraction

The chelex method was first described as being a useful and simple extraction procedure by Singer-Sam *et al*. 1989 and Walsh *et al*. 1991. Briefly, the method involves the use of a Chelex chelating resin. Typically the sample is heated in the presence of Proteinase K and the resin. Cells are lysed by the heat, Proteinase K digests the proteins while the resin binds magnesium and other cellular components that might interfere with downstream processes. The binding of magnesium is important as it inhibits nuclease activity. A negative aspect of this is that if the resin beads are carried over to a PCR reaction Taq polymerase activity will also be inhibited. The Chelex method has been shown to be an effective protocol for the extraction of DNA from saliva (Sweet *et al*. 1996). This has made it ideal for the extraction of reference samples collected with buccal swabs. However it has been observed by Hoff-Olsen *et al*. 1999 that when used on degraded tissue and ancient animal bones it fails to remove PCR inhibitors.

1.5.2. Phenol/Chloroform Extraction

The phenol/chloroform extraction is a classical organic DNA extraction method. The method has been used to extract DNA from ancient animal and human skeletal remains and decomposed human soft tissue (Hagelberg *et al.* 1989; Hagelberg & Clegg 1991 and 1993; Hagelberg *et al.* 1991, Hagelberg *et al.* 1994a, 1994b; Hoff-Olsen *et al*. 1999). Most variations of the method are based on the protocol presented in a widely used laboratory manual (Sambrook *et al.* 1989).

The material from which DNA is extracted is typically homogenised and exposed to an aqueous lysis buffer containing a detergent. After cell lysis DNA is separated from the lysate by a phenol extraction followed by a series of phenol chloroform extractions. Phenol causes phase separation when combined with the aqueous lysis buffer while the chloroform enhances the efficiency of the phase separation and removes any residual phenol. Therefore when combined with the lysate it causes the separation of proteins and other cell debris that are more soluble in phenol (organic phase) from the aqueous phase in which DNA is more soluble. DNA is either precipitated from the aqueous phase using ethanol or isopropanol or desalted and concentrated by dialysis centrifugation. The dialysis centrifugation method is preferred as less DNA is lost during the process.

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1.5.3. Silica Extraction

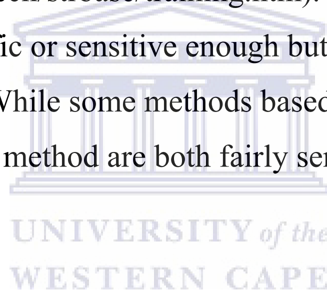
This method was first described by Boom *et al*. 1990. It exploits the tendency of DNA to bind to silica in the presence of chaotropic agents such as guanidinium thiocyanate (GuSCN). Boom *et al*. 1990 evaluated the use of the GuSCN, which has the ability to lyse cells, inactivate nucleases and facilitate the binding of DNA to silica. They observed that DNA from human serum and urine will bind to silica in the presence of sufficiently high GuSCN concentrations. This method was modified for use on ancient animal bones by Hoss and Paabo, 1993 and was found to overcome some of the challenges encountered using other methods. The principle of silica based purification systems has evolved into the modern commercial "spin-column" systems that are currently widely in use such as Qiagen's Qiaquick PCR purification and QIAamp DNA Blood Mini Kits.

1.6. DNA Quantitation

The accurate quantitation of DNA samples is required for human forensic casework. The human specific quantitation of samples is a federal requirement for crime labs in the United States of America. It’s important for the labs to show that the concentration of a crime scene reference sample is known and that non-human DNA from animals, plants, bacteria and fungi has not played a role in the quantitation. The accurate quantitation of samples is especially important when commercial STR kits are used since these kits are optimised for a narrow DNA input range usually 0.5-2ng.

1.6.1. Classical Methods

Sensitivity, dynamic range and human specificity are issues that have to be considered when assessing quantitation methods. Classical methods such as spectrometry (UV 260/280), yield gels, fluorescence and slot blots have been used but have limitations (http://www.cstl.nist.gov/biotech/strbase/training.htm). The UV 260/280 and yield gel methods are not human specific or sensitive enough but the yield gel does give an idea of the quality of the sample. While some methods based on fluorescent dyes (Pico Green assay) and the slot blot method are both fairly sensitive (150pg-10ng) they have a poor dynamic range.



1.6.2. End-Point PCR Methods

There are three stages to a PCR, the baseline stage where there is no significant increase in product, the exponential stage where there is an exponential increase in product and the final plateau stage where resources are limited and product synthesis ceases. End-point PCR quantitation is based on the use of the plateau stage, where the amount of product is not necessarily correlated with the amount of template in the initial reaction (Figure 1-6.)

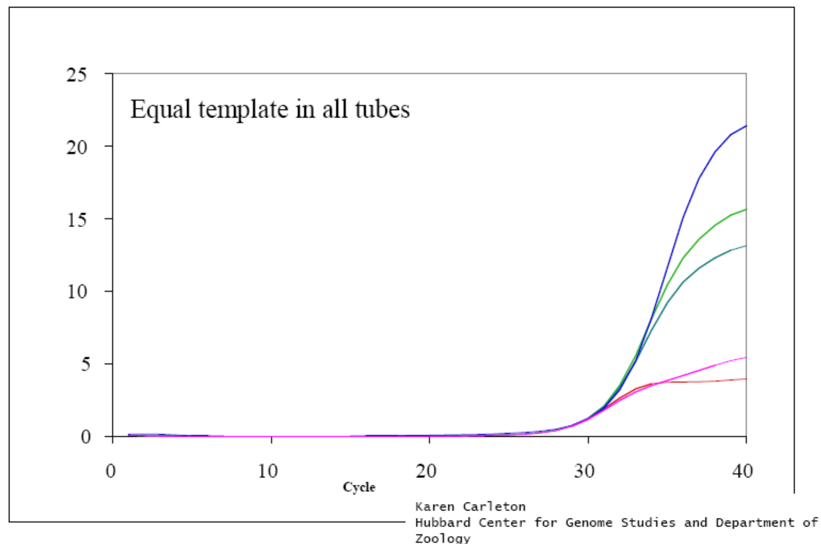


Figure 1-6. Problem of using end-point PCR as a quantitative tool. The final amount of product is not tightly correlated to the amount of template used. (http://www.cstl.nist.gov/biotech/strbase/training.htm)

Two End-point PCR methods however have been reported Sifis *et al*. 2002 and Holland *et al*. 2003. In the Sifis study an *Alu*-based quantitation method with a dynamic range of 2.5-100pg was developed. *Alu* sequences are a family of highly repetitive sequences that are primate specific and they are present at thousands of copies per cell. The method involves the use of *Alu* primers that are fluorescently labelled, which allows for product detection via an ABI PRISM™377 Genetic Analyser. A set of DNA standards of known concentration are run simultaneously with unknown samples and a standard curve is generated from the standards. The use of the ABI PRISM™377 Genetic Analyser and fluorescently labelled primers greatly increased the sensitivity of the end-point method.

In the Holland *et al*. 2003 study the authors used a quite different end-point method. It did not involve fluorescently labelled primers or an ABI PRISM™377 Genetic Analyser so the sensitivity was low but with a wider dynamic range 0.1ng-40ng. The method is referred to as the BodeQuant method and involved the amplification of unknown samples and a set of DNA standards of known concentration simultaneously with unlabelled TH01 primers. The concentrations of the unknowns are determined using an automated plate reader that measures the fluorescent signal of each sample when Pico-green is added to the TH01 products and compares it to a standard curve generated from the DNA standards.

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These methods overcome the problems of sensitivity and dynamic range but not the major problem associated with end-point methods namely that the product accumulated at the plateau stage is not always correlated with the initial amount of template.

1.7. DNA Typing Methods

DNA typing has become a vital component of human identification. It has been widely used to solve cases involving rape and murder, paternity issues and to identify the remains of victims of mass disasters. The array of markers and commercial typing technologies has increased the speed and ease with which DNA typing methods can be implemented.

1.7.1. History

DNA typing was first described in 1985 when it was discovered that DNA sequences at certain regions were tandemely repeated and varied between individuals (Butler *et al*. 2005). These regions later became known as variable number of tandem repeats (VNTRs) and were typed using restriction fragment length polymorphism analysis.

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The use of DNA typing in human identification has been widespread ever since PCR technology and information on human markers increased speed and discrimination capacity. At present hundreds of thousands of DNA tests are conducted by forensic and paternity labs around the world. These labs make use of a variety of DNA typing systems but the most commonly use autosomal short tandem repeats, Y-chromosome short tandem repeats and mtDNA.

1.7.2. Autosomal Short Tandem Repeats (STRs)

Studies by Edwards *et al*. 1991, 1992 were the first to describe autosomal short tandem repeats markers as being useful for human identification. Autosomal STRs are tandemely repeated units of 2-6bp that are found on the autosomal chromosomes. Short tandem repeats are useful for human identification because they are highly polymorphic and sets of loci can be selected which have independent chromosomal assortment and recombination. They can also be amplified and analysed using multiplex PCR .These properties have prompted the development of a number of commercial STR typing kits (Table 1-1).

Table 1-1. Summary of available commercial STR kits that are commonly used (Butler, 2006)

Kit Name	STR Loci Included	Random Match Probability with Author's Profile*
	Promega Corporation	
PowerPlex 1.1 and 1.2	CSF1PO, TPOX, TH01, VWA, D16S539, D13S317, D7S820, D5S818	7.4×10^{-10}
PowerPlex 2.1 (for Hitachi FMBIO users)	D3S1358, TH01, D21S11, D18S51, VWA, D8S1179, TPOX, FGA, Penta E	3.4×10^{-11}
PowerPlex ES	FGA, TH01, VWA, D3S1358, D8S1179, D18S51, D21S11, SE33, amelogenin	1.3×10^{-10}
PowerPlex 16	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, Penta D, Penta E, amelogenin	1.2×10^{-18}
PowerPlex 16 BIO (for Hitachi FMBIO users)	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, Penta D, Penta E, amelogenin	1.2×10^{-18}
	Applied Biosystems	
AmpFlSTR Blue	D3S1358, VWA, FGA	1.0×10^{-3}
AmpFlSTR Green I	Amelogenin, TH01, TPOX, CSF1PO	7.8×10^{-4}
AmpFlSTR Cofiler (CO)	D3S1358, D16S539, Amelogenin, TH01, TPOX, CSF1PO, D7S820	2.0×10^{-7}
AmpFlSTR Profiler Plus (Pro)	D3S1358, VWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820	2.4×10^{-11}
AmpFlSTR Profiler Plus ID	D3S1358, VWA, FGA, Amelogenin, D8S1179, D21S11, D18S51, D5S818, D13S317, D7S820 (extra unlabeled D8-R primer)	2.4×10^{-11}
AmpFlSTR Profiler	D3S1358, VWA, FGA, Amelogenin, TH01, TPOX, CSF1PO, D5S818, D13S317, D7S820	9.0×10^{-11}
AmpFlSTR SGM Plus (SGM)	D3S1358, VWA, D16S539, D2S1338, Amelogenin, D8S1179, D21S11, D18S51, D19S433, TH01, FGA	4.5×10^{-13}
AmpFlSTR Sefiler (SE)	FGA, TH01, VWA, D3S1358, D8S1179, D16S539, D18S51, D21S11, D2S1338, D19S433, SE33, amelogenin	5.1×10^{-15}
AmpFlSTR Identifiler (ID)	CSF1PO, FGA, TPOX, TH01, VWA, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, D2S1338, D19S433, amelogenin	7.2×10^{-19}

1.7.2.1 CODIS

The most commonly used STR markers are those which constitute the Combined DNA Index System (CODIS) has been in existence for approximately a decade, it uses DNA profiles generated for 13 core STR loci (Figure 1-7) from crime scenes and convicted offenders which are stored in databases to link suspects and repeat offenders unsolved cases (Butler, 2006). The selection of the markers involved the evaluation of 17 candidate loci by 22 DNA typing labs and ended in 1997. Of the 17 loci examined only 13 were chosen to be part of the CODIS set. Since its official launch in October 1998 over 2.8 million DNA profiles have been entered into the database and over 27000 investigations aided (http://www.cstl.nist.gov/biotech/strbase/training.htm).

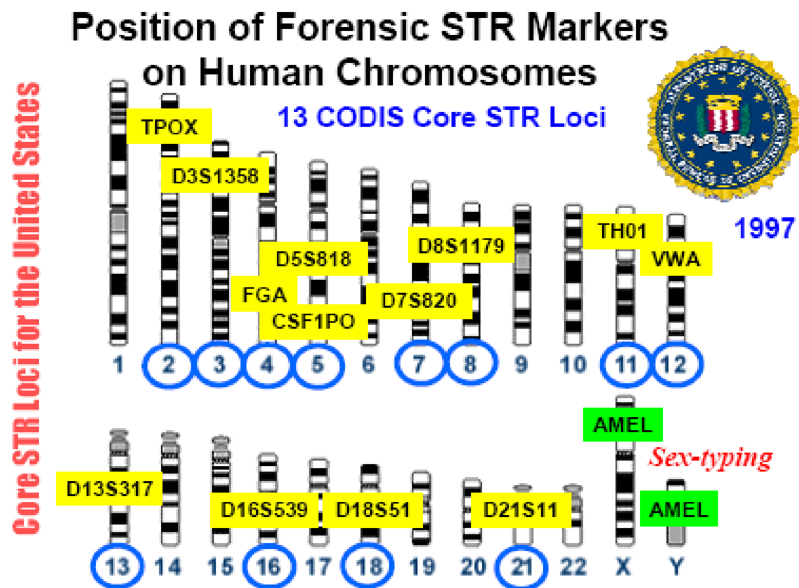

Figure1-7. CODIS Core Loci (http://www.cstl.nist.gov/biotech/strbase/training.htm)

The establishment of the CODIS loci motivated the development of commercial STR kits that could type the 13 core loci simultaneously. Initially two Applied Biosystems kits, Profiler Plus and COfiler were available which together covered the 13 CODIS loci while Promega developed the PowerPlex 1.1 and 2.1 kits (Table1-1) (Butler, 2006). In 2000 Promega released their PowerPlex 16 kit that amplifies the 13 CODIS loci, Penta D and Penta E as well as the sex determining marker amelogenin. This was followed in 2001 by Applied Biosystems release of their 16plex Identifiler kit amplifying the 13 CODIS loci and amelogenin but also includes D2S1338 and D19S433 (Figure 1-8). These kits are currently used by the majority of forensic and paternity labs around the world.

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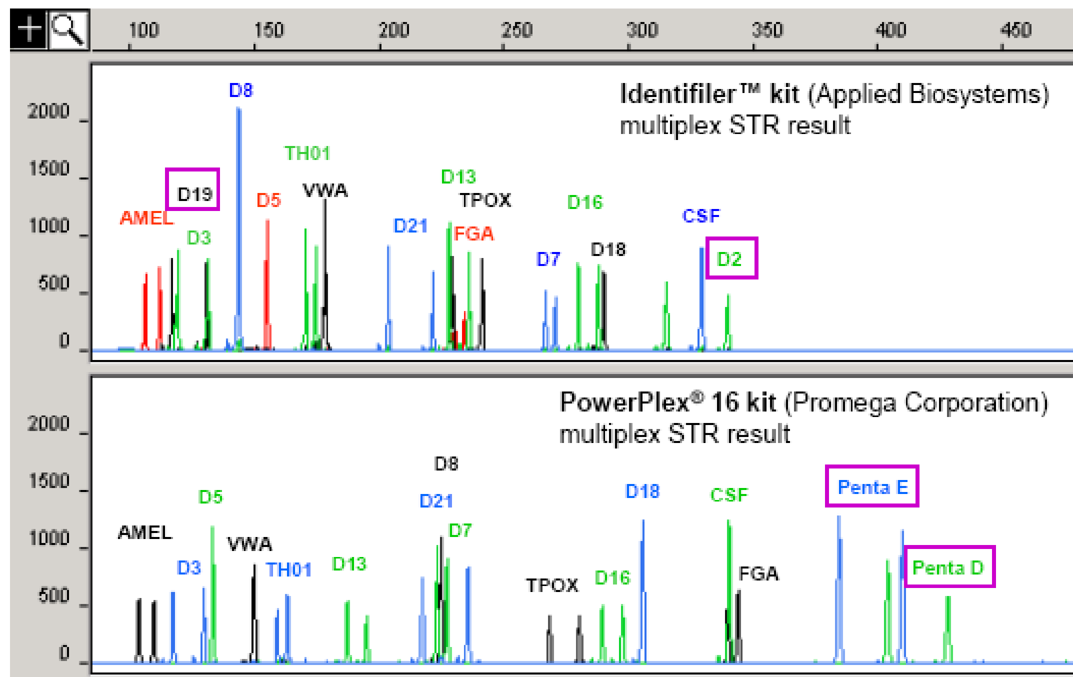

Figure 1-8. Comparison of the Applied Biosystems 16plex Identifiler and the Promega PowerPlex 16 kits (Butler, 2005).

1.7.3. Autosomal Single Nucleotide Polymorphisms (SNPs)

An alternative method for analysing degraded DNA is autosomal single nucleotide polymorphisms (SNPs) analysis. SNPs account for approximately 85% of the genetic variation in humans (Kashyap *et al*. 2004; Budowle *et al*. 2005). They occur at a rate of 1/100bp and are single base deletions, insertions or substitutions. Due to their bi-allelic nature a large panel (>50) are required to approach the discrimination capacity of commercial STR systems (Dixon *et al*. 2005; Budowle *et al*. 2005). However since SNP analysis requires the amplification of small products (40-70bp) highly degraded DNA samples can be typed.

SNP multiplexes have been developed but there is only one fully validated forensic multiplex available (Dixon *et al*. 2005). The Foren-SNP™ kit (The Forensic Science Service, UK) amplifies 21 loci simultaneously that can be analysed on an electrophoresis instrument (Dixon *et al*. 2005). The other available systems like the GenomeLab™ SNPStream (Beckman Coulter) and SNaPshot™ (Applied Biosystems) are not suitable for forensic purposes because they are multi-stage procedures and require large volumes of initial DNA or PCR template (Dixon *et al*. 2005; Budowle *et al*. 2005). These systems however are capable of targeting

thousands of SNPs and with optimisation and a few modifications in the future could play an important role in the identification of human skeletal remains (Dixon *et al*. 2005).

1.7.4. Y-STRs

Y-chromosome short tandem repeats (Y-STRs) are useful for human identification testing (Butler, 2006). Y-STRs are paternally inherited and do not undergo recombination. Therefore all paternal relatives, barring a mutation will share the same haplotype. There are certain disadvantages and advantages associated with this. The primary disadvantage is that paternal relatives cannot be distinguished from one another. The advantages of Y-STRs can be seen in their application in sexual assault cases, paternity testing, missing person's investigations, human migrations and evolutionary studies as well as historical and genealogical research (Butler, 2005). In sexual assault cases the time consuming process of differential extraction, which separates sperm from epithelial cells is required in order to use autosomal STRs. This can be avoided by using Y-STRs which target only the male fraction. In paternity testing it can be useful when the mother is unavailable as the male child will share an identical Y-STR profile with his father. This inheritance pattern is also useful in missing persons investigations as it allows any paternal male relative to be used as a reference sample. Due to the lack of Y-chromosome recombination, in human Y-STRs can be used to compare males separated by long time periods and in historical and genealogical research can make links where historical records are limited.

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The core set of Y-STRs has changed over the years but this has not stopped the establishment of a large database. The original core set referred to as the minimal haplotype loci (MHL) were chosen in the 1990s when only a few Y-STRs were characterised and consisted of seven loci (Table 1-2) (Butler, 2006). The MHL were then extended by the addition of the duplicated dinucleotide repeat locus YCA II and referred to as the extended haplotype. In 2003 the Scientific Working Group on DNA Analysis Methods (SWGDAM) recommended that DYS438 and DYS439 form part of a new extended haplotype and that YCA II be removed. These changes have not hindered the Y-Chromosome Haplotype Reference Database (YHRD) (http://www.yhrd.org), which as of 28 December 2006 contained 46831 haplotypes of which 17935 have been typed with the SWGDAM core.

Table 1-2. Y-STR Core loci

Minimal Haplotype	Extended Haplotype	SWGDAM Core
DYS19	DYS19	DYS19
DYS385 a/b	DYS385 a/b	DYS385 a/b
DYS389I/II	DYS389I/II	DYS389I/II
DYS390	DYS390	DYS390
DYS391	DYS391	DYS391
DYS392	DYS392	DYS392
DYS393	DYS393	DYS393
	YCA II	DYS438
		DYS439

Not long after the establishment of the SWGDAM core two commercial kits were released that amplified the core loci plus a few additional loci. Promega released their PowerPlex Y in September 2003 while Applied Biosystems released their AmpFlSTR Yfiler™ in December 2004 (see Figure 1-9 for comparison). These kits are widely used and kit specific databases have been established.
(See http://www.promega.com/techserv/tools/pplexy/ and http://www.appliedbiosystems.com/yfilerdatabase/)

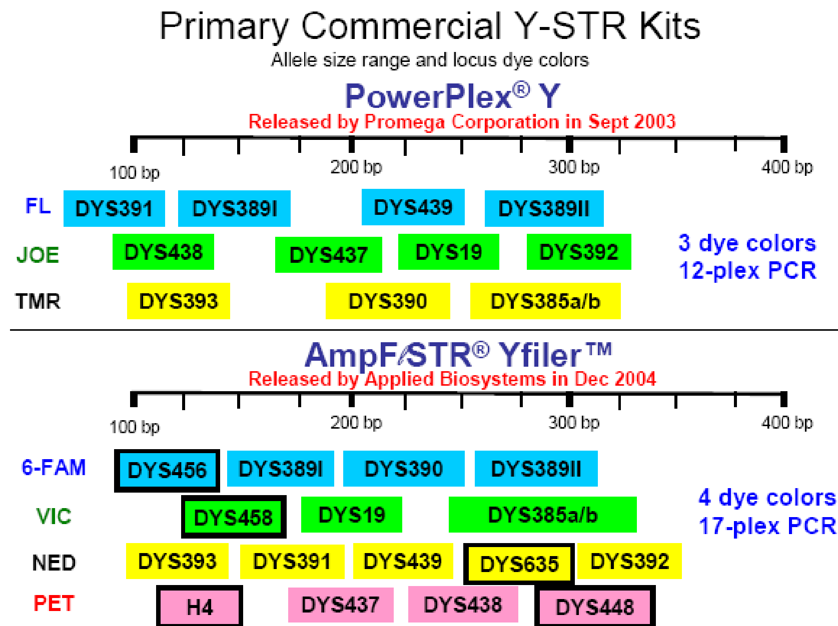

Figure 1-9. Schematic overview of the product size ranges for the loci included in commercial typing kits. (http://www.cstl.nist.gov/biotech/strbase/training.htm)

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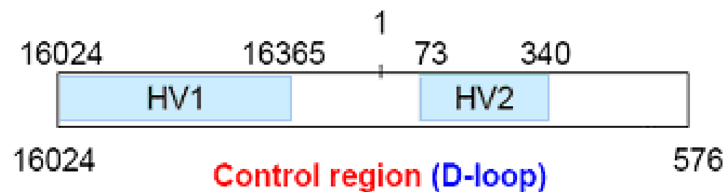
1.7.5. MtDNA

Mitochondrial DNA analysis is useful in the study of human evolution and migration as well as for the analysis of highly degraded forensic samples. Mitochondrial DNA is maternally inherited, does not undergo recombination and is present at over 1000 copies per cell (Holland and Parsons, 1999; Kashyap *et al*. 2004; Budowle *et al*. 2005).

These properties have disadvantages and advantages when used for human identity testing (http://www.cstl.nist.gov/biotech/strbase/training.htm). The absence of recombination and maternal inheritance means that all maternal relatives, barring a mutation will share the same haplotype. This limits the degree to which mtDNA analysis can discriminate between individuals. However the high copy number is useful when analyzing degraded samples containing low concentrations of DNA (Holland and Parsons, 1999; Kashyap *et al*. 2004; Budowle *et al*. 2005).

The identification of human skeletal remains often relies on mtDNA sequencing (Kashyap *et al*. 2004; Budowle *et al*. 2005). The regions that are the most variable among individuals are the hypervariable regions I and II (HVI and HVII) (Figure 1-10) (Holland and Parsons, 1999; Kashyap *et al*. 2004). When STR analysis has failed

a success rate of 95% has been achieved for mtDNA analysis of skeletal remains (Budowle *et al*. 2005). However typing success does not necessarily mean positive identification due to the low discrimination capacity and mode of inheritance of mtDNA.



Forensic Focus

Typically only **610 bases examined**

– (HVI: 16024-16365; HVII: 73-340)

Figure 1-10. Non-Coding Control Region (Adapted from http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm)

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1.7.6. MiniSTRs

MiniSTR analysis produces reduced sized STR amplicons due to the repositioning of its primers as close as possible to the STR target region (Figure1-11, comparison with conventional STRs), (Yoshida *et al*. 1997; Ricci *et al*. 1999; Wiegand and Kleiber, 2001; Tsukada *et al*. 2002 and Butler *et al*. 2003). This reduction in product size allows degraded DNA to be typed. MiniSTR analysis has been used to type human telogen hairs (Hellman *et al*. 2001); formalin fixed and mummified tissue (Wiegand and Kleiber, 2001); 17-26 year old bloodstains (Tsukada *et al*. 2002); human skeletal remains from the World Trade Center Disaster (Holland *et al*. 2003); enzymatically degraded DNA (Chung *et al*. 2004) and old bone remains from mass graves of the Spanish Civil War (Martin *et al*. 2006).

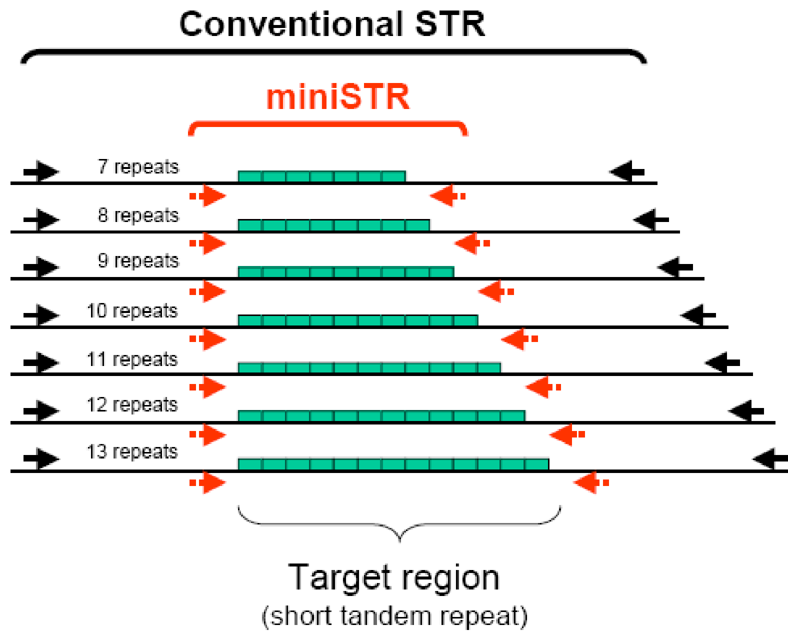

Figure 1-11. Comparison of conventional and miniSTRs. The black arrows represent the original conventional STR primer set while the red arrows represent the redesigned miniSTR primer set (http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm).

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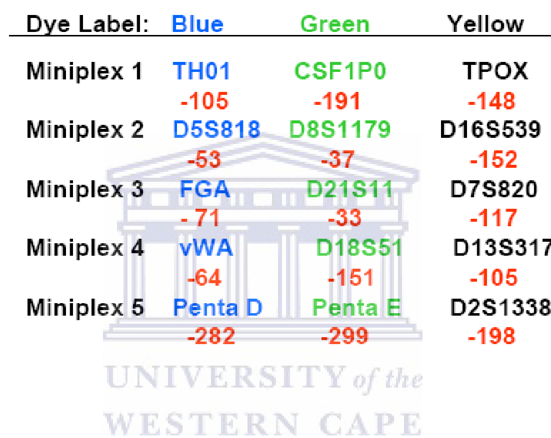
1.7.6.1. History of MiniSTR Typing Systems

The impetus to create miniSTR multiplex systems originated from research on the rapid analysis of STRs with time-of-flight mass spectrometry (Butler, 1999). In order to successfully perform STR typing using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) STR amplicon sizes had to be reduced. Primers for the 13 CODIS loci were among the first to be redesigned. The success observed using this new technology led to a patent being applied for and granted for this technology in July 2000 (http://www.cstl.nist.gov/biotech/strbase/miniSTR/timeline.htm).

The World Trade Center (WTC) disaster led to the acceleration of the development of the miniplexes for use in victim identification. Dr. Robert Shaler the director of forensic biology in the New York City's Office of the Chief Medical Examiner (NYC OCME) at the time was responsible for the identification of the victims of the disaster (Marchi, 2004). He asked John Butler from the US National Institute for Standards and Technology (NIST) to accelerate the development of these systems so that it could be used to assist in WTC victim identification (Marchi, 2004). This work led to the development of five miniplexes that incorporated fifteen loci including twelve of

the CODIS 13 (Table 1-3) (http://www.cstl.nist.gov/biotech/strbase/miniSTR/timeline.htm). Following these developments Robert Shaler asked the Bode Technology Group who were already involved in the WTC victim identification to build on these efforts (Marchi, 2004). The Bode Technology Group developed two miniplexes, BodePlex 1 and BodePlex 2 (Holland *et al*. 2003). The use of these systems more than tripled the success rate of results when compared to commercial systems (Holland *et al*. 2003).

Table 1-3. Original Miniplex Systems. The numbers in red indicate the size reduction relative to Applied Biosystems kits. When Miniplex 1 and 2 are combined they are referred to as the 'BigMini' (http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm).



Dye Label:	Blue	Green	Yellow
Miniplex 1	TH01	CSF1P0	TPOX
	-105	-191	-148
Miniplex 2	D5S818	D8S1179	D16S539
	-53	-37	-152
Miniplex 3	FGA	D21S11	D7S820
	- 71	-33	-117
Miniplex 4	vWA	D18S51	D13S317
	-64	-151	-105
Miniplex 5	Penta D	Penta E	D2S1338
	-282	-299	-198

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1.7.6.2. Degradation

The hypothesis that a reduction in PCR product size ranges would improve typing of degraded DNA was examined in Butler *et al*. 2003 and Chung *et al*. 2004. The initial study focused on comparing the typing of 92 Chelex extracted DNA samples using the commercial PowerPlex 16 system (Promega) and miniplexes 1, 2 and 3 (Butler *et al*. 2003). The DNA was extracted from bloodstains that were stored at room temperature for 14-15 years. Amplification efficiency improved when the miniplexes were used. The results showed that the larger loci (CSF1PO, Penta D) of the PowerPlex 16 kit (Promega) consistently amplified poorly while the miniplexes showed improved amplification (Figure 1-12).

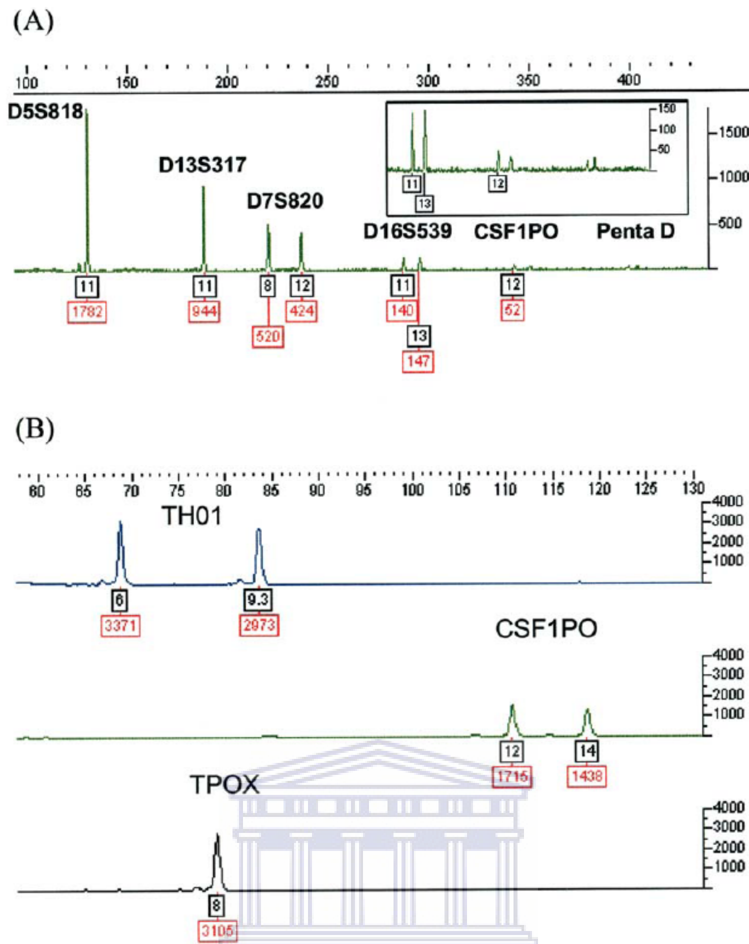
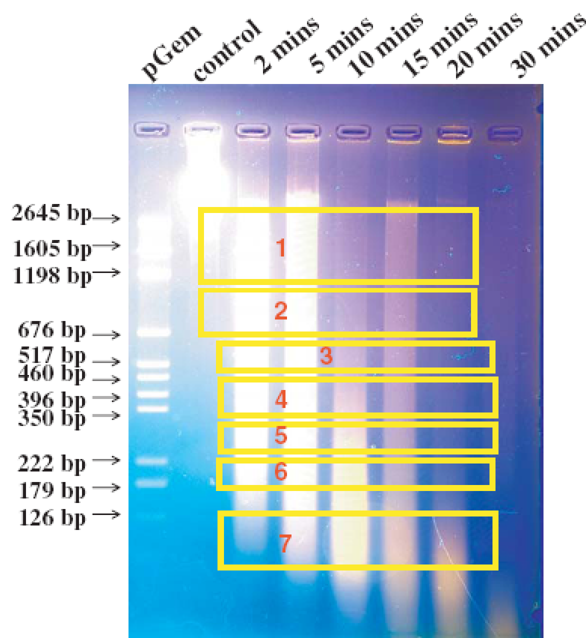


Figure 1-12. Comparison of STR allele peak heights obtained from PowerPlex® 16 (A) and Miniplex 1 loci (B) with equivalent amounts of the same aged bloodstain sample. Peak labels are allele calls and peak heights are in relative fluorescence units (RFUs). The second allele for CSF1PO with the PowerPlex® 16 kit is below the 50 RFU peak detection threshold and therefore not labelled by the software (Butler *et al*. 2003).

The hypothesis that reduced sized amplicons would improve the amplification efficiency of degraded DNA samples was further examined by Chung *et al*. 2004. In this study enzymatically degraded DNA was typed with miniplexes 2, 4 and “BigMini” as well as the commercial PowerPlex 16 kit (Promega). DNA was extracted from whole blood using the QIAamp Blood Maxi kit (Qiagen), and digested with DNase I for varying lengths of time (Chung *et al*.2004). The digests were run on an agarose gel to facilitate the purification of appropriately degraded DNA samples (Figure 1-13).



Legend: DNA length range (bp)

1. >1198
2. ~676-1198
3. ~460-517
4. ~350-460
5. ~222-350
6. ~179-222
7. <126

Figure 1-13. DNA degraded with DNase I over different time periods. pGEM_R DNA marker (Promega corporation, Madison, WI) was used as ladder (L). Lanes 1–7 were loaded with DNA incubated with DNase for several time periods: 0 (control), 2, 5, 10, 15, 20, and 30 min, respectively. DNA from different regions of the gel corresponding to fragment sizes of <126, ~179-222, ~222-350, ~350-460, ~460-517, ~676-1198, and >1198 base pairs were excised from the gel and amplified with the Miniplex primer sets and the commercial PowerPlex 16 kit (Chung *et al.*2004).

The results of the study clearly showed that as the template size decreased so did the amplification efficiency of the larger loci of the PowerPlex 16 kit (Figure 1-15). By contrast the miniplexes results showed a significant improvement in amplification efficiency overall but particularly of the loci that performed poorly with the commercial kit, as shown in figure 1-14.

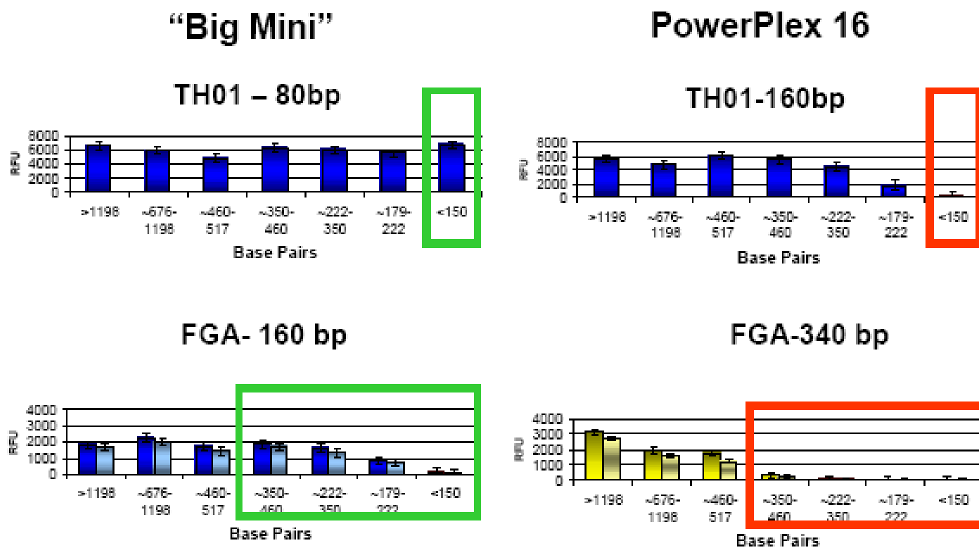


Figure 1-14. Amplification efficiency of the "BigMini" and commercial PowerPlex 16 typing systems. TH01 and FGA loci were amplified using degraded DNA as a template. The "BigMini" clearly improved the amplification efficiency of the degraded DNA (Chung *et al.*2004).

1.7.6.3. Sensitivity

The study conducted by Chung *et al*. 2004 also investigated how DNA template concentrations influenced miniplex amplification efficiency. This is important because highly degraded samples usually yield low DNA concentrations. In this study, sensitivity of Miniplex 2, 4 and the Big Mini were examined by amplifying DNA concentrations ranging from 31-500pg in 25µL reactions (Chung *et al*. 2004).

The sensitivity observed was generally superior to that reported for commercial kits. Miniplex 2 and 4 generated correct genotypes for most of the samples at concentrations of 31pg and 63pg/25µL (Chung *et al*. 2004). The allele dropout rate for Miniplex 2 for both concentrations was 8.33% while Miniplex 4 had a dropout rate of 30% for 31pg/25µL and 20% for 63pg/25µL. As for the Big Mini significant dropout was observed at both 31pg and 63pg/25µL. Template concentrations above 100pg in 25µL reactions were found to be ideal for Miniplex 2 and 4 while concentrations above 250pg/25µL were required for the Big Mini. The high degree of multiplexing was given as the reason for the lower performance of the Big Mini. The overall results show that the sensitivity of these Miniplex systems is better than commercial kits (Chung *et al*. 2004).

1.7.6.4. Concordance

Concordance between Miniplex and commercial kit results was examined by Butler *et al*. 2003 and Drabek *et al*. 2004. When the DNA profiles generated from 50 samples using the Miniplex systems were compared to those generated from the Profiler™, SGM Plus™, Profiler Plus™, COfiler™, and PowerPlex® 16 kits the only loci to produce discordant results were D5S818 and D13S317 (Butler *et al*. 2003). The reason given for the D13S317 discordance was the presence of a potential four base deletion sequence located upstream of the Miniplex primer binding region but within the binding region of the commercial primers. While the D5S818 discordance was believed to be due to a nucleotide polymorphism within the miniplex primer binding region (Butler *et al*. 2003). These mechanisms are presented schematically in figure 1-15.

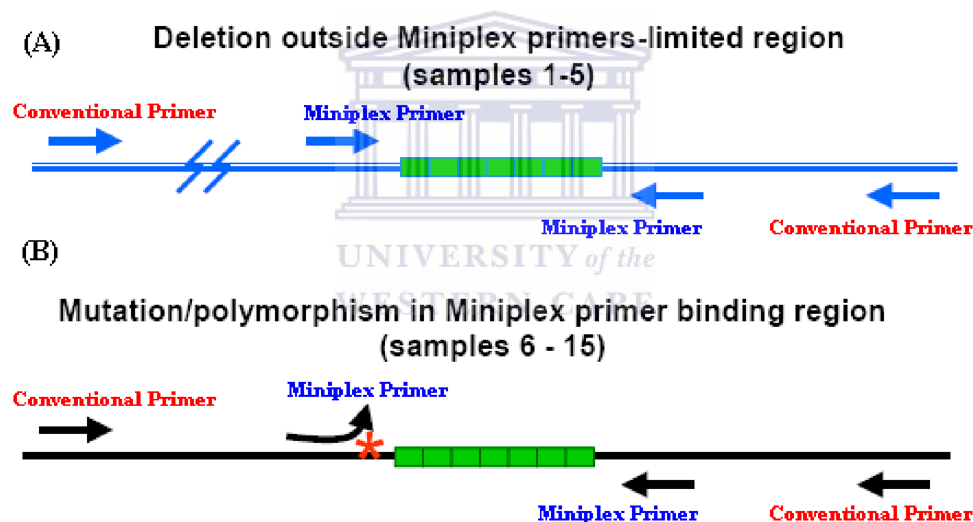


Figure 1-15. Proposed Mechanism behind discordant results. (A) If a deletion, for example a 4bp deletion occurs within the flanking region of the STR but in-between the primer binding sites of the MiniSTR primers and the commercial kit primers both systems will amplify but generate different allele calls. This is due to the conventional commercial kit primers binding outside of the 4bp deletion and therefore generating a product 4bp smaller thus causing 1 allele shift. (B) If a mutation occurs at the 3` end of the Miniplex primer binding site the Miniplex primers would not be able to bind thus resulting in allele dropout. The commercial kit primers however would bind and generate the correct allele calls. (http://www.cstl.nist.gov/biotech/strbase/NISTpub.htm)

A more comprehensive concordance study was conducted by Drabek *et al*. 2004. In this study 532 DNA samples were typed with Miniplex 2, 4 and the Big Mini as well as two commercial kits and the genotypes compared. The DNA samples originated from 2 Asian, 110 Hispanic, 208 Caucasians and 212 African American individuals (Drabek *et al*. 2004).

A mere 0.2% discordance rate was observed for all allele calls. The discordance originated from not only loci D3S317 and D5S818 but also from vWA (Drabek *et al*. 2004). A total of 15 differences were observed between the commercial AmpFlSTR™ Identifiler kit (Applied Biosystems) and the miniplex systems for those loci in African American and Hispanic samples. These differences were confirmed when the samples were typed with the PowerPlex® 16 kit (Promega). These differences and well as likely causes are presented in Table 1-4. These findings led to the redesigning of the D5S818 primers.

Table 1-4. Summary of 15 discordant STR profiling results: observed in this study between the Identifiler kit and the Miniplex assays for 12 different African American (AA) and 3 Hispanic (H) samples. PowerPlex 16 (PP16) results all agree with the Identifiler results for these 15 samples. Single allele shifts of 1 repeat in the D13S317 heterozygotes are likely due to a 4 base pair deletion in the flanking region outside of the Miniplex primer binding site. Allele dropout at D5S818, D13S317, and vWA are likely due to primer binding site mutations at specific alleles (Drabek *et al*. 2004).

	Locus	Origin	Miniplex	Identifiler	PP16	Likely Cause
1	D13S317	AA	**11**,13	10,13	10,13	deletion outside of allele 11
2	D13S317	H	**9**,14	8,14	8,14	deletion outside of allele 9
3	D13S317	AA	**10**,11	9,11	9,11	deletion outside of allele 10
4	D13S317	H	**10**,11	9,11	9,11	deletion outside of allele 10
5	D13S317	H	**10**,14	9,14	9,14	deletion outside of allele 10
6	D5S818	AA	11,**11**	11,12	11,12	primer binding site mutation
7	WA	AA	**16**,16	12,16	12,16	primer binding site mutation
8	WA	AA	**18**,18	13,18	13,18	primer binding site mutation
9	WA	AA	**15**,15	14,15	14,15	primer binding site mutation
10	WA	AA	**15**,15	14,15	14,15	primer binding site mutation
11	WA	AA	**17**,17	14,17	14,17	primer binding site mutation
12	WA	AA	**17**,17	14,17	14,17	primer binding site mutation
13	WA	AA	**19**,19	14,19	14,19	primer binding site mutation
14	WA	AA	**19**,19	14,19	14,19	primer binding site mutation
15	WA	AA	**19**,19	14,19	14,19	primer binding site mutation

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1.7.6.5. Analysis of DNA from Human Skeletal Remains

Studies conducted by Chung *et al*. 2003 and Coble and Butler, 2005 demonstrated that Miniplex systems could be used to analyse degraded DNA from human skeletal remains, however the first comprehensive study was conducted by Opel *et al*. 2006. In this study, Miniplex 2, 4 and the "BigMini Multiplex" as well as the commercial PowerPlex 16 kit (Promega) were used to type DNA extracted from 31 human bones that had been exposed to diverse environmental conditions. The set of bones consisted of 30 femurs and 1 tibia. Specimens were prepared and the DNA extracted and quantified as described in Chung *et al*. 2003.

The amplification efficacy observed for the Miniplex systems was much greater than that of the commercial kits. The PowerPlex kit (Promega) generated full DNA profiles for only 16% of the samples while the Miniplex systems generated full DNA profiles for 64% of the samples (Opel *et al*. 2006). As expected it was the larger loci of the commercial kit that amplified poorly. While the Miniplex systems often generated full profiles the larger loci of the Big Mini were the most likely to fail. Overall these results confirmed the findings of Chung *et al*. 2003 and Coble and Butler, 2005 but also highlighted the problem with the larger loci of the Big Mini.

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1.7.6.6. Beyond the use of CODIS STR Markers

A process of characterising new miniSTRs was initiated by Coble and Butler, 2005. The original miniSTR design plan was based on placing the primers adjacent to the repeat region thus making the amplicon as small as possible. However the large allele ranges and polymorphic nature of the flanking regions of some of the CODIS loci made this challenging (Coble and Butler, 2005). This is particularly evident with Miniplex 3, which contains loci which generate relatively large amplicons (FGA, D21S11 and D7S820).

The study conducted by Coble and Butler, 2005 screened a large number of novel miniSTR loci. The screening process considered characteristics of ideal miniSTR markers. These characteristics include a small allele range, flanking regions free of repeated elements, heterozygosity value above 0.70 and a tetranucleotide repeat unit which limits stuttering (Coble and Butler, 2005). Out of a total of 920 loci screened 18 produced amplicons generally below 110bp. The focus of the study was on six of

the18 markers identified for initial testing. The six new miniSTR were arranged into two triplexes, mini01 and mini02. Details of the screening process are presented in (Figure 1-16).

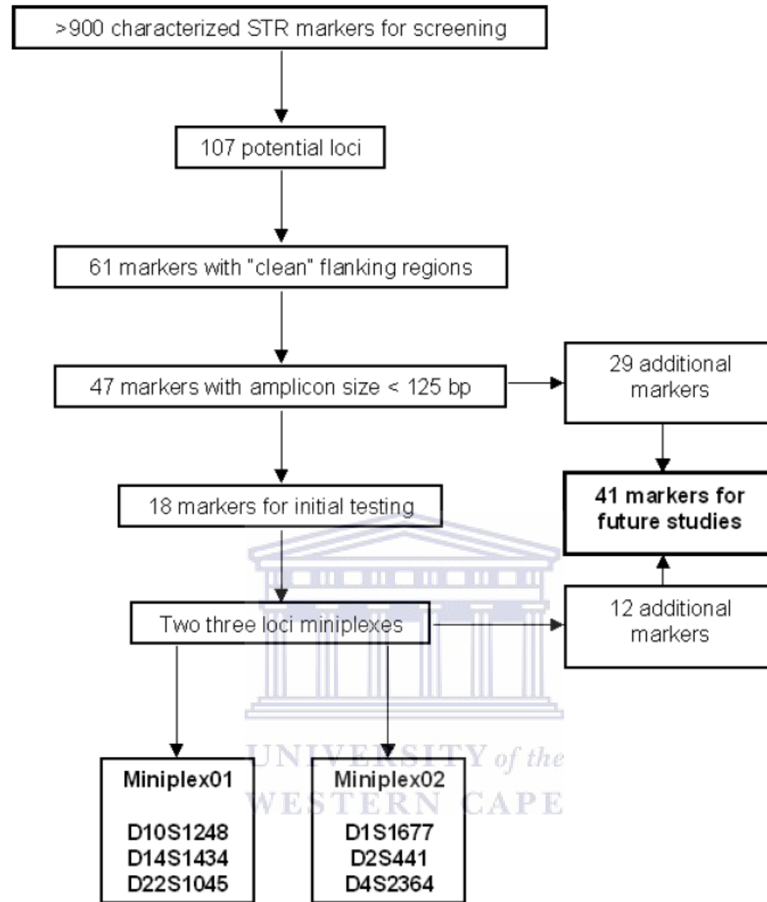

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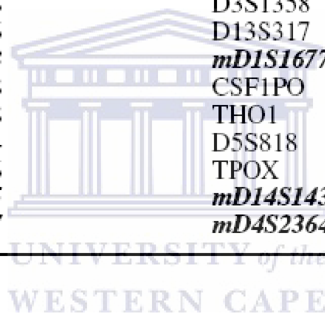
Figure 1-16. Overview of the screening process to identify miniSTR loci. Published STR markers were used to screen for "ideal" markers. Potential loci were selected for allele ranges less than 24 bp and heterozygosity values greater than 0.70 (Coble and Butler, 2005).

To evaluate the two triplexes, tests were conducted to assess locus heterozygosity values, sensitivity and ability to type degraded DNA from human skeletal remains (Coble and Butler, 2005). To assess the heterozygosity values a population study was conducted. The population samples consisted of DNA from 170 Caucasians, 164 African Americans and 140 Hispanic individuals. All these samples were typed with mini01, mini02 as well as the markers of the AmpFlSTR™ Identifiler kit (Applied Biosystems). The heterozygosity values for the novel miniSTR loci, D10S1248, D22S1045, D2S441 and D1S1677 compared well with equivalent values for the markers included in commercial kits (Table 1-5). Heterozygosity values for

D14S1434 and D4S2364 were relatively low and indicate a low polymorphic content for these markers.

Table 1-5. Comparison of heterozygosity values from 474 individuals: (164 African Americans, 170 Caucasians, and 140 Hispanics) using six miniSTR loci (denoted in bold and italics) and the 15 STR loci within the identifiler™ kit (Coble and Butler, 2005).

Heterozygosity	Marker
0.8784	D2S1338
0.8753	D18S51
0.8710	FGA
0.8393	D21S11
0.8245	vWA
0.8076	D7S820
0.7970	D19S433
0.7759	***mD10S1248 - mini01***
0.7759	D16S539
0.7674	***mD22S1045 - mini01***
0.7674	D8S1179
0.7590	***mD2S441 - mini02***
0.7548	D3S1358
0.7526	D13S317
0.7463	***mD1S1677 - mini02***
0.7378	CSF1PO
0.7378	THO1
0.7294	D5S818
0.7146	TPOX
0.6765	***mD14S1434 - mini01***
0.5307	***mD4S2364 - mini02***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The sensitivity of the new Miniplex assays was assessed by typing ten replicates of DNA with concentrations ranging from 5pg/μL to 500pg/μL and comparing each profile to that generated using 1ng/μl (Coble and Butler, 2005). When standard PCR conditions were utilised with 100pg DNA per reaction, full profiles were generated for all samples while allele dropout and dropin were observed at lower template concentrations. These results were slightly improved when higher Taq Gold polymerase concentrations were used and the number of PCR cycles increased (Figure 1-17).

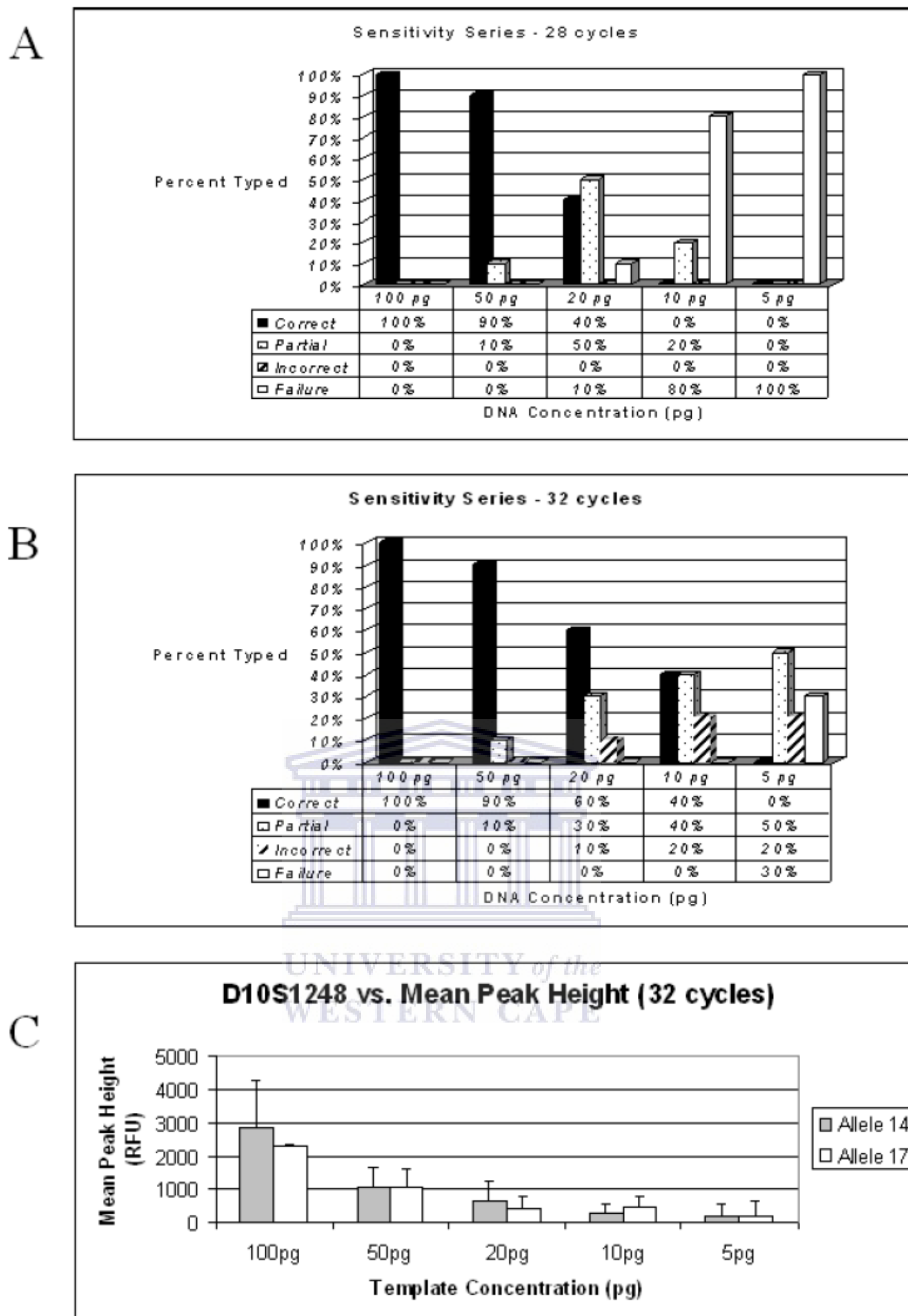

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Figure 1-17. Sensitivity results for DNA dilutions using the D10S1248 marker. Results are from 10 replicate PCR amplifications and were analyzed on the ABI 3100. An interpretational threshold of 50 relative fluorescent units (RFUs) was used for genotyping each dilution. Each replicate was typed as being either correct (matching the genotype at 1 ng amplification), partial (one allele in a heterozygote drops below 50 RFUs), incorrect (allele drop-in that creates a wrong genotype), or failure (no peaks were observed above 50 RFUs). (A) Results with 28 cycles of PCR amplification and 1U of Taq Gold polymerase. (B) Results with 32 cycles of PCR amplification and 2U of Taq Gold polymerase. These results show an increase in the successful typing at low template levels. However, the number of partial profiles and profiles having the incorrect typing can be increased with extra samples and extra polymerase. (C) Mean Peak Height values from samples amplified at 32 cycles with 2U AmpliTaq Gold polymerase for the D10S1248 marker (Coble and Butler, 2005).

To assess the performance of the novel triplexes on degraded DNA, tests were conducted on skeletal remains (Coble and Butler, 2005). DNA was extracted from 16 bone specimens in varying stages of decomposition. The extracts were typed with the novel triplexes and the PowerPlex 16 kit (Promega). One sample failed completely with all systems while the triplexes generated full profiles for the rest. The PowerPlex 16 kit (Promega) only produced one full profile. These results as well as the previous studies indicated that the novel miniSTR systems especially mini01 could be a useful addition to the current miniSTR sets.

The European DNA Profiling Group (EDNAP) and the European Network of Forensic Science Institutes (ENFSI) recently agreed on the inclusion of the mini01 loci in a group of core loci used by European laboratories. This was partly due to a collaborative study conducted by Dixon *et al*. 2005. In the study, the ability of typing systems to profile artificially degraded DNA samples was assessed by nine European and US labs. Each lab was supplied with a set of degraded DNA stains and DNA profiling systems. These included a Foren-SNP kit (The Forensic Science Service), the Miniplex systems and the preferred commercial kit of each laboratory.

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The mini-STR systems out performed the SNP kit as well as the common commercial kits. The NC01 mini-STR system gave the highest overall profile percentage and was the most consistent across all labs (Dixon *et al*. 2005). The consistency showed how robust miniSTR systems can be. While the SNP kit gave the lowest profile percentage and varied the most between labs. The reason given for this was the complexity of the SNP kit; it contained 65 separate primers which amplified 21 loci. These results led to the EDNAP and the ENFSI to adopt the NC01 loci as new core European loci and recommend that current core-loci be transformed into miniSTRs (Dixon *et al*. 2005; Gill *et al*. 2006).

1.7.6.7. Commercial MiniSTR Kits

Applied Biosystems has developed the first commercial miniSTR kit, the AmpFlSTR® MiniFiler™. The kit amplifies D13S317, D7S820, D2S1338, D21S11, D16S539, D18S51, CSF1PO, and FGA as well as the sex determining marker Amelogenin. The kit was launched in March 2007. An initial evaluation to examine

the AmpFlSTR® MiniFiler™ kits performance on human skeletal remains as well as other compromised samples has been conducted by Eisenberg *et al*. 2006. The study was part of a multi lab study and included a reproducibility, sensitivity and mixture study. In the reproducibility study four DNA samples were run in triplicate with a positive and negative control. While for the sensitivity study, positive control DNA dilutions with a concentration range of 31.25pg to1ng in a 25μL reaction were run in triplicate. For the mixture study two DNA samples were mixed as follows; 0:1, 15:1, 10:1, 7:1, 3:1, 1:1, 1:0 and run in triplicate.

The kit proved to be highly reproducible down to a concentration of 125pg/25μL (Eisenberg *et al*. 2006). Concentrations of 62pg/25μL and 32pg/25μL showed significant allele dropout and peak imbalance and limited data respectively. However full profiles were generated when 125pg/25μL was used, which was twice as sensitive as other commercial kits (Profiler Plus® *ID* and COfiler®) previously used in the lab. All mixtures could be typed, with major and minor contributors presented at all loci.

The AmpFlSTR® MiniFiler™ kit produced full profiles for degraded DNA samples from skeletal remains when commercial kits only produced partial profiles (Eisenberg *et al*. 2006). The Profiler Plus® *ID* and COfiler® kits consistently failed to amplify the following loci, FGA, D21S11, D18S51, D13S317, D7S820, D16S539, and CSF1PO when used on degraded DNA from skeletal remains. These are the larger loci of the kits and highlight the problem with using standard kits to analyse highly degraded DNA. The AmpFlSTR® MiniFiler™ kit produced full profiles when used on the same degraded samples. As can be seen the AmpFlSTR® MiniFiler™ kit has incorporated all the loci that have proven to be problematic with the commercial kits. Although the evaluation of the AmpFlSTR® MiniFiler™ kit is ongoing the overall results of the study indicate that it will be a useful addition to the miniSTR systems already in use to analyse degraded DNA from skeletal remains.

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1.8. Laboratories Involved in the Identification of Human Remains

There are academic, governmental, private and non-governmental laboratories worldwide that utilises DNA analysis to assist in the identification of human remains. Major laboratories included the following: the Center for Human Identification (CHI); the Armed Forces DNA Identification Laboratory (AFDIL); The Bode Technology Group and the International Commission on Missing Persons (ICMP). The Center for Human Identification is an academic laboratory located at the University of North Texas Health Science Center in the USA (Eisenberg *et al*. 2006; Ritter, 2007). It conducts DNA analysis on unidentified human skeletal remains, the relatives of missing persons and investigates new technologies that could aid in skeletal remains identification. The Armed Forces DNA Identification Laboratory is a governmental laboratory situated in Washington, USA (http://www.afip.org). It uses DNA analysis to identify US military personnel and civilians that have died in both recent and past conflicts around the world. The Bode Technology Group is a commercial service provider located in New York, USA (http://www.bodetech.com). To date it has analysed more than 40 000 forensic casework samples using both STR and mtDNA analysis and has assisted US labs in eliminating a backlog of 300 000 convicted offender samples. The International Commission on Missing Persons is a non-governmental organisation located in the former Yugoslavia (http://www.ic-mp.org). It's has a Forensic Science Department (FSD) that's responsible for tracing approximately 40 000 people that went missing as a result of conflicts in the former Yugoslavia. The forensic DNA laboratory is located in Banja Luka, Republika Srpska, Bosnia and Herzegovina. These activities of these laboratories are briefly considered below.

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1.8.1. Center for Human Identification

The Center for Human identification (CHI) provides free DNA testing of skeletal remains and missing persons direct or family reference samples as well as anthropological examinations of the skeletal remains to all law enforcement agencies in the USA (Eisenberg *et al*. 2006; Ritter, 2007). The CHI is located at the University of North Texas Health Science Center. It's able to provide free mtDNA, STR as well as forensic anthropological analysis to all law enforcement agencies due to significant funding by the National Institute of Justice (NIJ). This collaboration with the NIJ has also made it one of a few labs that has access to the FBI CODIS(mp) database, which

contains data on unidentified human remains and missing persons cases including nuclear and mitochondrial data (Ritter, 2007). This means that all law enforcement agencies in the US now have access to the database via the CHI.

The development and dissemination of DNA collection kits has led to an increase in samples being received by the lab (Ritter, 2007). A problem identified by the director of the CHI, Arthur Eisenberg was the lack of family reference data in the database. Reference sample data is essential to the identification process of human skeletal remains. Therefore the lab developed a family DNA reference sample collection kit as well as a human remains collection and transportation kit. These kits have been made freely available to the medico legal community as well as all law enforcement agencies in the US and have led to the lab receiving 1200 family reference samples and 680 unidentified human remains as of July 2006.

A major part of the funding received by the NIJ was to investigate new technologies, which include both CODIS and non-CODIS based miniSTR systems to aid in human skeletal remains identification (Eisenberg *et al*. 2006). The miniSTR systems NC01 and NC02 have both been evaluated by the lab and recently it has aided in the development and evaluation of the first commercial miniSTR kit, the AmpFlSTR® MiniFiler™ kit (Applied Biosystems).

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The CHI will soon be implementing a high throughput system to assist with their caseload, which is certain to increase dramatically in the coming years (Ritter, 2007). This involves implementing a robotic system. With each robot able to analyse over 17000 samples a year.

1.8.2. Armed Forces DNA Identification Lab

Since its inception in 1991 the Armed Forces DNA Identification Lab (AFDIL) has been regarded as a leader in the identification of human remains (http://www.afip.org). Its mission in partnership with the Office of the Armed Forces Medical Examiner (OAFME) and the Central Identification Laboratory, Hawaii (CILHI) is to identify US military personnel and civilians that have died in recent and past conflicts (Edson *et al*.2004). AFDIL has experience with samples which have been exposed to various conditions including humid jungles, mountaintops and even

drenched in jet fuel and saltwater. This has enabled AFDIL to develop highly effective protocols for the identification of human skeletal remains.

ADFIL consists of two sections, a Nuclear DNA (nucDNA) Section and a Mitochondrial DNA (mtDNA) Section. The nuclear DNA section handles current US military personnel and civilian death cases. The mtDNA sections priority is to identify skeletal remains from the Vietnam War, Korean War, and World War II. Since large scale mtDNA sequencing was implemented by AFDIL, it has had an 85% success rate (http://www.afip.org).

1.8.3. The Bode Technology Group

The Bode Technology Group is probably the most respected forensic DNA analysis laboratory worldwide. This respect has been gained by the role it has played in identifying victims of war including the Bosnian and Vietnam wars, natural disasters like the tsunami in Thailand and aircraft disasters but most of all for its work in the World Trade Center (WTC) attack (http://www.bodetech.com). To date it has analysed more than 40 000 forensic casework samples using both STR and mtDNA analysis and has assisted US laboratories in eliminating a backlog of 300 000 convicted offender samples. It offers these services not only to agencies and organisations in the US but worldwide.

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Bode developed a high quality, high throughput system to analyse DNA from skeletal remains from the WTC disaster (Holland *et al*. 2003). They recognised that conventional methods although robust were time consuming and labour intensive and therefore new approaches were needed to process the volume of samples from the WTC attack. The most time consuming and labour intensive step involved the initial sampling and cleaning of bone. A modified sampling protocol was implemented reducing the sampling time from 20 minutes to 3-4 minutes (Holland *et al*. 2003). In addition a new high throughput extraction method was also developed. A 96 well DNA extraction procedure was implemented by modifying the standard protocol for the QIAamp 96 DNA Blood Kit (Qiagen) (Holland *et al*. 2003). Overall this increased the DNA extraction success rate and allowed for the extraction of over 1000 samples per week.

The initial STR analysis of DNA samples focussed on the use of commercial kits but later included the use of miniSTR systems, which dramatically increased the success rate. Bode initially relied solely on the use of the Profiler Plus (Applied Biosystems), Cofiler (Applied Biosystems) and PowerPlex 16 kits (Promega) for STR analysis (Holland *et al*. 2003). For those samples that did amplify with the commercial kits it was observed that the larger loci of the kits amplified poorly and frequently dropped out. Therefore they developed proprietary miniSTR systems (BodePlex 1 and 2) incorporating those loci that were observed to be problematic. The use of the miniSTR systems tripled the amplification success rate of the problematic loci. Overall the development of the Bode high throughput system helped the WTC victim identification effort tremendously and together with work done by other labs 54% of the victims were identified as of March 2004 (Marchi, 2004).

1.8.4. International Commission on Missing Persons

The International Commission on Missing Persons (ICMP) was formed in 1996 and soon established a Forensic Science Department (FSD) to address the issue of approximately 40 000 people missing as a result of conflicts in the former Yugoslavia (http://www.ic-mp.org). Many of the missing were believed to be buried in mass graves. The ICMP had to setup a human remains identification program. It consists of three divisions: [1] the Excavations and Examination Program, which locates, recovers and undertakes anthropological examinations on remains; [2] the Identification Coordination Division, which collects family reference samples, does the initial sampling and cleaning of bones in preparation for DNA extraction and manages software as well as the storage of samples and DNA reports and [3] the DNA Laboratories program, which extracts DNA from samples, conducts DNA analysis and generates and reviews reports. To date it has identified 8000 of approximately 13000 bodies that have been recovered. Besides its work in the former Yugoslavia it also helped in the South-East Asia Tsunami and helps the Iraqi government as well as other countries that have a history of large numbers of missing persons.

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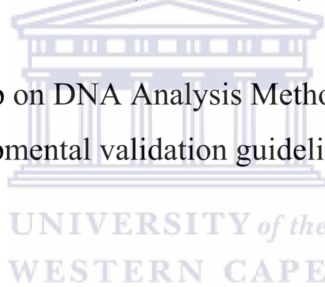
1.9. Validation

Validation is the process used by the Forensic Science community to demonstrate that a procedure is reproducible, reliable and robust (Butler, 2001). A validation study should be performed by a DNA laboratory or the scientific community before methods are put to routine use. It indicates whether the method is suitable for the intended purpose and identifies the limitations of the method and the aspects that are critical to its reliability. There are two kinds of validation, developmental and internal validation.

1.9.1. Developmental Validation

Developmental validation demonstrates that a novel method is suitable for its intended purpose by determining its conditions of reliability and its limitations. It's conducted by various groups including manufacturers, government laboratories, technical organizations and academic institutions (Butler, 2001).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The Scientific Working Group on DNA Analysis Methods (SWGDAM) has recommended a set of developmental validation guidelines which are presented in appendix I.

1.9.2. Internal Validation

An internal validation is the in-house demonstration of the limitations and the conditions of reliability of an established procedure (one that has undergone developmental validation) (Butler, 2001). It should be conducted by forensic DNA laboratories before the procedure is applied to casework.

SWGDAM suggests the following studies be conducted as part of an internal validation and comprise a minimum of 50 sample runs.

1. Known and non-probative evidence samples: The method must be evaluated and tested using known samples and, when possible, authentic case samples; otherwise, simulated case samples should be used. DNA profiles obtained from questioned items should be compared to those from reference samples. When previous typing results are available, consistency as to the inclusion or exclusion of suspects or victims within the limits of the respective assays should be assessed.

2. Reproducibility and precision: The laboratory must document the reproducibility and precision of the procedure using an appropriate control(s).

3. Match criteria: For procedures that entail separation of DNA molecules based on size, precision of sizing must be determined by repetitive analyses of appropriate samples to establish criteria for matching or allele designation.

4. Sensitivity and stochastic studies: The laboratory must conduct studies that ensure the reliability and integrity of results. For PCR-based assays, studies must address stochastic effects and sensitivity levels.

5. Mixture studies: When appropriate, forensic casework laboratories must define and mimic the range of detectable mixture ratios, including detection of major and minor components. Studies should be conducted using samples that mimic those typically encountered in casework.

6. Contamination: The laboratory must demonstrate that its procedures minimize contamination that would compromise the integrity of the results. A laboratory should employ appropriate controls and implement quality practices to assess contamination and demonstrate that its procedure minimizes contamination.

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7. Qualifying test: The method must be tested using a qualifying test. This may be accomplished through the use of proficiency test samples or types of samples that mimic those that the laboratory routinely analyzes. This qualifying test may be administered internally, externally, or collaboratively.

1.10. Investigation, Recovery and Analysis of Human Skeletal Remains in the Context of the Political Violence in South Africa

The Truth and Reconciliation Commission (TRC) was created in 1995 to uncover details about human rights violations committed during the period March 1960-May 1995 in South Africa (http://www.brandonhamber.com/publications/pap_khulumani.doc).
The TRC provided a forum where the testimony of survivors and perpetrators could be heard. Perpetrators who provided a full account of their actions were granted amnesty while the victims and families received reparations. By the end of the commission over 20 000 victims had made submissions and over 7000 perpetrators applied for amnesty.

The issue of missing persons was consistently encountered by the commission. It was realized that to fully investigate disappearances related to human rights abuses an investigation unit that comprised of experts in human rights investigations and an array of forensic fields was required. Therefore it turned to the National Prosecuting Authority (NPA). A team was formed within the NPA which had access to state archives including police and military as well as other required resources. The team was referred to as the South African Missing Persons Task Team (SAMPTT).

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The SAMPTT formed a strong partnership with the Argentine Forensic Anthropology Team (Equipo Argentino de Antropología Forense, EAAF). The EAAF were among the first to apply forensic anthropology and archaeology to human rights violations investigations (http://eaaf.typepad.com/). The EAAF has always been committed to training and building capacity in the countries in which it operates.

In 2005, the newly established Forensic DNA Laboratory of the University of the Western Cape was invited to attend one of the EAAF run training programs. The objective of the training program was to introduce South African professionals and students to the EAAF forensic investigation methods. The training consisted of lectures on the preliminary investigation, crime scene investigation and laboratory methods and analysis required for human rights violations investigations.

It was during this training program that the Forensic DNA Laboratory of the University of the Western Cape formed a relationship with both the SAMPTT as well as the EAAF.

1.11. Objectives of this Study

The objective of the study was to conduct an internal validation on miniSTR systems and apply it to cases received from the SAMPTT. This was prompted by the fact that miniSTR systems have been shown to out perform commercial kits and provide an alternative to mtDNA when analysing degraded DNA from skeletal remains and that the DNA extracted from skeletal remains received from the SAMPTT would be degraded due to the remains generally being fragmented or charred and buried for at least 20 years.

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Chapter 2: Validation Studies

2.1. Introduction

The recent demonstration of miniSTR systems as an effective tool for the analysis of degraded DNA prompted the Forensic DNA Laboratory at the University of the Western Cape (UWC) to conduct internal validation studies on miniSTR systems. At the time the Laboratory was conducting mtDNA analysis on skeletal remains received from the Missing Persons Unit of South Africa. The skeletal remains were of individuals thought to have been victims of South Africa's past political violence. The use of miniSTR systems in addition to mtDNA analysis would allow for more information to be gathered in each case. Internal validation studies were conducted to evaluate the performance of the miniSTR systems. As discussed in chapter 1, the original miniSTR systems consist of five multiplex reactions each amplifying three STR loci. The loci included in each multiplex are as follows. Miniplex 1: TH01, CSF1PO, TPOX, Miniplex 2: D5S818, D8S1179, D16S539, Miniplex 3: FGA, D21S11, D7S820, Miniplex 4: vWA, D18S51, D13S317 and Miniplex 5: Penta D, Penta E, D2S1338. Of these fifteen loci, twelve form part of the Combined DNA Index System (CODIS) loci set. Uniplex amplification of D3S1358 was required to obtain a full 13 locus CODIS genotype.

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2.2. Materials and Methods

2.2.1. DNA Extraction

One whole blood control sample was extracted following the QIAamp Blood Mini Kit protocol (Qiagen) according to the manufactures instructions (Appendix I).
Blood stained materials were extracted following the Chelex protocol described in the Applied Biosystems Profiler Plus User Manual (Appendix I).

2.2.2. DNA Quantification

DNA samples were quantified using a Nanodrop ND 1000 UV-Vis Spectrophotometer.

2.2.3. PCR Amplification

Amplifications were performed in a final reaction volume of 10µL. Unless otherwise specified the reactions contained 250pg genomic DNA, 1X Supertherm PCR buffer

with 15mM $MgCl_2$, 200µM dNTPs, 0.8 U of Supertherm GoldTaq, and Miniplex primers (Miniplex 1: 0.075µM TH01, 0.3µM CSF1PO, 0.2µM TPOX, Miniplex 2: 0.3µM D5S818, 0.5µM D8S1179, 0.1µM D16S539, Miniplex 3: 0.3µM FGA, 0.5µM D21S11, 0.5µM D7S820 and Miniplex 4: 0.2µM vWA, 0.4µM D18S51, 0.5µM D13S317). Primers were synthesized by Applied Biosystems using previously reported sequences (Butler *et al*. 2003). The standard operating procedure for preparing the primer mixtures can be found in Appendix I.

PCR amplification was performed using a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems). Thermal cycling conditions were:
1 cycle at 95°C for 10 minutes, 33 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by a final cycle at 60 ^{o}C for 45 minutes. Allelic ladders were prepared by re-amplifying diluted commercial kit ladders with Miniplex primers as described previously (Butler *et al*. 2003).
Modifications to the general protocol are indicated where relevant in the results and discussion section.

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2.2.4. Detection and Data Analysis

Amplicons were analysed using an ABI PRISM 377 DNA Sequencer. Samples were prepared for electrophoresis by adding 1µL loading mix (5µL HiDi formamide, 1.5µLGenescan ROX 500 size standard, 1.5µL Dextran blue loading dye Applied Biosystems) to1µL PCR product. Following denaturation of the samples at 95 ^{o}C for 5 to 9 minutes in a GeneAmp PCR System 9600 thermal cycler (Applied Biosystems), the samples were snap-cooled on ice for 2 minutes before loading 1µL of each sample on a 5% Long Ranger gel (BioWhittaker Molecular Applications) (Detailed protocols are included in Appendix I).

Electrophoresis was conducted and the data analysed according to manufacturer's instructions. The gel was run with filter set D for 2 ½ hours at 2400 scans per hour and the data collected with ABI 377 collection software and analyzed with GeneScan 3.0.0 software (Applied Biosystems). Allele sizes and allele designations were subsequently assigned using Genotyper 3.7 software (Applied Biosystems).

2.3. Results and Discussion

2.3.1. Reaction Volume Study

The reduction of PCR reaction volume saves valuable reagents and reduces the amount of DNA template required. DNA recovered from skeletal remains and crime scenes is often degraded and limited and therefore it has to be used efficiently.

The amplification efficiency of miniSTR systems was assessed for the following reaction volumes: 10, 12.5, 25 and 50µL. Reactions were tested with a constant DNA concentration of 40pg per µL (i.e. the 10µL reaction contained a total of 400pg DNA while the 50µL reaction contained a total of 2000pg). Reactions were run for 33 cycles. As expected, full profiles were observed for all reaction volumes tested. The reaction volumes of 10 and 12.5µL generating marginally better results in terms of average peak height than the 25 and 50µL reactions (Figure 2-1). There was no clear advantage in using 25 or 50µL reaction volumes for any of the Miniplex systems. Using these volumes would potentially waste of reagents and template DNA. A reaction volume of 10µL was therefore adopted for all subsequent Miniplex reactions.

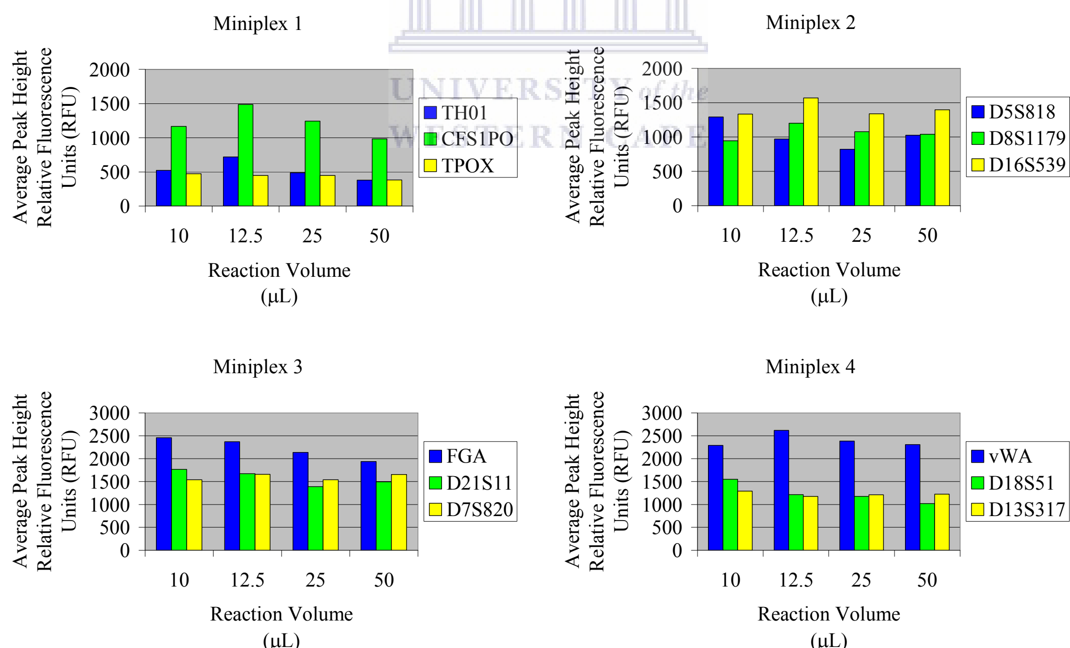

Figure 2-1. Reaction Volume Study. The change in fluorescent signal intensity as a function of reaction volume is shown for Miniplex 1, 2, 3 and 4. All samples were amplified at 33 cycles. The reaction volumes of 10 and 12.5µL generating marginally better results in terms of average peak height than the 25 and 50µL reactions. A reaction volume of 10µL was therefore adopted for all Miniplex systems. The RFU values for heterozygous loci were summed and then averaged.

2.3.2. Sensitivity Studies

Since the DNA recovered from degraded samples is often at a low concentration a typing system designed to amplify degraded DNA needs to have a high sensitivity.

To assess the sensitivity of the miniSTR systems, varying amounts of control DNA were added to 10μL Miniplex reactions for 33 cycles. The amounts of template DNA added were 10pg, 50pg, 100pg, 250pg, 500pg and 1000pg. At concentrations of 250pg and above full profiles were generated for all miniSTR systems (Figure 2-2). While at 100pg, allele dropout was evident for the TH01 and TPOX loci of Miniplex 1 and D8S1179 for Miniplex 2, however Miniplex 3 and 4 generated full profiles. At 50pg only Miniplex 3 generated a full profile while allele dropout was observed for Miniplex 1, 2 and 4. At 10pg complete allele dropout was observed for all miniSTR systems except one allele of vWA from Miniplex 3.

Overall these results indicate that of the concentrations tested at least 250pg/10μL is required in order to avoid allele dropout and to generate good quality profiles for all miniSTR systems at 33 cycles. However in situations where highly degraded DNA has to be analysed this might not be ideal. The DNA concentrations of highly degraded samples are often low and therefore it might be impossible to add a total of 250pg to each miniSTR system amplification reaction.

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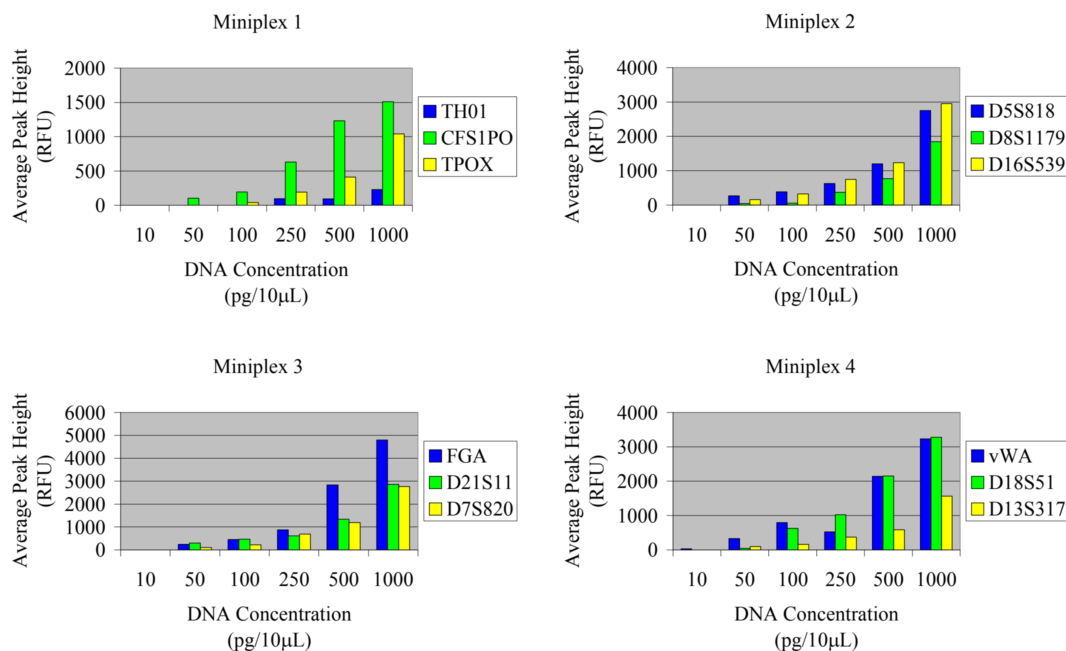

Figure 2-2. Sensitivity studies. The change in fluorescent signal intensity as a function of template concentration is shown for Miniplex 1, 2, 3 and 4. All samples were amplified at 33 cycles. At concentrations of 250pg and above full profiles were generated for all miniSTR systems.

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2.3.3. PCR Cycle Number Study

The sensitivity of any PCR-based method can be improved by increasing the PCR cycle number. The standard number of amplification cycles for multiplex systems is 28-30 (Gill, 2001). The range of sample types that can be analysed has been increased by increasing the PCR cycle number above the standard. DNA profiles have been obtained from epithelial cells originating from strangulation tools using 30-31 cycles (Wiegand *et al*. 2000), telogen hairs using 35-43 cycles (Barbaro *et al*. 2000), fingerprints from tools using 28-40 cycles (Van Hoofstat *et al*. 1999) and 70 year old bones from the Romanov family using 38-43 cycles (Gill *et al*. 1994). Gill *et al*. 2000 also demonstrated that with the SGM and AmpFlSTR SGM Plus kits (Applied Biosystems) it is possible to analyse samples with less than 100pg template DNA using 34 cycles.

The effect that cycle number has on the sensitivity of the miniSTR systems was evaluated by amplifying 50pg and 100pg control DNA samples per 10µL PCR reaction at 28, 33, 38 and 43 cycles (Figure 2-3). At 28 cycles, no amplification was observed for 50pg/10µL or 100pg/10µL for any of the miniSTR systems.

Miniplex 1 performed as follows. At 33 cycles, for 50pg/10μL, complete dropout was observed for the TH01 and TPOX loci (Figure 2-3). At 38 cycles, complete dropout was observed for TH01 but alleles were observed for TPOX, which failed at 33 cycles. At 43cycles, over-amplification was observed for all loci. At 33 cycles, for 100pg/10μL, the complete dropout of CFS1PO and one allele of TH01 were observed. At 38 cycles, a full profile was generated while over-amplification was observed at 43 cycles.

Miniplex 2 performed as follows. At 33 cycles, for 50pg/10μL, complete dropout was observed for D5S818 and D8S1179 (Figure 2-3). At 38 cycles, alleles were observed for D5S818 and D8S1179, which failed at 33 cycles. At 43 cycles, a full profile was generated. At 33 cycles, for 100pg/10μL, the drop out of one allele of D16S539 was observed. At 38 cycles, a full profile was generated. At 43 cycles, over-amplification was observed for all loci.

Miniplex 3 performed as follows. At 33 cycles, for 50pg/10μL, one allele of D7S820 and complete dropout was observed for D21S11. At 38 cycles, alleles were observed for D21S11 but the drop out of one allele of D7S820 was still observed. At 43 cycles, no significant improvement was observed. At 33 cycles, for 100pg/10μL, a full profile was generated. However at 38 cycles, the drop out of one allele of D7S820 was observed. The fact that this allele was observed at 33 cycles suggests that preferential amplification due to stochastic effects was the reason behind the allele drop out at 38 cycles. When low levels of DNA (100pg or less) are used in PCR reactions an unequal sampling of the two alleles present from a heterozygous individual can occur, this is what is referred to as a stochastic sampling effect. This results in the preferential amplification of one allele, which can cause drop out of the other allele or severe allele imbalance. At 43 cycles, over-amplification was observed for all loci.

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Miniplex 4 performed as follows. At 33 and 38 cycles, for 50pg/10μL drop out was observed for D18S51 and D13S317 (Figure 2-3). At 43 cycles, no significant improvement was observed. At 33 and 38 cycles, for 100pg/10μL, full profiles were generated. At 43 cycles, over-amplification was observed.

Overall an increase in cycle number increased the sensitivity of the Miniplex systems. For 50pg/10μL at 33 cycles, 43% (10 out of 23) of the expected control DNA profile alleles were observed. While at 38 cycles, this increased to 65% (15 out of 23). For 100pg/10μL at 33 cycles, 82.60% (19 out of 23) of the expected control DNA profile alleles were observed. While at 38 cycles, this increased to 95.65% (22 out of 23). Miniplex 1 and 2 exhibited sensitivity down to 100pg at 38 cycles while Miniplex 3 and 4 exhibited sensitivity down to 100pg at 33 cycles. However the high amplification of certain alleles (RFU>5000-6000) for Miniplex 1 and 2 at 38 cycles suggests that lower cycle numbers could be used successfully. Therefore when a DNA concentration of less than 250pg is used then a cycle number in the range of 34-37 cycles is recommended.

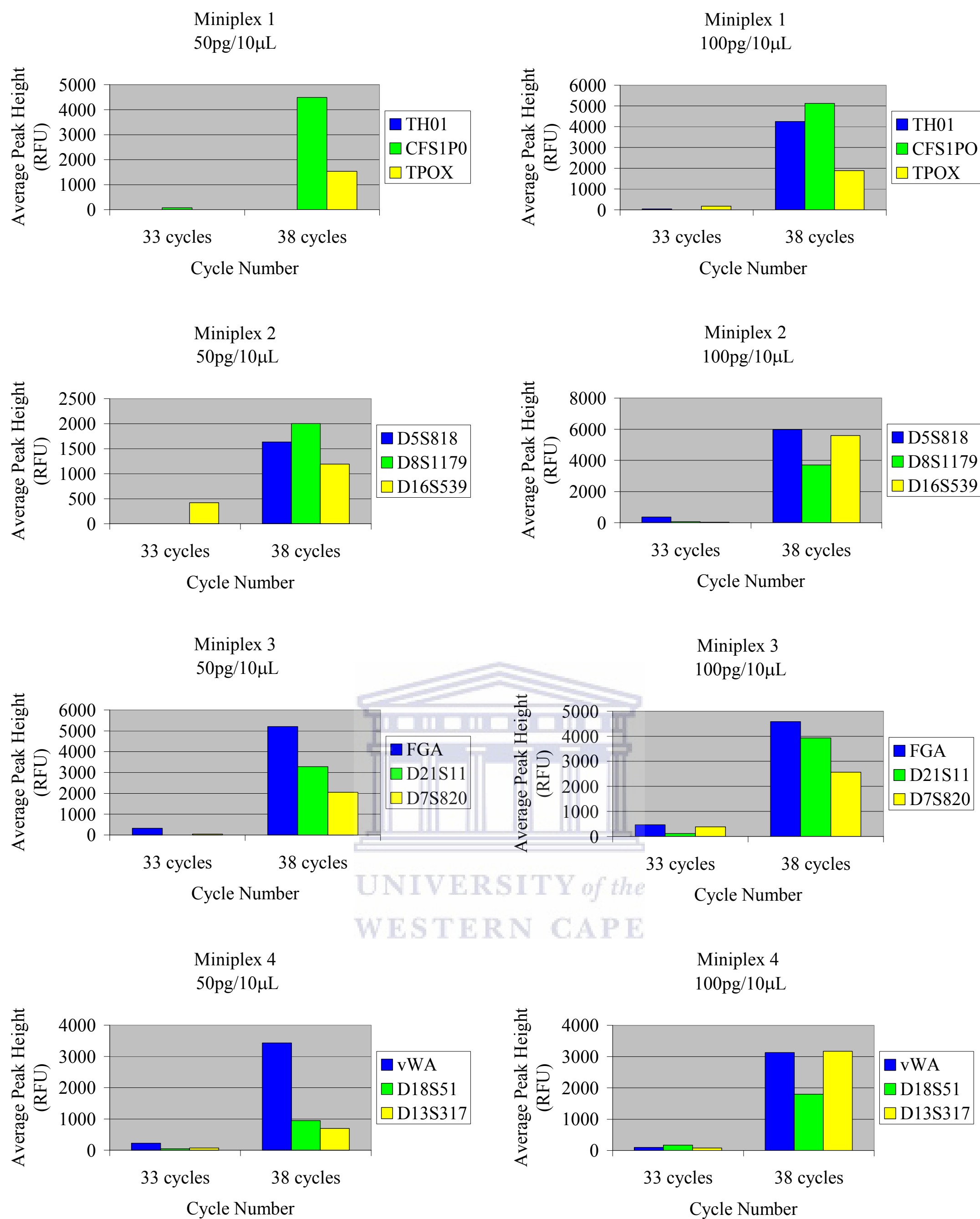

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Figure 2-3. Cycle number study. The change in fluorescent signal intensity as a function of cycle number and template concentration is shown for Miniplex 1, 2, 3 and 4. Miniplex 1 and 2 exhibited sensitivity down to 100pg at 38 cycles while Miniplex 3 and 4 exhibited sensitivity down to 100pg at 33 cycles.

2.3.4. Reproducibility and Precision

To ensure that the miniSTR systems generated consistent genotypes from DNA samples of the same donors, a reproducibility and precision study was conducted. DNA samples that were extracted from the blood of two individuals were utilised in this study. To test the reproducibility, the samples were amplified and analysed on two separate occasions using the standard conditions except that the miniSTR D3S1358 was included so that all 13 CODIS loci would be amplified. To test the precision of allele sizing, one of the samples was analysed an additional three times. The average allele size and standard deviation was determined.

For the reproducibility study, consistent genotypes were obtained for both samples and high precision was observed. No differences were observed between the genotypes determined for the first and second amplifications.

For the precision study, the standard deviation did not exceed 0.158 bases (Table 2-1), a reasonable value given that a standard deviation of 0.2 bases is considered acceptable (http://www.cstl.nist.gov/biotech/strbase/training.htm). However forensic laboratories that utilise capillary-based genetic analysers and DNA sequencers generally report standard deviation values under 0.15bp. In this study the gel-based ABI PRISM 377 DNA Sequencer (Applied Biosystems), which is generally believed to be less precise than the capillary-based genetic analysers and DNA sequencers was utilised. The study demonstrated that the miniSTR systems could generate reproducible results with high precision.

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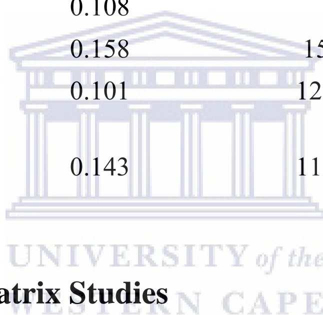
Table 2-1. Precision Study. To test the precision of allele sizing, one control sample was analysed four times and the average allele size and standard deviation determined.

Locus	Mean Size Allele 1 (bp)	Standard Deviation Allele 1 (bp)	Mean Size Allele 2 (bp)	Standard Deviation Allele 2 (bp)
TH01	81.298	0.072	88.432	0.127
CFS1PO	113.414	0.085	117.414	0.059
TPOX	84.032	0.079	100.426	0.06
D5S818	126.714	0.045	130.836	0.053
D8S1179	106.646	0.111	110.586	0.15
D16S539	105.124	0.097	113	0.06
FGA	157.032	0.114	160.97	0.157
D21S11	175.182	0.148	179.196	0.097
D7S820	164.678	0.064	172.594	0.079
vWA	120.38	0.108		
D18S51	143.18	0.158	155.41	0.104
D13S317	119.176	0.101	123.184	0.066
D3S1358	100.214	0.143	112.302	0.05



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2.3.5. Environmental and Matrix Studies

DNA samples recovered from crime scenes are often deposited on a variety of substrates that can negatively affect the quality of the samples. Depending on the environmental conditions and substrate, DNA degradation and PCR inhibition can be a problem. Many clothes and other materials commonly found at crime scenes contain pigments and dyes that can inhibit PCR reactions.

For this study, two to three drops of blood were deposited onto three substrates commonly found at crime scenes and extracted using the Chelex extraction method. Two sets of the following substrates were used: denim, cotton and leather. After being allowed to dry one set was stored in a cupboard for a month while the second set was stored outside the laboratory, totally exposed (but sheltered from rain) to the environment for a month prior to analysis .

The amplification efficiency of the samples stored in the cupboard was affected more than those stored outdoors. For the outdoor samples no allele dropout was observed

for any of the Miniplex systems and only the vWA locus produced an average peak height below 3000 RFU (Figure 2-4). Any DNA degradation that may have occurred during its exposure to the environment or any inhibitors that may have been co-extracted with the DNA as is evident from the results, certainly did not influence the Miniplex systems amplification efficiency. However the amplification efficiency of certain loci of the indoor samples was severely reduced. The average peak heights of TH01 and TPOX of Miniplex 1 and D5S818 and D8S1179 of Miniplex 2 of the denim were markedly lower than that of the outdoor denim (Figure 2-4). For the cotton, a reduction in amplification efficiency of all loci of Miniplex 1 and 3 as well as D8S1179 and D16S539 of Miniplex 2 and D18S51 and D13S317of Miniplex 4 were observed. While for the leather sample the dropout of one TH01 allele of Miniplex 1 and the reduction in amplification efficiency of D8S1179 and D16S539 of Miniplex 2 and D18S51 of Miniplex 4 was observed.

DNA degradation and PCR inhibition are two possible reasons for the results observed for the indoor samples. It would be expected that the outdoor samples would be affected more by degradation than those stored indoors. The results observed for Miniplex 3 of the denim and leather samples certainly suggests that DNA degradation was not responsible for the reduction in amplification efficiency observed for these samples because it generates the largest sized alleles (+/- 157-179bp) of the control DNA profile. If degradation was responsible then it would be expected that Miniplex 3 would be the most severely effected of all Miniplex systems. The denim and leather results could therefore be attributed to inhibitors from the denim and leather that were co-extracted with the DNA. For the cotton results, however both Miniplex 1(+/- 81-118bp) and 3 (+/- 157-179bp) were significantly affected. If Miniplex 1 was affected by DNA degradation then it would be expected that Miniplex 3 would exhibit a lower amplification efficiency and allele dropout. In this case, however the amplification efficiency of Miniplex 3 was higher than Miniplex 1, which suggests that inhibitors may have been responsible for the reduction in amplification efficiency of Miniplex 1. The reduction in amplification efficiency of Miniplex 3 could be a result of either DNA degradation or inhibitors.

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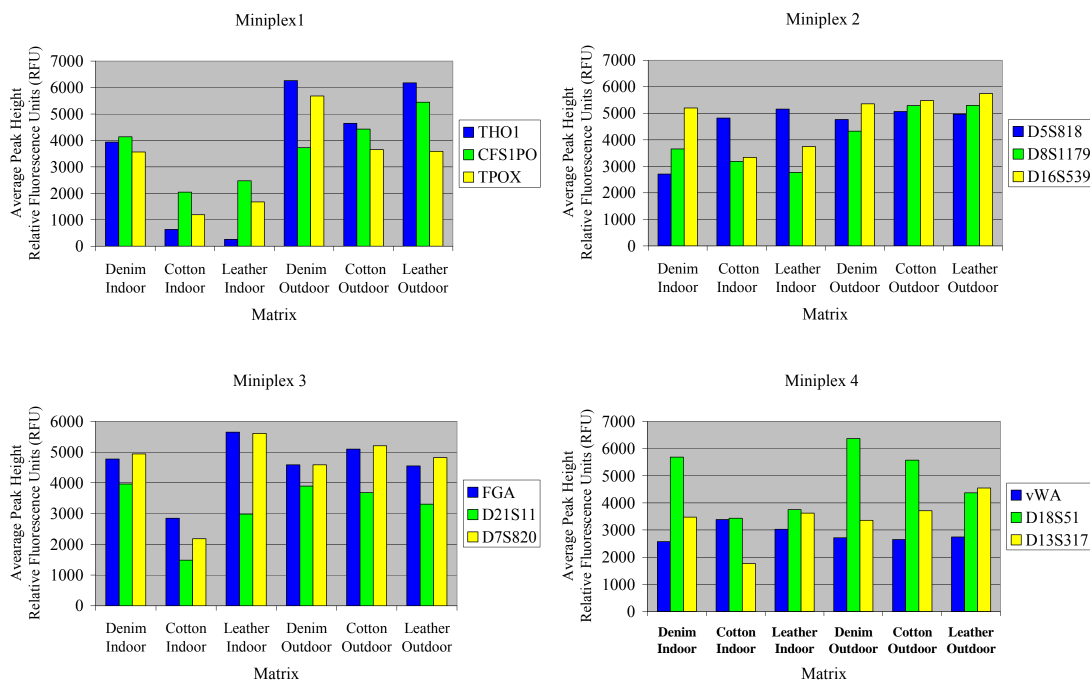

Figure 2-4. Environmental and Matrix Studies. The change in fluorescent signal intensity as a function of environmental and substrate exposure is shown for Miniplex 1, 2, 3 and 4. Two to three drops of blood were deposited onto three substrates commonly found at crime scenes. Two sets of the following substrates were used: denim, cotton and leather. After being allowed to dry one set was stored in a cupboard for a month while the second set was stored outside the laboratory, totally exposed (but sheltered from rain) to the environment for a month prior to amplification. All samples were amplified using 200pg template DNA in 10µL reactions for 35 cycles. The amplification efficiency of the samples stored in the cupboard was affected more than those stored outdoors.

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2.3.6. Mixture Studies

In many casework studies, DNA mixtures have to be analysed. Samples obtained from crime scenes often contain DNA from multiple donors and therefore the ability of STR multiplex systems to differentiate between the minor and major components of samples is important.

Peak ratio analysis can be of great help in these situations as it can help determine the quantity of DNA from the minor or major donors. The ratio of the minor to major component for a locus can be determined by dividing the average peak height of the minor alleles by the average peak height of the major alleles.

In this study, two control samples were mixed in the following ratios: 1/100, 1/10, 1/5 and 1/1 while keeping the template concentration of the major component constant at 250pg/10µL. The average peak height ratios of minor to major peaks were determined

for the following loci: CFS1PO (Miniplex 1), D16S539 (Miniplex 2), FGA (Miniplex 3) and D18S51 (Miniplex 4) (Figure 2-5). These loci were chosen because both DNA samples were heterozygous at each locus. The samples only shared one common allele and that was at the locus CFS1PO. At a ratio of 1/100, the minor component is present at 1% of the total amount of DNA template and therefore the average peak height ratio of minor to major peak is expected to be 0.01. At a ratio of 1/10, the minor component is present at 10% of the total amount of DNA template and therefore the average peak height ratio of minor to major peak is expected to be 0.1. At a ratio of 1/5, the minor component is present at 20% of the total amount of DNA template and therefore the average peak height ratio of minor to major peak is expected to be 0.2.

For the CFS1PO locus, the ratio of the minor to major component alleles reflected the ratio of input DNA (Figure 2-5). The average peak height of the minor component was above the laboratories Relative Fluorescence Units (RFU) threshold (50RFU) at all mixture ratios except 1/100. For the D16S539 locus, the ratio of the minor components above a 1/100 ratio was below what was expected (Figure 2-5). However the average peak height of the minor component was above the laboratories RFU threshold at all mixture ratios. For the FGA locus, the ratio of the minor component reflected the ratio of input DNA only at 1/1, while at 1/5 and 1/10 it was below and at 1/100 it was above the expected ratio (Figure 2-5). The average peak height of the minor component was above the laboratories RFU threshold at all mixture ratios except 1/100. For the D18S51 locus, the ratio of the minor component at no point reflected the ratio of input DNA and the average peak height of the minor component was below the laboratories RFU threshold at mixture ratios below 1/5 (Figure 2-5).

Overall the results of this study show that at a mixture ratio of 1/10 the minor components of the CFS1PO, D16S539 and FGA loci are detectable (RFU>50). As for D18S51, it is only detectable when the minor component is present at 20% (1/5 ratio) of the total DNA template.

This is a limited study and therefore a more detailed study is suggested in order to obain a better understanding of the overall ability of the miniSTR systems to differentiate between the minor and major components of samples. The study could

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involve the use of a number of control samples, which have no shared alleles for the loci being studied. This would provide a better understanding of the overall ability of the miniSTR systems to differentiate between the minor and major components.

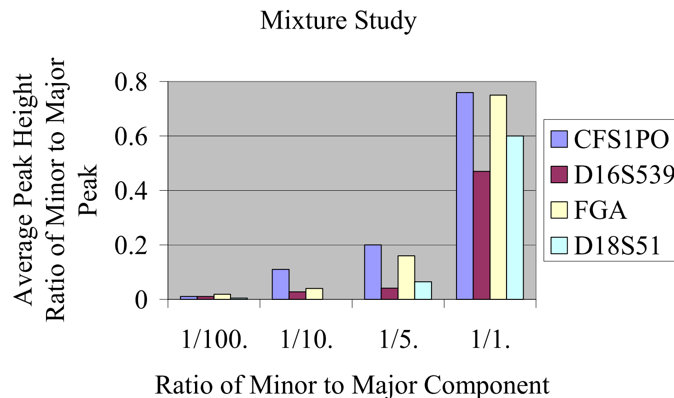

Figure 2-5. Mixture study. Two control samples were mixed in the following ratios: 1/100, 1/10, 1/5 and 1/1 while keeping the template concentration of the major component constant at 250pg/10μL. At a mixture ratio of 1/10 the minor components of the CFS1PO, D16S539 and FGA loci are detectable (RFU>50). As for D18S51, it is only detectable when the minor component is present at 20% (1/5 ratio) of the total DNA template.

2.3.8. Concordance Studies

In this study, nine samples that had previously been typed using the PowerPlex 16 kit (Promega) were typed using the Miniplex systems to examine the concordance between the two systems. Of the nine, one sample was a commercial standard DNA template (9947A) and the other eight were family reference samples that were previously typed by The Bode Technology Group. The nine samples were amplified and the results analysed using the standard conditions except that the miniSTR D3S1358 was included so that all 13 CODIS loci would be covered. Concordance was observed in 99% (200 out of 202) of all allele calls made. The two discrepancies were both observed for the locus D21S11 (Table 2-2).

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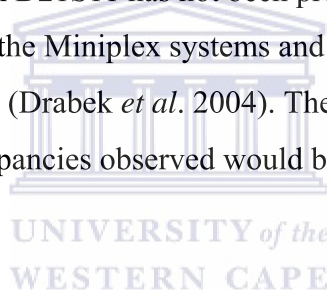
Table 2-2. Discordant STR profiling results observed between the PowerPlex 16 kit (Promega) and the Miniplex systems.

	Locus	Bode PowerPlex 16		Miniplex		Likely Cause
1	D21S11	35	**35.2**	35	**35**	allele 35.2 primer binding site or insert mutation
2	D21S11	**24.2**	27	**27**	27	allele 24.2 primer binding site mutation

There are three possible reasons for the observed discrepancies, firstly an insertion mutation could be present between the Miniplex primer binding site and the commercial kits binding site. An insert mutation present between the Miniplex primer binding site and the commercial kits binding site would not hinder amplification of either system but result in the systems generating different allele calls. The insertion would cause the commercial kit to generate a larger product for a given allele. Secondly, a mutation in the middle of a primer binding site would hinder primer binding and cause preferential amplification of an allele and result in allele peak height imbalance. An allele might be present but not called due to its peak height being below a laboratory RFU threshold. Thirdly, a mutation at the 3` end of a primer binding site would prevent the primer from binding and result in allele dropout.

It's difficult to determine exactly what mechanism could be responsible for the results as discordance associated with D21S11 has not been previously reported in concordance studies between the Miniplex systems and the PowerPlex16 (Promega) or AmpFlSTR Identifiler kits (Drabek *et al*. 2004). The only way to determine what was responsible for the discrepancies observed would be to sequence the relevant alleles.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Chapter 3: Case Study 1: DNA Analysis of 25 year old and Fragmented and Charred 20 year old Human Skeletal Remains

3.1. Introduction

This study involved two cases that were related to political violence and human rights abuses. The first case involved an uMkhonto weSizwe (MK) operative who was killed during a police ambush outside the town of Piet Retief in 1982. Remains thought to be those of the operative were exhumed in December 2005 and provided for DNA analysis.

The second case involved a commingled grave containing the fragmented remains of four adults. The remains were thought to be those of four activists abducted and murdered by the Northern Transvaal Security Police in June and July 1987. The individuals were tortured and their bodies dismembered with explosives. Fragmented skeletal remains were exhumed from an alleged grave site.

These remains were highly fragmented. This made it difficult for the Argentine Forensic Anthropology Team (EAAF) to identify the victims using conventional forensic anthropology. Therefore samples were sent to the Forensic DNA Laboratory at the University of the Western Cape (UWC) for analysis. This particular study was conducted to confirm earlier DNA analysis.

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3.2. Materials and Methods

3.2.1. Laboratory Setup

Skeletal remains preparation, DNA extraction and PCR amplification setup was conducted in a dedicated ancient DNA laboratory. This laboratory was physically and logistically separated from the Modern-DNA pre-PCR lab where reference samples were extracted and PCR amplifications were setup. Both pre-PCR areas were exposed to UV irradiation overnight and all surfaces were treated with bleach regularly. Disposable hair nets, lab coats and gloves as well as boots were always worn in the ancient DNA lab while standard protective equipment was worn in the Modern-DNA lab. This minimised the risk of contamination.

3.2.2. Sample Collection and Preparation

Family reference samples and skeletal remains were received from the South African Missing Persons Task Team (SAMPTT). For the first case, 1 family reference swab sample and 1 femur was received while for the second case, 13 family reference swab samples and 4 femur fragments were received. The femur fragments were obtained from remains that had been analyzed by the EAAF as part of their work for the SAMPTT. All femurs were stored in paper bags at room temperature prior to sampling, cleaning and milling.

The sampling procedure was the most time consuming. A dremmel tool was used to cut an appropriately sized sample from the femur. Surface material was then removed by sanding using the dremmel tool. Once the surface was removed the sample was photographed and then cut into smaller pieces to assist the milling processes. Each bone was sampled separately and the area dedicated to sampling was treated with bleach during and between each sampling session. This minimised the chances of cross contamination.

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Before the samples were milled they were cleaned to ensure the removal of contaminant DNA. The bone samples stored in the 50mL falcon tubes were immersed in bleach and rinsed with deionised water and ethanol before drying in a UV irradiated laminar flow hood.

Once the samples were cleaned they were ready to be milled using a 6750 freezer mill (Spex Certiprep). The milling began with 15 min of pre-cooling followed by 3 cycles of 2-min grinding and 2-min resting, with an impact frequency of 15. The standard operating procedures for bone sampling and cleaning as well as milling can be found in Appendix I.

3.2.3. DNA Extraction

DNA was extracted from reference buccal swabs using the Chelex protocol described in the Applied Biosystems Profiler Plus User Manual. The step by step protocol for this method can be found in Appendix I.

DNA was extracted from the milled bone samples using the organic extraction method described previously (Hagelberg *et al.* 1989). One to two grams of milled bone powder was incubated overnight at 37^0 C in 10 ml of Lysis buffer (0.5 M EDTA pH 8-8.5, 0.5 ml 10% *N*-lauroyl sarcosine and 200µL of the 20 mg/mL Proteinase K) in a shaking incubator. Water-saturated phenol (10 ml) was added and the solution vortexed and centrifuged for 10 minutes at 3000 r.p.m. The aqueous phase was then extracted twice with an equal mixture of water-saturated phenol and chloroform and the solution vortexed and centrifuged as before. Residual phenol was removed by one chloroform (10ml) extraction. The aqueous phase was recovered and concentrated using dialysis centrifugation. This was achieved using Centriplus 30 tubes centrifuged at max speed until the solution was concentrated down to 100-200ul. The extract was then washed twice with 10ml SABAX water to remove salt. The extracts were stored at -20^0 C. The step by step protocol for this method can be found in Appendix I.

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3.2.4. DNA Quantification

The family reference samples and bone DNA samples were quantified using an in-house developed TH01 quantification system. The system relies on the amplification of the TH01 locus from unknown and standard DNA samples and was adopted from a previously published protocol (Sifis *et al*. 2001). Instead of amplifying the samples with *Alu* primers and using quantitation standards with a range of 1pg to 100pg, samples were amplified with miniSTR TH01 primers and quantified against standards ranging from 3.33pg/µL to 100pg/µL.

Amplifications were performed in a final reaction volume of 10µL containing 3µL DNA, 10 x Supertherm PCR buffer with 15mM $MgCl_2$, 200µM dNTPs, 0.8 U of Supertherm Gold, and Miniplex primers (0.075µM TH01). Primers were synthesized by Applied Biosystems using previously reported sequences (Butler *et al*. 2003).

PCR amplification was performed using a GeneAmp 2700 or 2720 thermal cycler (Applied Biosystems). Thermal cycling conditions were:
1 cycle at 95°C for 10 minutes, 33 cycles at 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, followed by a final cycle at 60 °C for 45 minutes.

Amplicons were analysed using an ABI PRISM 377 DNA Sequencer. Samples were prepared for electrophoresis by mixing1µL PCR product with 1µL loading mix, which consisted of the following components at the indicated ratio: 5µL HiDi formamide, 1.5µL ROX 500: 1.5µL Dextran Blue loading dye (all Applied Biosystems). Following denaturation of the samples at 95 °C for 5 to 9 minutes in a GeneAmp PCR System 2700 or 2720 thermal cycler (Applied Biosystems), the samples were snap-cooled on ice for 2 minutes before loading 1µL of each sample on a 5% Long Ranger gel (BioWhittaker Molecular Applications) (The step by step protocol for the gel preparation and pouring can be found in Appendix I).

Electrophoresis was conducted and the data analysed according to manufacturer's instructions. The gel was run with filter set D for 2 ½ hours at 2400 scans per hour and the data collected with the ABI 377 collection software (Applied Biosystems), analyzed using GeneScan 3.0.0 software (Applied Biosystems) to determine peak heights. The construction of the standard curve and the quantitation of the extracts were achieved as described (Sifis *et al*. 2001).

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3.2.5. MiniSTR PCR Amplification

The family reference samples were amplified as previously described (Chapter 2, section 2.2.3). Specific modifications made to the reactions are considered in the results and discussion section.

3.2.6. Mitochondrial DNA PCR Amplification and Sequencing

Amplifications were performed in a final reaction volume of 30μL containing 600pg genomic DNA (Family reference samples) or 3μL Bone DNA extract, 1X Supertherm PCR buffer with 15mM $MgCl_2$, 0.16μg/mL BSA, 200μM dNTPs, 3U of Supertherm GoldTaq and the mtDNA HVI and HVII primers (Table 3-1) at 0.5μM each. The primers were synthesized by Applied Biosystems using previously reported sequences (Vigilant *et al*. 1989).

PCR amplification was performed using a GeneAmp PCR System 2700 or 2720 thermal cycler (Applied Biosystems). Thermal cycling conditions were:
1 cycle at 94°C for 10 minutes, 30 cycles (Family reference samples) or 35-40 cycles (Bone specimens) at 94°C for 45 seconds, 62°C for 45seconds, and 72°C for 45 seconds, followed by a final cycle at 72 °C for 5 minutes.

Table 3-1. mtDNA amplification and sequencing primers

Primer	Primer Sequence	Final Concentration	Product size
HVI_L15996	5' CTC CAC CAT TAG CAC CCA AAG C 3'	0.5μM	
HVI_H16405	5' CGG GAT ATT GAT TTC ACG GAG GA T 3'	0.5μM	454bp
HVII_L00029	5' GGT CTA TCA CCC TAT TAA CCA C 3'	0.5μM	
HVII_H00408	5' CTG TTA AAA GTG CAT ACC GCC A 3'	0.5μM	422bp

Agarose gel electrophoresis was used to verify PCR products. An aliquot of 5μL of each product was run on a 1.2% agarose gel stained with 0.1μg/mL Ethidium Bromide.

Following verification, the products were sequenced in the forward and reverse directions. The products were purified using ExoSAP-IT (USB Corporation) according to the manufacturer's instructions. The SAP (shrimp alkaline phosphatase) digests unincorporated dNTPs while the exonuclease digests residual primers. Aliquots of 2μL of ExoSAP-IT were mixed with 5μL of each product and incubated at 37 °C for 15min followed by 15min of denaturation at 80°C.

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Sequencing was performed using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to manufacturer's instructions. Sequencing was performed in a final reaction volume of 10µL containing 4.0µL ready reaction mix, 1µL mtDNA primer at 0.5µM each, and 1.5µL Sabax water and 3.5µL template DNA (ExoSAP-IT treated). The primers used for sequencing were identical to those used for amplification.

Cycle sequencing was performed using a GeneAmp PCR System 2700 or 2720 thermal cycler (Applied Biosystems). Thermal cycling conditions were:
1 cycle at 96°C for 1 minute, 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, followed by a hold at 4 ^{o}C.

The sequencing products were purified and precipitated using Ethanol/EDTA/Sodium Acetate Precipitation as described by the manufacturers. Briefly, 125mM EDTA, 3M Sodium Acetate and 100% Ethanol were added to each reaction prior to an incubation for 15 minutes at room temperature. The samples were then centrifuged for 30 minutes and the supernatant removed prior to the addition of 70% Ethanol and centrifugation for 15minutes. The supernatant was again removed and the samples incubated at room temperature for 40 minutes.

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Amplicons were analysed using an ABI PRISM 377 DNA Sequencer. In preparation for running, the samples were resuspended in 1.5µL loading mix (5µL formamide to 1µL Dextran Blue Loading Dye). Following denaturation of the samples at 95 ^{o}C for 5 to 9 minutes in a GeneAmp PCR System 2700 or 2720 thermal cycler (Applied Biosystems), the samples were snap-cooled on ice for 2 minutes before loading 0.8-1.2µL of each sample on a 5% Long Ranger gel (BioWhittaker Molecular Applications)

Electrophoresis was conducted according to manufacturer's instructions. The gel was run using filter set E for 10½ hours at 1200 scans per hour.

3.2.7. Detection and Data Analysis

The basic analysis of raw data was conducted using Genescan and Sequence analysis software (Applied Biosystems). Genotypes were then assigned manually. Victim and relative genotypes were compared both manually and using the excel program STR_MatchSamples that was developed by David Duewer, Analytical Chemical Division, National Institute for Standards and Technology.

3.3. Results and Discussion

Note: Names have been coded to protect the identities of the individuals involved. For further details about the cases contact the Forensic DNA Laboratory at the University of the Western Cape.

3.3.1. Forensic Specimens

The UWC laboratory was provided with five bone specimens THKY 01-G3, AA06, AA17, AA32 and AA35 for two cases. The femur specimen THKY 01-G3 was very well preserved (Figure 3-1). The shaft of the femur was not fragmented and had no fungal or mold growth. The femur specimens AA06 and AA17 were not well preserved. They were charred, severely fragmented and had fungal and mold growth (Figure 3-2). The femur specimens AA32 and AA35 although better preserved than specimens AA06 and AA17 were also charred, fragmented and had fungal and mold growth (Figure 3-2).

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Figure 3-1. Femur specimen THKY 01-G3 received from the SAMPTT for DNA analysis

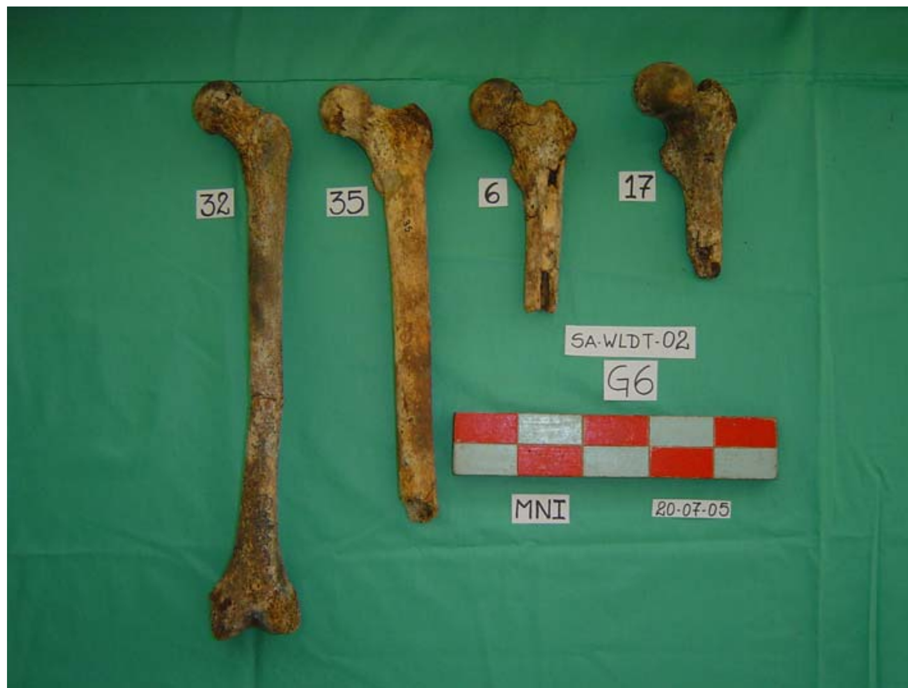

Figure 3-2. Femur specimens AA06, AA17, AA32 and AA35 received from the SAMPTT for DNA analysis

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3.3.2. DNA Quantification

The DNA concentrations of all the family reference samples as well as the bone samples were determined. Table 3-2 presents the DNA concentrations of family reference buccal swab DNA samples and Table 3-3 presents the DNA concentrations of the bone DNA samples. The DNA concentrations of the family reference samples show just how much the DNA yield from buccal swabs can vary. The highest concentration (15.5ng/µL) was observed for CM while the lowest concentration (2.2ng/µL) was observed for JM. The DNA concentrations of the bone specimens show the negative impact of high temperatures and bone fragmentation on DNA survival. Although bone specimen THKY 01-G3 was buried before bone specimens AA32 and AA35 it yielded a DNA concentration of 120pg/µL while the latter yielded DNA concentrations below 12pg/µL possibly because of exposure to high temperatures and explosive fragmentation.

Table 3-2. DNA Concentrations of Family Reference Buccal Swab DNA Samples

Victim	Relatives Samples	Kinship	DNA Concentration (ng/µL)
OF	1. RF	Mother	10
JAM	2. EM	Mother	9.6
	3. SM	Brother	NQ
HS	4. MT	Sister	NQ
	5. MOT	Nephew (Martha's Son)	NQ
	6. LS	Wife	5.7
	7. LOS	Daughter	4.9
AM	8. LAM	Maternal cousin	NQ
	9. MM	Wife	4.8
	10. KM	Daughter	3.6
	11. LM	Daughter	2.9
JUM	12. JM	Father	2.2
	13. CM	Sister	15.5
	14. JOM	Sister	7.6

(NQ) Not quantified as part of this study.

Table 3-3. DNA Concentrations of DNA Samples extracted from bone

Bone Samples	DNA Concentration (pg/µL)
1. AA06	NQ
2. AA17	NQ
3. AA32	8.74
4. AA35	11.78
5. THKY 01-G3	120

(NQ) Not quantified as part of this study.

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3.3.3. MiniSTR PCR Amplification

<u>Case 1</u>

<u>Background:</u> Mr OF was an uMkhonto weSizwe (MK) operative and a member of a MK squad based in Swaziland who was killed by security police during an ambush near Piet Retief in 1982 after returning from Swaziland (www.doj.gov.za/trc/media/1998/9805/s980506d.htm). It's believed that he was on his way to Durban on a secret mission when the ambush occurred. He boarded a taxi to Durban and revealed his political affiliation to the taxi driver during the trip. The driver is alleged to have contacted the security police who arranged an ambush. OF was killed during the ambush and buried in an unmarked grave.

Remains thought to be those of the operative were exhumed by the EAAF in December 2005 and a bone specimen (THKY 01-G3) was sent to the Forensic DNA Laboratory at UWC for DNA analysis. Full CODIS miniSTR profiles were determined for the bone specimen THKY 01-G3 and the relative's reference swab sample RF. The STR data is consistent with a parent-child relationship between the reference swab sample RF (the mother of OF) and the bone specimen THKY 01-G3 (Table 3-4).

The miniSTR systems worked well in this case. The quality of the profile generated for the bone specimen THKY 01-G3 was as good as the profile generated for the reference swab sample.

Table 3-4. Full CODIS miniSTR profiles of bone specimen THKY 01-G3 and reference swab RF. The STR data is consistent with a parent-child relationship between RF the mother of OF and bone specimen THKY 01-G3.

Locus	RF Mother of OF		Bone Specimen THKY 01-G3		Result
TH01	7	9	7	7	Not excluded
CFS1PO	11	13	10	11	Not excluded
TPOX	9	11	9	11	Not excluded
D5S818	10	13	11	13	Not excluded
D8S1179	14	14	12	14	Not excluded
D16S539	9	13	9	9	Not excluded
FGA	18	21	18	23	Not excluded
D21S11	28	32.2	28	29	Not excluded
D7S820	9	10	10	11	Not excluded
vWA	15	16	15	18	Not excluded
D18S51	18	19	15	18	Not excluded
D13S317	11	12	11	12	Not excluded
D3S1358	16	17	15	17	Not excluded

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Case 2

<u>Background:</u> This case involved the activists, JAM, AM, HS and JUM who were abducted, tortured and murdered by the Northern Transvaal Security Police in two separate incidents during June and July 1987. The incidents that led to there deaths and how their remains arrived in a single grave are relevant to the analysis and are considered below.

In the first incident, JAM was recruited by the Northern Transvaal Security Police but was suspected of being a double agent (www.stanford.edu/class/history48q/Documents/EMBARGO/2chap3.htm). He was abducted and taken to a deserted farm owned by the Pretoria Portland Cement Mine and interrogated. He denied the accusation but after torture, in the form of electric shocks, confessed that he was working for the ANC and gave the name of AM as his uMkhonto weSizwe (MK) contact (http://www.khulumani.net/content/view/620/164/) After confirming that AM was a courier for the ANC by checking Security Branch office files, AM was also abducted, and interrogated (www.stanford.edu/class/history48q/Documents/EMBARGO/2chap3.htm). AM in turn gave the name of HS as an MK operative. AM was forced to phone HS and arrange a meeting to facilitate his abduction. During subsequent interrogations all three activists were electrocuted. The bodies were transported to a remote area of Bophuthatswana and dismembered with landmine explosions.

In the second incident, JUM was abducted by Security Police in Eesterust, Pretoria and taken to a private farm, Klipdrift, north of Pretoria where he was tortured and interrogated for a week. In this time it became clear that he was not going to provide information, so a decision was made to kill him. It's believed that he was drugged, his head smashed with a spade and his body driven to a remote rural road near Phokeng, Rustenberg. Here landmines were attached to his body and detonated.

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To locate the remains of these activists an extensive investigation was conducted by the South African Missing Persons Task Team (SAMPTT). Careful analysis of the state mortuary registers for 1986 and 1987 revealed that a total of sixteen paupers including fragmented remains from four individuals were removed for pauper burials at Winterveld Cemetery near Pretoria on 22 September 1987. The remains of JAM, AM and HS were thought to be part of this group. Records also indicated that the remains of JUM were buried as part of this group.

Although there were sixteen paupers, only eight burial sites were paid for. The four fragmented remains cases were among those for which no sites were purchased. It was therefore hypothesized that although two separate incidents led to the deaths of the activists, their fragmented remains were buried together.

During the exhumations a single commingled grave was identified containing remains thought to be those of the four activists (JAM, AM, HS and JUM).This case was particularly challenging because it involved a commingled grave, which contained the charred fragmented remains of at least four adults. Due to this poor preservation, only low concentrations of DNA (<12pg/µL) could be extracted from the specimens. Due to the low concentrations, 6µL of bone DNA and 35 cycles were used for amplification.

The following bone DNA and family reference DNA samples were analysed: AA06, AA17, AA32, AA35, JM, CM, JOM, EM, MM, KM, LM and LS, LOS.

Full CODIS miniSTR profiles were determined for all relatives and two of the four bone specimens (AA32 and AA35). The STR data is consistent with parent-child relationships between bone specimen AA32 and the daughters of AM (reference swabs KM and LM) (Table 3-5, 3-6). The STR evidence is also consistent with a parent-child relationship between bone specimen AA35 and the daughter of HS (reference swab LOS) (Table 3-7). Only 11 out of 13 loci of bone specimen AA06 are consistent with a parent-child relationship between the father of JUM (reference swab JM) and bone specimen AA06 (Table 3-8). It is important to note that the profile generated for bone specimen AA06 was rather poor. It is entirely possible that the failure of the two loci to match between bone specimen AA06 and reference swab JM reflects an allele dropout event for bone specimen AA06. No reliable data was recovered from bone specimen AA17.

In an effort to resolve the situation with bone specimen AA06, the sisters of JUM (reference swabs CM and JOM) were also typed and compared with their alleged father JM. The STR data generated is inconsistent with a parent-child relationship between JM and CM (Table 3-9) but consistent with a parent-child relationship between JM and JOM (Table 3-10). At best CM is the half-sister of JOM and JUM. One way to resolve this would be to use the mothers STR data but unfortunately her DNA was not available. Overall the miniSTR systems were only completely useful for two out of the four specimens and therefore mtDNA typing was employed in the hope of resolving the case.

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Table 3-5. Full CODIS miniSTR profiles of bone specimen AA32 and reference swab KM. The STR data is consistent with a parent-child relationship between bone specimen AA32 and KM the daughter of AM.

Locus	MM Wife of AM		KM Daughter of AM		Bone Specimen AA32		Result
TH01	7	7	7	7	7	10	Not excluded
CFS1PO	10	12	6	10	6	11	Not excluded
TPOX	8	10	8	10	8	11	Not excluded
D5S818	12	12	11	12	11	12	Not excluded
D8S1179	12	15	15	15	12	15	Not excluded
D16S539	10	11	11	11	11	11	Not excluded
FGA	23	25	23	24	23	24	Not excluded
D21S11	28	29	28	34.2	32.2	34.2	Not excluded
D7S820	8	10	10	13	13	13	Not excluded
vWA	16	18	15	16	15	17	Not excluded
D18S51	17	18	13	18	13	15	Not excluded
D13S317	11	13	13	14	11	14	Not excluded
D3S1358	15	16	15	16	15	15	Not excluded

Table 3-6. Full CODIS miniSTR profiles of bone specimen AA32 and reference swab LM. The STR data is consistent with a parent-child relationship between bone specimen AA32 and LM the daughter of AM.

Locus	MM Wife of AM		LM Daughter of AM		Bone Specimen AA32		Result
TH01	7	7	7	10	7	7	Not excluded
CFS1PO	10	12	6	11	6	10	Not excluded
TPOX	8	10	8	11	8	10	Not excluded
D5S818	12	12	11	12	11	12	Not excluded
D8S1179	12	15	12	15	12	15	Not excluded
D16S539	10	11	11	11	10	11	Not excluded
FGA	23	25	23	24	24	25	Not excluded
D21S11	28	29	32.2	34.2	29	32.2	Not excluded
D7S820	8	10	13	13	8	13	Not excluded
vWA	16	18	15	17	15	18	Not excluded
D18S51	17	18	13	15	15	17	Not excluded
D13S317	11	13	11	14	11	14	Not excluded
D3S1358	15	16	15	15	15	16	Not excluded

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Table 3-7. Full CODIS miniSTR profiles of bone specimen AA35 and reference swab LOS. The STR data is consistent with a parent-child relationship between bone specimen AA35 and LOS the daughter of HS.

Locus	LS Wife of HS		LOS Daughter of HS		Bone Specimen AA35		Result
TH01	6	8	8	8	8	8	Not excluded
CFS1PO	7	7	7	13	12	13	Not excluded
TPOX	9	9	9	12	11	12	Not excluded
D5S818	12	12	12	13	12	13	Not excluded
D8S1179	14	15	14	15	14	14	Not excluded
D16S539	12	13	11	13	11	11	Not excluded
FGA	20	22	19	22	19	21	Not excluded
D21S11	27	28	27	32.2	29	32.2	Not excluded
D7S820	8	10	8	11	10	11	Not excluded
vWA	14	17	14	16	14	16	Not excluded
D18S51	16	17	16	20	17	20	Not excluded
D13S317	11	11	11	11	11	12	Not excluded
D3S1358	15	16	16	17	16	17	Not excluded

Table 3-8. Full CODIS miniSTR profile of reference swab JM and partial profile of bone specimen AA06. Only 11 out of 13 loci are consistent with a parent-child relationship between JM the alleged father of JUM and bone specimen AA06.

Locus	JM Alleged father of JUM		Bone Specimen AA06		Result
TH01	7	8	7	8	Not excluded
CFS1PO	10	12	11	12	Not excluded
TPOX	8	12	8	8	Not excluded
D5S818	11	13	11	12	Not excluded
D8S1179	13	13	13	13	Not excluded
D16S539	11	12	11	12	Not excluded
FGA	20	22	22	24	Not excluded
D21S11	31	32.2		31.2	Excluded
D7S820	9	10		8	Excluded
vWA	15	17	17	17	Not excluded
D18S51	20	20	19	20	Not excluded
D13S317	11	12	11	13	Not excluded
D3S1358	16	16	16	16	Not excluded

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Table 3-9. Full CODIS miniSTR profiles of reference swabs JM and CM. The STR data is inconsistent with a parent-child relationship between JM and CM.

Locus	CM		JM Alleged father of CM		Result
TH01	8	8	7	8	Not excluded
CFS1PO	6	10	10	12	Not excluded
TPOX	8	9	8	12	Not excluded
D5S818	12	12	11	13	Excluded
D8S1179	12	13	13	13	Not excluded
D16S539	10	11	11	12	Not excluded
FGA	20	26	20	22	Not excluded
D21S11	31	31.2	31	32.2	Not excluded
D7S820	8	11	9	10	Excluded
vWA	18	18	15	17	Excluded
D18S51	16	18	20	20	Excluded
D13S317	13	13	11	12	Excluded
D3S1358	15	15	16	16	Excluded

Table 3-10. Full CODIS miniSTR profiles of reference swabs JM and JOM. The STR data is consistent with a parent-child relationship between JM and JOM.

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Locus	JOM		JM Alleged father of JOM		Result
TH01	8	8	7	8	Not excluded
CFS1PO	10	10	10	12	Not excluded
TPOX	9	12	8	12	Not excluded
D5S818	8	13	11	13	Not excluded
D8S1179	13	16	13	13	Not excluded
D16S539	11	11	11	12	Not excluded
FGA	22	24	20	22	Not excluded
D21S11	28	31	31	32.2	Not excluded
D7S820	8	9	9	10	Not excluded
vWA	16	17	15	17	Not excluded
D18S51	18	20	20	20	Not excluded
D13S317	12	13	11	12	Not excluded
D3S1358	15	16	16	16	Not excluded

3.3.4. Mitochondrial DNA PCR Amplification and Sequencing

Due to the fact that the miniSTR systems were only completely useful for two out of the four specimens in the body parts case, mtDNA typing was employed in the hope of resolving the case. Typing of mtDNA is often more successful than typing autosomal DNA, when dealing with degraded samples. This is due to the high copy number of mtDNA in human cells. The drawback however is that mtDNA typing does not have the same discriminating capacity as autosomal typing systems.

The following bone DNA and family reference swab DNA samples were subjected to mtDNA analysis of hypervariable regions one and two: AA06, AA17, AA32, AA35, EM, SM, MT, MOT, LAM, CM and JOM.

Complete mtDNA haplotypes were determined for all samples. The mtDNA data of bone specimen AA17 matches haplotypes determined for a maternal relative of JAM (reference swab SM) and is consistent with a parent-child relationship between reference swab EM and bone specimen AA17 (Table 3-11). Bone specimen AA32 mtDNA data matches the haplotype determined for a maternal relative of AM (reference swab LAM) (Table 3-12). While both the AA06 and AA35 bone specimens' data matches haplotypes determined for maternal relatives of HS (reference swabs MT and MOT) and JUM (reference swabs CM and JOM) (Table 3-13).

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The results demonstrate both the advantages and disadvantages of utilising mtDNA for forensic casework. The advantages of mtDNA are its high copy number per cell, which allows for the typing of highly degraded and low quantities of DNA and its maternal inheritance pattern, which extends the possible reference samples beyond one generation. These advantages were demonstrated by the fact that full mtDNA profiles were generated from the bone specimens AA06, AA17, AA32 and AA35, which had been exposed to high temperatures and buried for approximately 20 years. Comparisons could then be made with a variety of maternal relatives such as EM (Mother of JAM), SM (Brother of JAM), LAM (Cousin of AM), MT (Sister of HS), MOT (Nephew of HS and MT's Son), CM and JOM (Sisters of JUM). However the disadvantage of mtDNA being its low power of discrimination can be seen in the

results of bone specimens AA06 and AA35, and associated relatives who all share the same haplotype (Table 3-13).

Table 3-11. Mitochondrial DNA haplotypes for HVI (16024 to 16365) and HVII (73 to 340) of bone specimen AA17 and maternal relatives of JAM. The mtDNA data matches haplotypes determined for a maternal relative of JAM (SM) and is consistent with a parent-child relationship between EM and bone specimen AA17.

Bone Specimen AA17		EM Mother of JAM		SM Brother of JAM	
HVI (16024 to 16365)	**HVII (73 to 340)**	**HVI (16024 to 16365)**	**HVII (73 to 340)**	**HVI (16024 to 16365)**	**HVII (73 to 340)**
16148 C-T	93 A-G	16148 C-T	93 A-G	16148 C-T	93 A-G
16172 T-C	152 T-C	16172 T-C	152 T-C	16172 T-C	152 T-C
16187 C-T	189 A-G	16187 C-T	189 A-G	16187 C-T	189 A-G
16188 C-G	204 T-C	16188 C-G	204 T-C	16188 C-G	204 T-C
16189 T-C	207 G-A	16189 T-C	207 G-A	16189 T-C	207 G-A
16223 C-T	236 T-C	16223 C-T	236 T-C	16223 C-T	236 T-C
16230 A-G	247 G-A	16230 A-G	247 G-A	16230 A-G	247 G-A
16311 T-C	263 A-G	16311 T-C	263 A-G	16311 T-C	263 A-G
16320 C-T	315.1C	16320 C-T	315.1C	16320 C-T	315.1C

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Table 3-12. Mitochondrial DNA haplotypes for HVI (16024 to 16365) and HVII (73 to 340) of bone specimen AA32 and a maternal relative of AM. The mtDNA data of bone specimen AA32 matches the haplotype determined for a maternal relative of AM (LAM).

Bone Specimen AA32		LAM Maternal Cousin of AM	
HVI (16024 to 16365)	**HVII (73 to 340)**	**HVI (16024 to 16365)**	**HVII (73 to 340)**
16187 C-T	73 A-G	16187 C-T	73 A-G
16189 T-C	146 T-C	16189 T-C	146 T-C
16223 C-T	152 T-C	16223 C-T	152 T-C
16230 A-G	195 T-C	16230 A-G	195 T-C
16234 C-T	247 G-A	16234 C-T	247 G-A
16243 T-C	315.2C	16243 T-C	315.2C
16249 T-C		16249 T-C	
16311 T-C		16311 T-C	

Table 3-13. Mitochondrial DNA haplotypes for HVI (16024 to 16365) and HVII (73 to 340) of bone specimens AA06, AA35 and maternal relatives of HS and JUM. The mtDNA data of bone specimens AA06 and AA35 matches haplotypes determined for maternal relatives of HS (MT and MOT) and of JUM (CM and JOM).

Bone Specimen AA06		**CM Sister of JUM**		**JOM Sister of JUM**	
HVI	**HVII**	**HVI**	**HVII**	**HVI**	**HVII**
(16024 to 16365)	**(73 to 340)**	**(16024 to 16365)**	**(73 to 340)**	**(16024 to 16365)**	**(73 to 340)**
16223 C-T	73 A-G	16223 C-T	73 A-G	16223 C-T	73 A-G
16239 C-T	150 C-T	16239 C-T	150 C-T	16239 C-T	150 C-T
16324.-T	185 G-A	16324.-T	185 G-A	16324.-T	185 G-A
	189 A-G		189 A-G		189 A-G
	263 A-G		263 A-G		263 A-G
	309.1C		309.1C		309.1C
	315.1C		315.1C		315.1C
Bone Specimen AA35		**MT Sister of HS**		**MOT Nephew of HS**	
HVI	**HVII**	**HVI**	**HVII**	**HVI**	**HVII**
(16024 to 16365)	**(73 to 340)**	**(16024 to 16365)**	**(73 to 340)**	**(16024 to 16365)**	**(73 to 340)**
16223 C-T	73 A-G	16223 C-T	73 A-G	16223 C-T	73 A-G
16239 C-T	150 C-T	16239 C-T	150 C-T	16239 C-T	150 C-T
16324. -T	185 G-A	16324. -T	185 G-A	16324. -T	185 G-A
	189 A-G		189 A-G		189 A-G
	263 A-G		263 A-G		263 A-G
	309.1C		309.1C		309.1C
	315.1C		315.1C		315.1C

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3.4. Conclusion

The UWC laboratory was provided with five forensic bone specimens THKY 01-G3, AA06, AA17, AA32, AA35 and fourteen reference swab samples for two cases. The first case involved bone specimen THKY 01-G3 and a reference swab sample from the mother of OF. The second case was a closed case and involved bone specimens AA06, AA17, AA32 and AA35. The reference swab samples included samples from the father and two sisters of JUM, the mother and brother of JAM, the maternal cousin, wife and two daughters of AM and the sister, nephew, wife and daughter of HS.

The forensic DNA study involved a miniSTR and mtDNA analysis. In the first case (OF), the miniSTR analysis provided data which was consistent with a parent–child relationship between the mother of OF (reference swab RF) and the bone specimen

THKY 01-G3. This data augmented the non-DNA evidence supporting the hypothesis that bone specimen THKY 01-G3 was derived from OF.

In the second case (Body Parts), the miniSTR analysis could only resolve the identities of two (AA32 and AA35) of the four bone specimens. The miniSTR data was consistent with a parent–child relationship between bone specimen AA32 and the daughters of AM (reference swabs KM and LM). The data was also consistent with a parent-child relationship between bone specimen AA35 and the daughter of HS (reference swab LOS). The data generated for bone specimen AA06 was ambiguous and no reliable data was recovered from bone specimen AA17.

To further resolve the case mtDNA analysis was conducted. For bone specimens AA17 and AA32 the mtDNA data indicates relationships which were consistent with the results for the miniSTR analysis. For AA06 and AA35 a shared haplotype was observed. This haplotype was also shared with the relatives of JUM and HS. MiniSTR data had already indicated that AA35 was derived from HS. By a process of elimination this suggested that AA06 was derived from JUM. These data augmented the non-DNA evidence supporting the hypothesis that the bone specimens AA06, AA17, AA32 and AA35 were derived from JUM, JAM, AM and HS.

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Chapter 4: Case Study 2: DNA Analysis of 44 year old Human Skeletal Remains

Note: Names have been coded to protect the identities of the individuals involved. For further details about the cases contact the Forensic DNA Laboratory at the University of the Western Cape.

4.1. Introduction

LN was born on May 22, 1922, in Langa Cape Town and grew up in KwaZali village in the Eastern Cape and became the first detainee to die under the 90-Day Detention Act in 1963 (http://www.cosatu.org.za/press/2006/sept/press6.htm, http://www.star.co.za). LN was a significant activist yet his role is not widely appreciated. Therefore it seems appropriate to place the case in context by considering his political life, the events that led to his death and the 44 year search for his remains.

LN joined the ANC in the 1950s and in 1961 when the ANC established a military wing, uMkhonto weSizwe (MK), he became an MK commander. He joined the ANC after he witnessed forced removals and the use of bulldozers to demolish black-owned houses to create space for white suburbs. There was a marked change in his perspective. Politics consumed his life and when the ANC established a military wing (uMkhonto weSizwe) it was a natural step for him to join the armed struggle.

LN became the MK commander of the Western Cape and soon started recruiting operatives and training them. He and a friend DG, a technical officer, started a training camp at Mamre. The camp taught recruits how to make electric circuits for bombs, how to read a compass and first aid. The unit successfully carried out various acts of sabotage.

The police had been monitoring LN’s movements for years because of information presented in his speeches. This information made its way to Justice Minister John Vorster and a focus was placed on LN. He was constantly harassed by security police and was jailed on several occasions. However these incidents required both a charge and court appearance within two days of arrest.

On May 1, 1963, Justice Minister John Vorster introduced the 90-day Detention Act. The detention act provided the security police and military intelligence with greater opportunity to interrogate and torture suspects. Abuse was common because the act allowed for the solitary confinement of detainees without charge or trial for renewable periods, without access to family or lawyers (http://www.star.co.za).

LN went into hiding when the high command of MK was captured just two months after the introduction of the detention act. However information from an informer led to him being arrested on August 19, 1963. He was taken to Caledon Square police station in Cape Town and then transferred to Pretoria Central Prison a few days later. LN was tortured and died shortly after arriving in Pretoria.

An inquest was held into the death of LN by his families' lawyers. At the inquest a District Surgeon as well as the security police claimed that LN committed suicide. The lawyers argued that if he had committed suicide then it would surely have been as a result of extensive and severe torture (http://www.info.gov.za/speeches/2007/07030215151001.htm). This claim was denied by the surgeon and the police. The Detention Act had served its purpose by removing the influence of the courts. The situation for the family was further exacerbated by continued police harassment.

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The family continued the search for LN's remains. By the time the Truth and Reconciliation Commission (TRC) was setup they had been searching for over thirty years. At the TRC, GM was the only key figure to testify. The two had been held in Pretoria Central Prison at the same time. In a chance meeting LN had confided details of his abuse in a note to GM. No one else came forward with information or applied for amnesty for the incident. LN's widow Beauty then requested that the TRC set up an investigation and search for her husbands remains. This investigation led nowhere and the family were no closer to finding his remains.

In 2007 the Missing Persons Task Team reinvestigated the whereabouts of LN's remains. After an extensive search a grave register was discovered with LN's name, a grave number and a cemetery map. The map indicated that his grave was located in the middle of the Mamelodi West Cemetery near Pretoria. A Land Surveyor was used

by the team to identify the grave according to the cemetery map. The remains were exhumed and a preliminary anthropological assessment of the skeleton suggested that its age was consistent with that of LN at the time of his death. To augment the existing evidence, the remains were sent to the Forensic DNA Laboratory at UWC where genotyping was carried out. The genotyping presented in this chapter was duplicated by another investigator and a consensus has been presented below.

4.2. Materials and Methods

4.2.1. Sample Collection and Preparation

Family reference samples and skeletal remains were sent to UWC by the South African Missing Persons Task Team (SAMPTT). A total of 6 reference buccal swabs and one femur were received. The reference samples included LN's two sons and his brother and three unrelated controls. The femur was stored in a paper bag at room temperature prior to sampling, cleaning and DNA analysis. The femur was prepared for DNA extraction as previously described (Chapter 3, section 3.2.2).

4.2.2. DNA Extraction

The family reference buccal swab samples were either extracted using the Chelex extraction method or the Epicenter buccal swab extraction kit used according to the manufactures instructions. Reference buccal swabs samples were extracted following the Chelex protocol described in the Applied Biosystems Profiler Plus User Manual. The step by step protocol for this method can be found in Appendix I. DNA was extracted from the milled bone samples using the organic extraction method previously described (Hagelberg *et al.* 1989).

4.2.3. DNA Quantification

All samples were quantified using a previously published assay (Nicklas *et al*. 2003). The assay relies on the amplification of a 124bp ALU fragment. The assay was conducted on a Roche LightCycler 1 instrument using the LightCycler Fast Start SYBR GREEN kit.

4.2.4. MiniSTR PCR Amplification

Samples were amplified as previously described (Chapter 3, section 3.2.5).

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4.2.5. Y-STR PCR Amplification

A Y-STR multiplex assay designed by Dr. Maria Eugenia D'Amato from the Forensic DNA Laboratory at the University of the Western Cape (UWC) was used. The assay includes the markers DYS385, DYS518, DYS449, DYS504, DYS626, DYS447, DYS644, DYS612, DYS481, DYS710 and DYS710I. The development and validation of this assay will form the basis of a separate publication by the UWC forensic laboratory.

4.2.6. Detection and Data Analysis

Amplicons were analysed using an ABI PRISM 377 DNA Sequencer. The data was collected with the ABI 377 collection software (Applied Biosystems), analyzed using GeneScan 3.0.0 software (Applied Biosystems). Allele designations were automatically assigned using Genotyper 3.7 software (Applied Biosystems). The profiles were evaluated both manually and using the excel program STR_MatchSamples that was developed by David Duewer, Analytical Chemical Division, NIST. The methods were previously described in Chapter 2, section 2.2.4.

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4.3. Results and Discussion

4.3.1. DNA Quantification

The DNA concentrations of all the family reference swab samples as well as the bone samples were determined. Table 4-1 presents the DNA concentrations of reference buccal swab DNA samples and Table 4-2 presents the DNA concentration of the bone DNA sample.

Table 4-1. DNA Concentrations of Reference Buccal Swab DNA Samples

Victim	Reference Swab Samples	DNA Concentration (ng/ul)
LN	1. DNA_01_03_07	14.08
	2. DNA_02_03_07	1.72
	3. DNA_03_03_07	14.32
	4. DNA_04_03_07	11.40
	5. DNA_05_03_07	27.28
	6. DNA_06_03_07	8.36

Table 4-2. DNA Concentration of Bone DNA Sample

Bone Sample	DNA Concentration (pg/μL)
1. Mam07_001_5910_body 2	35

4.3.2. MiniSTR PCR Amplification

The coded reference samples were amplified using the standard miniSTR amplification conditions but for the bone DNA sample (Figure 4-1), 3μL DNA extract and 35 cycles were used.

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Figure 4-1. Femur specimen Mam07_001_5910_body 2 received from the SAMPTT for DNA analysis

The miniSTR systems worked extremely well. Full CODIS miniSTR profiles were determined for all reference samples as well as the bone specimen. Surprisingly the quality of the profile generated from the 44 year old bone specimen (Mam07_001_5910_body 2) was as good as the profiles generated for the reference samples. The STR data was consistent with a parent-child relationship between bone specimen Mam07_001_5910_body 2 and reference swab samples DNA_02_03_07 and DNA_04_03_07 (Tables 4-3 and 4-4). This data was sent to the SAMPTT who confirmed that reference swab samples DNA_02_03_07 and DNA_04_03_07 belonged to the sons of LN.

Table 4-3. Full CODIS miniSTR profiles of bone specimen Mam07_001_5910_body 2 and Swab DNA_02_03_07. The STR data is consistent with a parent-child relationship between bone specimen Mam07_001_5910_body 2 and DNA_02_03_07.

Locus	Swab DNA_02_03_07		Bone Specimen Mam07_001_5910_body 2		Result
TH01	6	7	6	9	Not excluded
CFS1PO	11	12	11	12	Not excluded
TPOX	6	8	6	8	Not excluded
D5S818	8	12	12	13	Not excluded
D8S1179	13	15	13	13	Not excluded
D16S539	11	12	10	12	Not excluded
FGA	24	25	22	24	Not excluded
D21S11	28	31	28	29	Not excluded
D7S820	10	10	10	10	Not excluded
vWA	16	17	15	16	Not excluded
D18S51	17	21	15	21	Not excluded
D13S317	13	14	12	13	Not excluded
D3S1358	14	16	14	17	Not excluded

Table 4-4. Full CODIS miniSTR profiles of bone specimen Mam07_001_5910_body 2 and Swab DNA_04_03_07. The STR data is consistent with a parent-child relationship between specimen Mam07_001_5910_body 2 and DNA_04_03_07.

Locus	Swab DNA_04_03_07		Bone Specimen Mam07_001_5910_body 2		Result
TH01	7	9	6	9	Not excluded
CFS1PO	11	12	11	12	Not excluded
TPOX	6	6	6	8	Not excluded
D5S818	12	13	12	13	Not excluded
D8S1179	13	15	13	13	Not excluded
D16S539	10	11	10	12	Not excluded
FGA	23	24	22	24	Not excluded
D21S11	29	31	28	29	Not excluded
D7S820	10	10	10	10	Not excluded
vWA	16	17	15	16	Not excluded
D18S51	18	21	15	21	Not excluded
D13S317	11	13	12	13	Not excluded
D3S1358	14	16	14	17	Not excluded

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4.3.3. Y-STR PCR Amplification

As previously indicated, the six coded reference samples included three unrelated controls, two swabs from his sons and one from his brother. This meant that three of the reference samples shared a paternal lineage with LN. The autosomal miniSTR analysis was useful in confirming the parent-child relationships between specimen Mam07_001_5910 and the two reference swab samples from LN's sons (DNA_02_03_07 and DNA_04_03_07). However, the relationship between Mam07_001_5910 and the sample from LN's brother had yet to be established. To address this Y-chromosome STR (Y-STR) analysis was used. The Y-STR analysis would identify all references samples sharing a paternal lineage.

The Y-STR testing system being used is the subject of a parallel project in the Forensic DNA laboratory at the UWC. The system has been developed to include a highly discriminatory set of Y-Chromosome markers: DYS385, DYS518, DYS449, DYS504, DYS626, DYS447, DYS644, DYS612, DYS481, DYS710 and DYS710I.

Full Y-STR profiles were determined for all reference samples as well as the bone specimen. The data indicated that bone specimen Mam07_001_5910_body 2 shared a paternal lineage with reference swab samples DNA_02_03_07, DNA_04_03_07 and DNA_05_03_07 (Tables 4-5 to 4-7). This suggested that the reference swab sample DNA_05_03_07 was from the brother of specimen Mam07_001_5910_body 2. This was confirmed by the South African Missing Persons Task Team (SAMPTT).

Table 4-5. Full Y-STR profiles of bone specimen Mam07_001_5910_body 2 and Swab DNA_02_03_07. The STR data is consistent with a paternal relationship between bone specimen Mam07_001_5910_body 2 and Swab DNA_02_03_07. Alleles are considered as identical if their sizes are within 0.5bp of one another.

Locus	Swab DNA_02_03_07	Bone Specimen Mam07_001_5910_body 2	Result
	Allele Size (bp)	Allele Size (bp)	
DYS 385	196.65	196.54	Not excluded
DYS 518	218.56	218.43	Not excluded
DYS 449	284.82	284.83	Not excluded
DYS 504	195.59	195.55	Not excluded
DYS 626	246.48	246.96	Not excluded
DYS 447	155.09	154.98	Not excluded
DYS 644	260.23	260.04	Not excluded
DYS 612	310.68	310.77	Not excluded
DYS 481	125.29	125.31	Not excluded
DYS 710	232.26	232.07	Not excluded
DYS710I	281.62	281.45	Not excluded

Table 4-6. Full Y-STR profiles of bone specimen Mam07_001_5910_body 2 and Swab DNA_04_03_07. The STR data is consistent with a paternal relationship between bone specimen Mam07_001_5910_body 2 and Swab DNA_04_03_07. As above.

Locus	Swab DNA_04_03_07	Bone Specimen Mam07_001_5910_body 2	Result
	Allele Size (bp)	Allele Size (bp)	
DYS 385	196.71	196.54	Not excluded
DYS 518	218.41	218.43	Not excluded
DYS 449	284.81	284.83	Not excluded
DYS 504	195.64	195.55	Not excluded
DYS 626	246.87	246.96	Not excluded
DYS 447	155.06	154.98	Not excluded
DYS 644	260.14	260.04	Not excluded
DYS 612	310.73	310.77	Not excluded
DYS 481	125.27	125.31	Not excluded
DYS 710	232.19	232.07	Not excluded
DYS710I	281.60	281.45	Not excluded

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Table 4-7. Full Y-STR profiles of bone specimen Mam07_001_5910_body 2 and Swab DNA_05_03_07. The STR data is consistent with a paternal relationship between bone specimen Mam07_001_5910_body 2 and Swab DNA_05_03_07. As above.

Locus	Swab DNA_05_03_07	Bone Sample Mam07_001_5910_body 2	Result
	Allele Size (bp)	**Allele Size (bp)**	
DYS 385	196.65	196.54	Not excluded
DYS 518	218.32	218.43	Not excluded
DYS 449	284.82	284.83	Not excluded
DYS 504	195.66	195.55	Not excluded
DYS 626	246.87	246.96	Not excluded
DYS 447	155.02	154.98	Not excluded
DYS 644	260.15	260.04	Not excluded
DYS 612	310.73	310.77	Not excluded
DYS 481	125.17	125.31	Not excluded
DYS 710	232.18	232.07	Not excluded
DYS710I	281.53	281.45	Not excluded

4.4. Conclusion

The UWC laboratory was provided with a forensic bone specimen Mam07_001_5910_body 2 and six reference samples. Three of the reference samples were relatives of LN and included his two sons and one brother. The remaining three reference samples were from unrelated control donors.

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The forensic DNA study involved a miniSTR and Y-STR analysis. The miniSTR analysis provided data which was consistent with a parent –child relationship between the bone specimen and reference swab samples DNA_02_03_07 and DNA_04_03_07. The Y-STR analysis provided data which indicated that the forensic bone specimen shared a paternal lineage with swab samples DNA_02_03_07, DNA_04_03_07 and DNA_05_03_07. These data are consistent with the known relationships for each of the reference samples. The DNA evidence did not suggest a relationship between the bone specimen and the three unrelated control donors. These data augmented the non-DNA evidence supporting the hypothesis that bone specimen Mam07_001_5910_body 2 was derived from LN.

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Electronic Supplementary Resources

http://www.ypatent.com/DNArepair.htm

http://www.bio.miami.edu/dana/250/25005_9.html

http://www.cstl.nist.gov/biotech/strbase/training.htm

http://www.promega.com/techserv/tools/pplexy/

http://www.appliedbiosystems.com/yfilerdatabase/

http://www.yhrd.org

http://www.cstl.nist.gov/biotech/strbase/miniSTR/timeline.htm

http://www.afip.org

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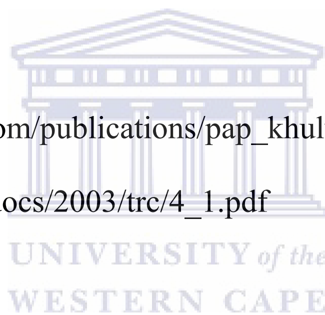
http://www.ic-mp.org

http://www.brandonhamber.com/publications/pap_khulumani.doc

http://www.info.gov.za/otherdocs/2003/trc/4_1.pdf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http://eaaf.typepad.com/

http://eaaf.typepad.com/founding_of_eaaf/

http://www.khulumani.net/content/view/620/164/

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Appendices

Appendix I: Protocols

Developmental Validation

1. Characterization of genetic markers: The basic characteristics (described below) of a genetic marker must be determined and documented.

1.1. Inheritance: The mode of inheritance of DNA markers demonstrated through family studies.

1.2. Mapping: The chromosomal location of the genetic marker (submitted to or recorded with the Nomenclature Committee of the Human Genome Organization).

1.3. Detection: Technological basis for identifying the genetic marker.

1.4. Polymorphism: Type of variation analyzed.

2. Species specificity: For techniques designed to type human DNA, the potential to detect DNA from forensically relevant nonhuman species should be evaluated. For techniques in which a species other than human is targeted for DNA analysis, the ability to detect DNA profiles from non-targeted species should be determined. The presence of an amplification product in the non-targeted species does not necessarily invalidate the use of the assay.

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3. Sensitivity studies: When appropriate, the range of DNA quantities able to produce reliable typing results should be determined.

4. Stability studies: The ability to obtain results from DNA recovered from biological samples deposited on various substrates and subjected to various environmental and chemical insults has been extensively documented. In most instances, assessment of the effects of these factors on new forensic DNA procedures is not required. However, if substrates and/or environmental and/or chemical insults could potentially affect the analytical process, then the process should be evaluated using known samples to determine the effects of such factors.

5. Reproducibility: The technique should be evaluated in the laboratory and among different laboratories to ensure the consistency of results. Specimens obtained from donors of known types should be evaluated.

6. Case-type samples: The ability to obtain reliable results should be evaluated using samples that are representative of those typically encountered by the testing laboratory. When possible, consistency of typing results should be demonstrated by comparing results from the previous procedures to those obtained using the new procedure.

7. Population studies: The distribution of genetic markers in populations should be determined in relevant population groups. When appropriate, databases should be tested for independence expectations.

8. Mixture studies: The ability to obtain reliable results from mixed source samples should be determined.

9. Precision and accuracy: The extent to which a given set of measurements of the same sample agree with their mean and the extent to which these measurements match the actual values being measured should be determined.

10. PCR-based procedures: Publication of the sequence of individual primers is not required in order to appropriately demonstrate the accuracy, precision, reproducibility, and limitations of PCR-based technologies.

10.1. The reaction conditions needed to provide the required degree of specificity and robustness must be determined. These include thermocycling parameters, the concentration of primers, magnesium chloride, DNA polymerase, and other critical reagents.

10.2. The potential for differential amplification among loci, preferential amplification of alleles in a locus, and stochastic amplification must be assessed.

10.3. When more than one locus is co-amplified, the effects of co-amplification must be assessed (e.g., presence of artifacts).

10.4. Positive and negative controls must be validated for use.

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10.5. Detection of PCR product

10.5.1. Characterization without hybridization

10.5.1.1. When PCR product is characterized directly, appropriate measurement standards (qualitative and/or quantitative) for characterizing the alleles or resulting DNA product must be established.

10.5.1.2. When PCR product is characterized by DNA sequencing, appropriate standards for characterizing the sequence data must be established.

10.5.2. Characterization with hybridization

10.5.2.1. Hybridization and wash conditions necessary to provide the required degree of specificity must be determined.

10.5.2.2. For assays in which the probe is bound to the matrix, a mechanism must be employed to demonstrate whether adequate amplified DNA is present in the sample (e.g., a probe that reacts with an amplified allele(s) or a product yield gel).

Bone cleaning

Step 1: Remove a section of bone from the main sample by sawing using a dremel tool. A typical sample weighs approx 2grams.

Step 2: Clean surface using a dremel tool.

Step 3: Immerse bone in 3.2% (w/v) bleach for 1hour.

Step 4: Wash 4X with sabax water and 2X with ethanol.

Step 5: Place under UV radiation for 15-30 minutes and let the sample dry.

Step 6: Place the bone sample into the Freezer mill sample vial as soon as possible.

The bone sample is now ready to be milled.

Tooth Cleaning

Step 1: Remove teeth from a jaw by using a pliers or by sawing using a dremel tool. A typical sample weighs approx 2grams.

Step 2: Remove any attached material from the tooth with a toothbrush and clean with sabax water. If all material cannot be removed with a toothbrush a razor blade may be used.

Step 3: Immerse tooth in 3.2% (w/v) bleach for 1 hour.

Step 4: Wash 4X with sabax water and 2X with ethanol.

Step 5: Place under UV radiation for 15-30 minutes and let the sample dry.

Step 6: Place the sample into the Freezer mill sample vial as soon as possible.

The tooth sample is now ready to be milled.

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Milling of Bones

Settings:

Pre-cooling: 15 minutes .

Grinding: 2 minutes X 3 cycles

Resting: 2 minutes .

Impact Frequency: 15

Cleaning of 6750 Freezer Mill sample vials and end pieces

Step 1: Decon for 30min-1 hour in a ziplock bag or suitable container

Step 2: Bleach for 1 hour

Step 3: Wash 4X with sabax water

Step 4: Wash 2X with ethanol

Sample vials and end pieces now ready for milling of next sample

DNA Extraction from oral swabs using Chelex Extraction Method

1. Suspend the swab sample in 1mL of deionized water in a 1.5mL microcentrifuge tube.

2. Incubate at room temperature for 30 minutes to rehydrate the sample.

3. Use an autoclaved toothpick to tease the fibers apart on the inside of the tube. Twirl the swab and toothpick for 2 minutes to release the cells from the swab.

4. Remove the substrate and toothpick. Spin in a microcentrifuge for 2 minutes at 10, 000-15,000 x g at room temperature.

5. Without disturbing the pellet, remove and discard all but 25µL of the pellet.

6. Resuspend the pellet in the remaining 25µL by stirring with an autoclaved pipette tip.

7. Add 5% Chelex to final volume of 200µL.

8. Add 2µL of 10mg/mL Proteinase K. Mix gently.

9. Incubate the sample at 56°C for 15-30 minutes.

10. Vortex the sample at high speed for 5-10 minutes.

11. Spin the sample in a microcentrifuge for 10-20 seconds at 10, 000-15, 000 x g at room temperature.

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12. Incubate the sample in a boiling water bath for 8 minutes.

13. Vortex the sample at high speed for 5-10 seconds.

14. Spin the sample in a microcentrifuge for 2-3 minutes at 10, 000-15, 000 x g at room temperature. The sample is now ready for DNA quantitation and the PCR amplification process.

15. Store the remainder of the sample at either 2-6 ^{o}C or -15 ^{o}C to -25 ^{o}C.

16. To reuse, thaw the sample at room temperature and repeat steps 7-8.

DNA Extraction using Phenol Chloroform Method

1. Wash the bone powder with 10 ml 0.25 M EDTA pH 8-8.5 to remove loose impurities.

2. Centrifuge immediately to pellet the bone powder and discard the supernatant
The washing step can be repeated once or twice.

3. Add 10 ml of Bone Lysis buffer (0.5 M EDTA pH 8-8.5, 0.5 ml 10% *N*-lauroyl sarcosine and 200 µl of the 20 $mgml^{-1}$ Proteinase K) to each tube containing the bone powder.
Make sure that the tubes are closed firmly and wrapped with Parafilm to avoid leaks.

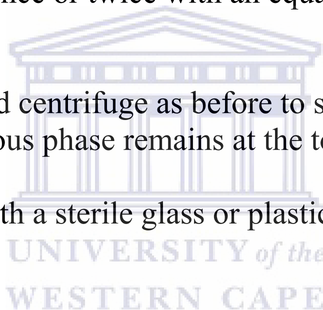
4. Incubate overnight at 37^0 C or 50^0 C for three hours. The important thing at this point is to ensure that the content of the tubes is thoroughly agitated so the bone powder does not settle out.

5. After the lysis step, use a glass pipette to add 10 ml of water-saturated phenol to each tube.

6. Mix solution thoroughly and centrifuged for 10 minutes at 3000 r.p.m as soon as possible to separate the two phases. Attention: the aqueous phase remains in the bottom in this step.

7. After centrifugation, remove the top (phenol) layer with a sterile glass Pasteur pipette and discarded into a suitable waste container.

8. Extract the aqueous phase once or twice with an equal mixture of water-saturated phenol and chloroform.



9. Mix solution thoroughly and centrifuge as before to separate the phases.
After centrifugation, the aqueous phase remains at the top of the organic phase.

10. Remove aqueous phase with a sterile glass or plastic Pasteur pipette and placed in a clean Falcon tube.

11. Remove residual phenol by one chloroform (10ml) extraction.

12. Recover aqueous phase and place into Falcon tube, it typically consists of 7-8 ml.

13. Add the 7-8ml aqueous solution to Centriplus 30 tubes and centrifuge at max speed until solution reduced/concentrated down to 100-200ul.

14. Wash extract at least twice with 7-8ml sterile water to remove the salt.

Extracts can be stored at -20^0 C, although long-term storage is preferable at -70^0 C.

The DNA is ready to amplify, although further purification might be necessary or desirable.

DNA Purification using QIAamp® Blood Mini Kit

1. Add an equal volume of Buffer AL to the sample. Mix by pulse-vortexing for 15 s. e.g., a 400 µl sample will require 400 µl Buffer AL

2. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from lid

3. Carefully apply the mixture to the QIAamp Spin Column without wetting the rim, close the cap.

4. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

5. Add 500 µl Buffer AW1 to the column.

6. Centrifuge at 6000 x *g* (8000 rpm) for 1 min. Place the QIAamp Spin Column in a clean 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

7. Add 500 µl Buffer AW2 to the column. Close the cap and centrifuge at full speed (20,000 x *g*; 14,000 rpm) for 3 min.

8. Place the QIAamp Spin Column in a clean 1.5 ml microcentrifuge tube (not provided), and discard the collection tube containing the filtrate. Carefully open the QIAamp Spin Column and add 60µl Buffer AE or distilled water.

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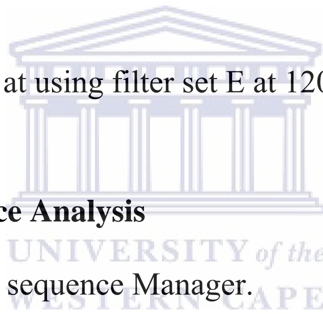
9. Incubate at room temperature (15–25°C) for 5 min, and then centrifuge at 6000 x *g* (8000 rpm) for 1 min.
Samples are now ready for PCR amplification

Precipitation and cleanup of sequencing reactions

1. 125mM EDTA mixed in equal parts with 3M Sodium Acetate.
2. 2ul added to each 10ul sequencing reaction.
3. 25ul of 100% EtOH added to each sequencing reaction.
4. Incubated at room temp for 15min
5. Spun at max for 30min (room temp)
6. Supernatant removed
7. 70ul 70% EtOH added.
8. Spun at max for 15 min (room temp)
9. Supernatant removed and air dried for +-40min in fume hood.
10. Resuspended in 1.5ul loading mix (5ul formamide to 1ul Blue Dye).
11. Denatured for 4 min.
12. Snap cooled on ice.
13. Load around 0.8 to 1.2ul.
14. Run on standard 36cm gel at using filter set E at 1200 scans per hour.



Mitochondrial DNA Sequence Analysis

1. Process raw data using ABI sequence Manager.
2. Reverse compliment heavy chain sequences.
3. Conduct pairwise comparison of heavy and light chains for each sequence.
4. Identify discrepancies between heavy and light chain sequences.
5. Examine corresponding electropherograms and attempt to identify source of discrepancy.
6. Correct sequence if one strand has very clear electropherogram (otherwise repeat sequencing)
7. Copy corrected sequences in fasta format to a text file. (Include Cambridge Reference Sequence (CRS))
8. Use clustal to conduct a multiple sequence alignment and render alignment using Genedoc.
9. Identify positions at which each sequence differs from CRS.
10. Confirm bases at these positions on the relevant electropherograms.

Epicenter extraction protocol for buccal swabs.

1. Place swab fragment in 250ul of Epicenter extraction buffer
2. Vortex for 15 seconds
3. Heat at 65C for five minutes
4. Vortex for 15 seconds
5. Heat at 98C for 2 minutes
6. Vortex for 15 seconds.

Genotyping with the ABI377

Pouring the gel

1. Place a clean set of assembled plates in the gel pouring area. Ensure that the glass plates have been moved as far down the cassette as possible. All the cassette clamps should be in place except the one that holds the beam trap. Ensure that the 48 tooth well forming comb is clean and accessible.

2. Ensure all glassware and syringes have been rinsed with deionised water.

3. Transfer 25ml of gel mix to a clean 50ml beaker. This allows for easy access to the gel mix with a 50ml syringe.

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4. Suck up gel mix with the 50ml syringe and filter through a 0.22 micron filter directly into the side-arm flask.

5. Degas gel mix with intermittent gentle agitation. Degas for +/- 5 min or until no bubbles appear after agitation. The pump must be running first. Always ensure that the rubber stopper is in place. Set or break the vacuum only at the rubber hose/side arm junction.

6. Transfer from side arm flask to a clean 50ml beaker.

7. Add 125µL (AMPS) and 17.5µL N, N, N', N' Tetramethyl-EthyleneDiamine (TEMED) to opposite sides of the gel mix in the beaker. You should mix soon after adding the AMPS and TEMED but make sure you don't introduce bubbles into the mix. Once mixed the gel takes a long time to set so there is no rush.

8. Pour gel.

9. Insert comb and clamp.

10. Seal bottom of the plates with cling-wrap.

11. Clamp the beam trap down.

12. Let the gel polymerise for 2hrs.

Running the ABI377

1. Start by turning the ABI377 on and let the instrument go through its start up routine.

2. Check that the laser fan is blowing.

3. Once the ABI377 has gone through its start up routine turn on the PC.

4. Start cleaning the gel so that it can be loaded onto the ABI. Remove the clamps but leave the comb in place. Remove the cling-wrap from the bottom of the gel.

5. Remove the gel from the cassette for cleaning. Pour deionised water over the bottom and top of the gel to remove any residual gel. After the initial rinse remove the comb drawing it backwards in the plane of the gel. Doing this helps draw any polyacrylamide away from the wells. Use paper towel to dab the residual polyacrylamide from the gel.

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6. Once the gel has been thoroughly rinsed, dry it with paper towel. Place it back in the cassette.

7. Lift the beam trap and clean the laser scan area.

8. Place the cleaned gel into the instrument. Clip it into place and ensure that it is correctly aligned.

9. Ensure the collection software is open. Open Genescan Run dialogue box. Set the Prerun options tab to PlateCheck X.

10. Click the PlateCheck Run option. With any of the run options if the instrument does not comply simple cancel the run and restart it.

11. Examine the scan and gel windows and cancel the run. If you're happy with the result continue with the rest of the setup. If not, clean the scan area again and repeat the plate check.

12. If you're satisfied with the plate check, place the upper buffer chamber onto the instrument. Pour 1X TBE into the chamber and check for leaks.

13. If no leaks occur then add the heat plate to the gel assembly.

14. Pour 1X TBE buffer into the lower buffer chamber.

15. Flush the well area and insert the 48 tooth comb so that approximately 1.5mm of the arches are exposed.

16. Put the lid of the upper chamber on and close the instrument door.

17. Select GS PRF 2400 X from the Prerun options tab. Click on the prerun tab.

18. Open the status window and check that the 35 second initialisation has been initiated. If not cancel the run and click on the prerun tab again.

19. The moment you hear the water pump activate, pause the run, open the instrument door and check that there are no leaks.

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20. If there is no leaking simply close the instrument door and allow it to heat up to 50°C.

21. While the instrument is heating up the samples can be prepared for loading.

Preparation of samples for loading on the ABI377

1. Prepare a sample sheet with the names of each sample.

2. Place the amplification-tubes in one continuous row.

3. In front of each amplification-tube place a clean 0.2ml PCR tube (loading tubes). Number the tubes with the relevant well number.

4. Prepare sufficient loading mixture (loading mixture in the following ratio: 5µL Formamide, 1.5µL ROX internal size ladder, 1.5µL Blue dye).

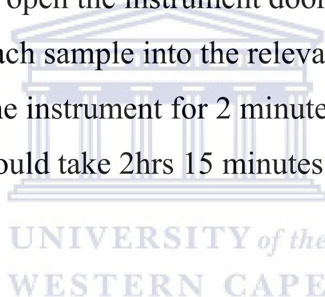
5. Place 1μL loading dye in each loading tube.

6. Transfer 1μL of each amplified sample to the appropriate loading tubes.

7. Place the loading tubes into the GeneAmp 2700 Thermocycler for 5 min to denature. Place odd numbered tubes consecutively into an appropriate number of rows. Then place the even numbered tubes consecutively into an appropriate number of rows. This simplifies the loading process when odd wells are loaded first, run in then even wells are loaded.

8. During this time the paused prerun will brought the gel to 50°C. Cancel the prerun and set up the Run parameters (48 tooth comb, GS RUN 2400 X module, Operator, Filter set, Sample Sheet). Click the run tab. Pause the instrument as soon as you hear the water pump.

9. With the instrument paused open the instrument door and flush the wells with a 5μL syringe. Load 0.8μL of each sample into the relevant well. Load all odd numbered samples first, run the instrument for 2 minutes, pause it and load the even samples. Restart the run. It should take 2hrs 15 minutes for all the relevant bands to be detected.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10. As soon you've seen the 400bp band you can terminate the run and process the results.

Processing the results from the ABI377

1. Open the Gel processor software. Auto track the lanes. Each lane should be visually inspected and any tracking errors corrected.

2. Once tracked the lanes can be extracted.

3. Open Genescan, start a new project and import the relevant run files. Before analyzing each sample you'll need to install a matrix file and an appropriate size standard. Good initial peak height threshold settings for the colour channels are B: 100, G: 100, Y: 100, R: 50.

4. Click the analyse tab to analyse the samples.

5. Once the initial processing is done you'll need to check each sample individually to see that all the peaks have been detected. Where necessary raise the peak height cut off threshold values to avoid stutter or lower the threshold to make sure that all allele peaks are detected.

6. Once satisfied, the project file can be saved and is now ready to be typed.

Genotyping of Samples

The samples can either be typed manually using a ladder or by using the Genotyper Software as follows:

1. Open Genotyper Macro

2. Click File, Import from Gensecan files. Search for appropriate project file and add it to the Genotyper document and save.

3. Click inside the Dye/Lanes window, click Edit and Select all.

4. Double click on the Label Peak Sizes macro.

5. Select each colour and ensure that the peaks have been properly labelled with sizes as follows: Click on one of the coloured squares next to the Dye/Lanes window and draw a virtual box around the peaks. Press Ctrl R to zoom in on peaks and Ctrl Y to show individual plots. If peaks have been missed or labelled incorrectly, change this by clicking on the appropriate peaks. Press Ctrl H to zoom back out. Do this for all other colours as well and save.

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6. Click inside the Dye/Lanes window, click Edit and Select all.

7. Double click on the Label Peaks Categories macro.

8. Select each colour and ensure that the peaks have been properly labelled with the allele numbers as follows: Click on one of the coloured squares next to the Dye/Lanes window and draw a virtual box around the peaks. Press Ctrl R to zoom in on peaks and Ctrl Y to show individual plots and check. Press Ctrl H to zoom back out. Do this for all other colours as well and save.

9. Double click on the Make Table macro and save. All samples are now typed.

SOP: MiniSTR-reactions

Preparing primer mixtures from 100µM stocks:

10x primer mix for Miniplex 1

Blue	TH01	0.75 µM	(dilute 100uM ¼ and take	3µl)
Green	CSF1PO	3 µM		3µl
Yellow	TPOX	2 µM	(dilute 100uM ¼ and take	8ul)

Dilute in 86ul H_20

10x primer mix for Miniplex 2

Blue	D5S818	3 µM		3µl
Green	D8S1179	5 µM		5µl
Yellow	D16S539	1 µM	(dilute 100uM ¼ and take	4ul)

Dilute in 88 H_20

10 x primer mix for Miniplex 3

Blue	FGA	3 µM	3 µl
Green	D21S11	5 µM	5 µl
Yellow	D7S820	5 µM	5 µl

Dilute in 87ul H_20

10 x primer mix for Miniplex 4

Blue	vWA	2 µM	2ul
Green	D18S51	4 µM	4 µl
Yellow	D13S317	5 µM	5 µl

Dilute in 89ul H_20

10 x primer mix for D3S1358

D3S1358	5 µM	5 µl

Dilute in 95µl H_20

Appendix II. Composition of Buffers and Solutions

0.5 M EDTA pH 8-8.5 Commercial Stock

10% (w/v) *N*-lauroyl sarcosine (50mL)

5g *N*-lauroyl sarcosine

Add *N*-lauroyl sarcosine to 50mL DI H_2O.

20 mgmL^{-1} Proteinase K (5mL)

100g Proteinase K

Add Proteinase K to 5mL DI H_2O.

Bone Lysis Buffer 0.465 M EDTA pH 8-8.5, 0.5% *N*-lauroyl sarcosine, 0.4mgmL^{-1} Proteinase K (50mL)

2.5mL 10% (w/v) *N*-lauroyl sarcosine
1mL 20 mgmL^{-1} Proteinase K

Add *N*-lauroyl sarcosine and Proteinase K to 46.5mL of 0.5 M EDTA pH 8-8.5.

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5% (w/v) Chelex (50mL)

2.5g Chelex

Add Chelex to 50mL DI H_2O.

10X TBE Buffer (1L)

108g Tris Base
EDTA
~55g Boric Acid

Add Tris Base, EDTA and Boric Acid to 800mL DI H_2O and mix to dissolve. Adjust to 1L with additional DI H_2O once dissolved.

1X TBE Buffer (1L)

100mL 10X TBE Buffer

Add TBE to 900mL DI H_2O.

Polyacrylamide Gel Mix (50mL)

18g Urea
5mL Long-Ranger Gel Solution
5mL 10X TBE

Add required DI H_2O to Urea, Long-Ranger Gel Solution and 10X TBE get 50mL.