Investigation into DNA recovered from human teeth for forensic applications

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Declaration

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Abstract

In South Africa, there is a burden of unidentified deceased individuals in forensic mortuaries. When human remains are severely compromised, hard tissues may provide the only DNA source for identification. The QIAamp® DNA Investigator Kit is used in forensic laboratories worldwide, including in South Africa, to extract DNA for identification purposes. However, in local forensic casework, the DNA recovered from teeth is often of insufficient quantity and quality for generating a DNA profile. The phenol-chloroform DNA extraction method has demonstrated improved, yet inconsistent results, when used on hard tissues. Therefore, this study assessed DNA recovery from 52 human control teeth from three deceased individuals, using an optimised phenol-chloroform method. This method involved an overnight demineralisation, two additions of phenol/chloroform/isoamyl alcohol (25:24:1) and an ethanol precipitation, as used by the Australian Federal Police. Quantitative PCR (Quantifiler™ Trio DNA Quantification Kit) and DNA profiling (PowerPlex® ESI 16 System) were then used to assess DNA quantity and quality. Results were compared to those obtained from the same teeth but extracted using the QIAamp® DNA Investigator Kit. The phenol-chloroform method recovered DNA with significantly higher yields ($p = 0.0454$) and significantly less degradation ($p < 0.0001$). Despite this improvement, there was no significant difference in DNA profiling success. This study also did a preliminary analysis of other factors affecting results and suggested that premolars might be the best tooth type with regards to DNA quantity, quality and profiling. Furthermore, dental disease and jawbone had a significant impact on results from teeth. Lastly, the phenol-chloroform method was applied to six teeth from a marine decomposition case to assess its performance in a local forensic setting. DNA metrics were particularly poor in this casework example, highlighting how different forensic and control environments are and the need for further optimisation. Overall, this study supports the use of the phenol-chloroform method and has provided a preliminary suggestion of the best tooth type, jawbone and tooth condition for DNA analysis for forensic human identification.

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Abbreviations

µ	micro
%	percentage
° C	degrees Celsius
®	registered trademark
™	trademark
A	adenine
AFP	Australian Federal Police
ANOVA	analysis of variance
bp	base pair
C	cytosine
C_T	threshold cycle
DHA	Department of Home Affairs
DI	degradation index
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FSL	Forensic Science Laboratory
G	guanine
g	gram
HCl	hydrochloric acid
HREC	Human Research Ethics Committee
ICMP	International Commission on Missing Persons
IPC	internal positive control
l	litre
m	milli
M	molar
mtDNA	mitochondrial DNA
n	nano
NaCl	sodium chloride
nDNA	nuclear DNA
NGS	next generation sequencing
p	pico

PCIA	phenol/chloroform/isoamyl alcohol
PCR	polymerase chain reaction
pH	potential of hydrogen
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
SAPS	South African Police Service
SDS	sodium dodecyl sulphate
SRM	Salt River Medico-legal Mortuary
STR	short tandem repeat
SNP	single nucleotide polymorphism
T	thymine
TE	Tris-EDTA
Tris-HCl	trisaminomethane hydrochloride
w/v	weight per volume

Chapter 1: Introduction and literature review

1.1 Background

1.1.1 Human identification

South Africa's medico-legal mortuaries are burdened by ever-increasing numbers of deceased individuals, which necessitates the improvement of current forensic workflows. When a person dies due to unnatural causes it is required by South African law (as per the Inquests Act No.58 of 1959) that a medico-legal autopsy is performed to determine cause of death. During the medico-legal autopsy it is the imperative of the Forensic Pathology Services to determine cause of death, while the identification of the decedent falls under the purview of the South African Police Service (SAPS).

Identification of the decedent is an essential part of the medico-legal death investigation as there are many social, legal and financial implications (Dix & Graham, 1999; Hanzlick & Smith, 2006). Identification is often critical in providing closure for surviving family and friends, as well as assisting in the investigation (Dix & Graham, 1999). When individuals remain unidentified, the cost of storage and eventual disposal of the body is substantial, which increases the burden on already-strained government resources (Evert, 2011). The burden of unidentified individuals is a challenge globally; however, in this minor dissertation there will be a focus on this challenge in a South African context. A study by Evert (2011) found that between 7 % and 10 % of bodies remain unidentified at the Pretoria Medico-legal Laboratory. Furthermore, more than 3 500 cases of suspected unnatural death are admitted annually to Salt River Medico-legal Mortuary (SRM) in Cape Town, with approximately 9 % remaining unidentified (Reid, Martin & Heathfield, 2019).

One major challenge with identification is when human remains are skeletonised, decomposed, dismembered or severely burned. In cases where visual or fingerprint identification is impossible, anthropological identification methods can be used, because the skeleton, including teeth, will usually survive longer than other identifiable features (Christensen & Anderson, 2013). However, if the skeleton is incomplete or extensively fragmented, DNA-based identification may be the only method available for identification (Sweet, Hildebrand & Phillips, 1999). Unlike fingerprints or dental features, DNA is not limited to a specific body

part, and DNA-based identification is currently the gold standard for identification of victims in forensic cases where remains are extensively fragmented or decomposed (Butler, 2006; Hughes-Stamm, 2012; Ziętkiewicz *et al*., 2012; Watherston *et al*., 2018).

1.1.2 DNA profiling

DNA is located in the nucleus and mitochondria of cells; therefore, excluding some specialised cells that lack these organelles, every cell in the body contains DNA. Approximately 99.7 % of the nuclear genome is identical among people; therefore, to identify an individual, forensic scientists focus on the 0.3 % of the genome that exhibits a high amount of variability (Butler, 2012). Variant regions of nuclear DNA can occur as repeat sequences that are made up of a core unit that is repeated multiple times. These core units occur in many different sizes with short tandem repeats (STRs) having a core unit of 2 to 6 base pairs (bp), and with tetranucleotides (a core repeat unit of 4 bp) being the most common in human identification applications (Butler, 2005).

DNA profiling is a technique used for human identification that is based on a matching principle and involves the comparison of an unknown profile to a known reference profile. A DNA profile represents a combination of STR loci (Butler, 2005). The number of times a core repeat unit occurs at each locus is variable between individuals and this gives STRs a high discriminatory power. The STRs that are used in forensic DNA profiling are in non-coding regions, so they do not "contain any information on the health or medical condition or mental characteristic of a person or the predisposition or physical information of the person other than the sex of that person" (*Criminal Law (Forensic Procedures) Amendment Act, No. 37 of 2013*, 2014:s36A). The selection of STR loci for profiling is based on their level of variation and the number of loci used varies based on the country.

There is a need to optimise DNA extraction workflows in forensic laboratories, to increase chances of obtaining forensically usable DNA from degraded samples and identifying the remains. The primary aim of this minor dissertation is to evaluate the performance of the organic phenol-chloroform method on human teeth and challenging remains, in terms of its potential to aid in forensic human identification and to compare this method to the commercial QIAamp® DNA Investigator Kit currently being used in SAPS Forensic Science Laboratory (FSL). The secondary aim is to preliminarily assess the impact of tooth morphotype, dental disease and dental treatment on DNA recovery using the phenol-chloroform method. To provide suitable background for the aims and objectives of this study (section 1.6),

decomposition and how it affects the identification process will be discussed. This will be followed by teeth as a source of DNA and DNA extraction methods for hard tissues.

1.2 Taphonomy

Forensic taphonomy is the study of what happens to human remains after death. It is used to provide answers by evaluating the state of decomposition and context the remains were found in (Gibbon, 2018). One taphonomic process is known as decomposition, which is a complex process that involves the disintegration of the soft tissues until skeletonisation is reached (Forbes, 2008). An understanding of the decomposition process and the factors that influence it is integral for the interpretation of human remains (Gunn, 2009).

Two major processes that influence the amount and rate of human decomposition are autolysis and putrefaction. During autolysis, the digestion enzymes break down the cellular and nuclear membrane and release the cell's contents into the extracellular matrix (Gill-King, 1997). These enzymes include nucleases, which degrade nucleic acids. Nucleic acids are macromolecules that contain genetic information and include DNA and RNA (Perry *et al.*, 1988). Therefore, DNA becomes increasingly more fragmented as biological tissues decompose (Fondevila *et al*., 2008). The anaerobic environment produced by autolysis initiates putrefaction, which is the breakdown of tissues by microorganisms. Several key factors affect decomposition, including humidity, animal scavenging and trauma (Mann, Bass & Meadows, 1990), with the single most important factor being temperature (Mann, Bass & Meadows, 1990; Megyesi, Nawrocki & Haskell, 2005; Simmons, Adlam & Moffat, 2010).

1.2.1 Molecular taphonomy

Generating DNA profiles from human remains can be difficult as DNA begins to degrade immediately after cell death. Cell death by autolysis takes place when a cell no longer has access to the body's circulating oxygen supply (Latham & Madonna, 2014). Following an organism's death, cells undergo metabolic and homeostatic breakdown, and since DNA repair processes cease to be functional, DNA becomes more vulnerable to damage through enzymatic attack, hydrolysis and oxidation (see Figure 1.1) (Alaeddini, Walsh & Abbas, 2010).

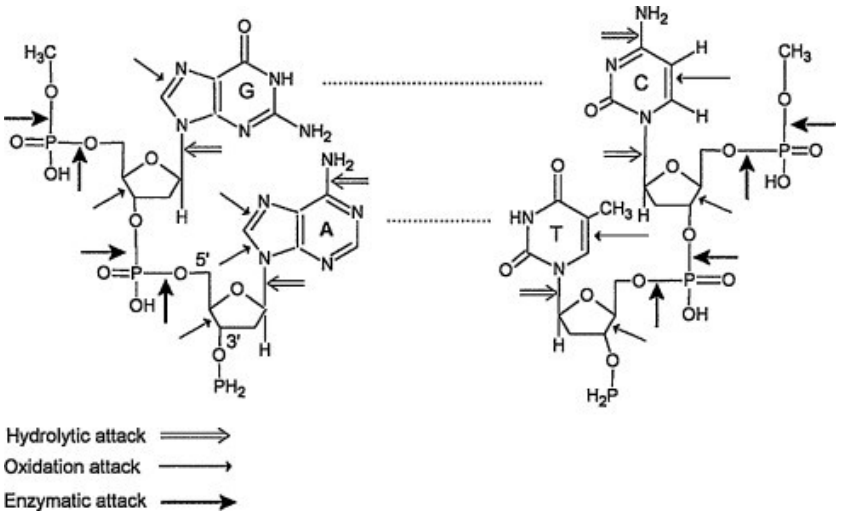

Figure 1.1: Oxidation, hydrolytic and enzymatic attacks associated with DNA degradation. A: adenine, G: guanine, C: cytosine, T: thymine. (Image taken from Alaeddini, Walsh & Abbas, 2010:152).

DNA is degraded by endogenous nucleases released by cells or exogenous nucleases released by microorganisms and invertebrates in the environment (Alaeddini, Walsh & Abbas, 2010). Endogenous nucleases are one of the first agents to start DNA fragmentation in the post-mortem period. These enzymatic attacks are followed at a much slower rate by the spontaneous degradation processes of hydrolysis and oxidation (Lindahl, 1993; Gill-King, 1997; Hofreiter *et al*., 2001).

Hydrolytic reactions cause fragmentation of DNA strands. Water breaks the glycosidic bond between the nitrogenous bases and the sugar backbone resulting in the cleavage of the DNA strand (Alaeddini, Walsh & Abbas, 2010). Additionally, direct hydrolytic cleavage of the phosphodiester bonds in the phosphate-sugar backbone results in a build-up of single-stranded cuts and increasing fragmentation. Hydrolytic reactions also cause the deamination of nitrogenous bases, which creates miscoding lesions that cause incorrect bases to be incorporated during PCR amplification (Hansen *et al*., 2001).

Oxygen-derived species are produced through ionising radiation or the metabolic processes of aerobic microorganisms that colonise tissues post-mortem. Oxidative damage mostly includes changes in sugar residues, conversion of cytosine and thymine to hydantoins, removal of bases and cross linkages. The conversion of cytosine and thymine into hydantoins by oxidation creates a blocking lesion that prevents PCR amplification (Hoss *et al*., 1996).

Therefore, during the decomposition process DNA becomes increasingly fragmented and chemically modified, which decreases the amount of intact target fragments for molecular analyses. The amount of biological degradation depends mainly on two factors: time and environmental conditions (Burger *et al*., 1999). Degradative processes accumulate with time, while environmental conditions (temperature, humidity, pH, soil chemistry) change the speed and degree of degradation. When skeletal remains have been in extreme environmental conditions (*e.g*. heat, humidity, and soil) for long periods of time, recovery of sufficient quantities of nuclear DNA for amplification becomes less likely (Byard, Both & Simpson, 2008).

1.3 Teeth as a source of DNA for human identification

Teeth have often been used as a source of DNA when all other tissues are unavailable or have not produced enough DNA for identification purposes (Hughes-Stamm, 2012). Teeth are the hardest tissue in the human body (Malaver & Yunis, 2003) and are resistant to unfavourable conditions, such as, humidity, high temperatures and microbial action (Alvarez García *et al.*, 1996; Marjanović *et al*., 2007). The limited porosity of teeth and their protected location physically limits the actions of exogenous nucleases released by microorganisms or invertebrates. Therefore, teeth are a preferred, high quality, skeletal DNA source due to their unique composition and location within the jaw bones, which protects them from environmental and physical conditions that accelerate post-mortem decomposition and DNA degradation (Higgins & Austin, 2013).

Anatomically a human tooth can be divided into two parts – the crown, which is exposed to the mouth and the root, which is enclosed in the jawbone (Figure 1.2). The durability of teeth is due to their composition being largely of dentine, which is a highly calcified connective tissue (Gunn, 2009). The crown is covered by enamel, which is about 95 % mineralised, is acellular and contains no DNA. The crown provides a physical barrier between the inner tooth and the external environment (Pinchi *et al*., 2011).

The root consists of cementum and the dentine/pulp complex, which are the DNA-rich regions within the tooth (Higgins & Austin, 2013). Cementum encloses the roots of teeth and is a thin bone-like layer that attaches the tooth to the bone via the periodontal ligament (Gunn, 2009). It can be classified into two types based on the presence or absence of cells. Cellular cementum contains nucleated cells and is a source of DNA (Avery & Chiego, 2006). The dentine/pulp

complex makes up most of the tooth. Dentine is 65 % mineralised and generally does not contain any nucleated cell bodies (Nanci, 2003). However, given that pulp is highly cellular, the dentine/pulp complex provides the richest DNA source in teeth.

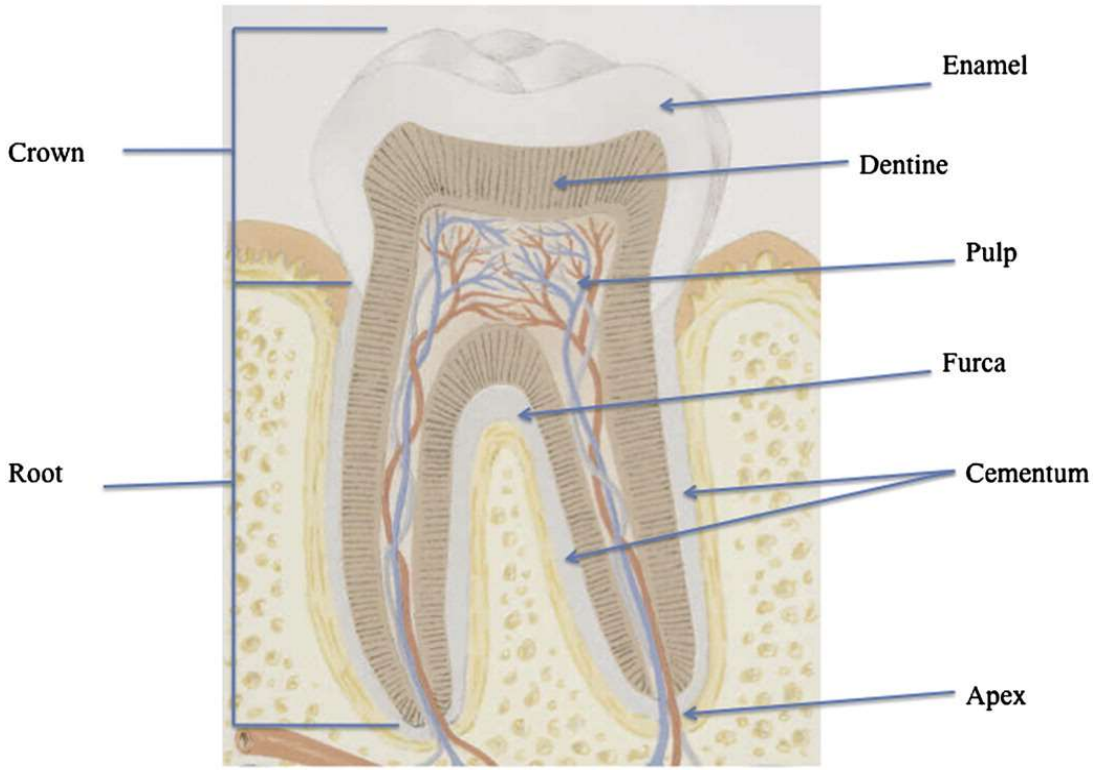

Figure 1.2: Tooth structure of a human mandibular molar (Image taken from Higgins & Austin, 2013:434).

Some studies have investigated which tooth type and portion of the tooth provides the highest DNA yield; however, these studies used mostly healthy unrestored teeth or non-human teeth (Smith *et al*., 1993; De Leo, Turrina & Marigo, 2000; Gaytmenn & Sweet, 2003). When human remains are found it is highly likely that not all the teeth will be available for analysis, and that the teeth that are present may have been altered or be diseased (Higgins *et al.*, 2011). According to Smith *et al*. (1993), one of the factors affecting the quantity of DNA available from teeth is the volume of the pulp chamber, which varies according to tooth type. Smith *et al.* (1993) then demonstrated that in adult teeth, the third mandibular molar has the largest average pulp volume. However, the results of a study by De Leo, Turrina & Marigo (2000) showed a greater DNA yield from premolars compared to molars and canines. Nevertheless, a consistent

correlation between tooth type and the quantity of extracted DNA has not yet been demonstrated.

Some of the other factors that may affect DNA quantity include tooth disease state (Higgins *et al*., 2011) and post-mortem cellular degradation, especially in moist environments (Alvarez García *et al.*, 1996; Higgins *et al*., 2011; Higgins *et al*., 2013). Dental diseases have been reported to negatively affect the DNA content of human teeth (Higgins *et al*., 2011). This is because dental caries, a bacterial disease, causes localised destruction of the calcified tissues of teeth, which helps bacteria access the pulp, resulting in cell death (Yu & Abbott, 2007). Eventually dental caries can cause total loss of pulp or loss of the tooth itself (Smith *et al*., 1993).

The influence of environmental factors on DNA preservation in teeth has not been fully elucidated, but studies on ancient DNA have highlighted some of the effects of these factors. Burger *et al.* (1999) showed that temperature is one of the most important factors affecting DNA, with low temperatures and no microbial contamination favouring preservation. Previous studies investigating the post-mortem degradation of pulp have shown that it decomposes more slowly than other soft tissues (Boy, Bernitz & Van Heerden, 2003; Caviedes-Bucheli *et al*., 2006). A dry environment will encourage the desiccation of pulp, protecting the DNA from hydrolytic damage, whereas a wet environment will increase the destruction of the pulp (Duffy, Skinner & Waterfield, 1991; Pötsch *et al*., 1992). Some additional factors that are hypothesised to affect DNA recovery are jawbone, intact roots, and dental treatments, *i.e.* fillings. Studies investigating DNA recovery from human teeth have not taken these factors into account or did not stratify the data accordingly (De Leo, Turrina & Marigo, 2000; Gaytmenn & Sweet, 2003; Higgins *et al*., 2011; Rubio *et al*., 2018).

This limitation of previous studies means that the impact of these factors on DNA recovery is unknown. Based on the available literature, it cannot be definitively concluded which tooth morphotype is the best, and there is a definite need for further investigation into the tooth type that is best suited for DNA analysis. This highlights a gap in the literature regarding DNA recovery from teeth. In cases where multiple teeth are available, it would be beneficial for the pathologist, odontologist or anthropologist, to have guidelines on which tooth would be best for DNA recovery to optimise the use of time and resources. The following section will look at some of the extraction methods available to recover DNA from hard tissues such as teeth and bones.

1.4 DNA extraction methods for hard tissues

The generation of a DNA profile from bones and teeth is an integral step in the identification process in both mass disasters and unidentified individuals. Bones and teeth are often the only biological tissues that remain following exposure to environmental conditions, extreme heat, traumatic events, and in cases of extended post-mortem interval (Latham & Miller, 2019). Therefore, being able to recover large amounts of DNA from hard tissues would be useful in a forensic context (Latham & Miller, 2019).

While investigations into DNA recovery from hard tissues using current DNA extraction workflows have been done, the results have been inconsistent and there remains scope for further research, as well as, possible improvements (Davoren *et al*., 2007; Pagan *et al*., 2012; Iyavoo, Hadi & Goodwin, 2013; Kuś, Ossowski & Zielińska, 2016). The physical and chemical properties that make bones and teeth resistant to degradation can make it difficult to extract DNA from them (Holland *et al*., 2003; Loreille *et al*., 2007; Miloš *et al*., 2007). Furthermore, DNA degradation and intrinsic PCR inhibitors make it important to extract the maximum amount of intact DNA, while also removing PCR inhibitors (Alaeddini, Walsh & Abbas, 2010).

There are two main categories of DNA extraction: liquid-phase extraction and solid-phase extraction. The organic phenol-chloroform method is an example of a liquid-phase extraction as it is based on the principle of the differential partitioning of molecules between two immiscible phases, organic and aqueous (Figure 1.3).

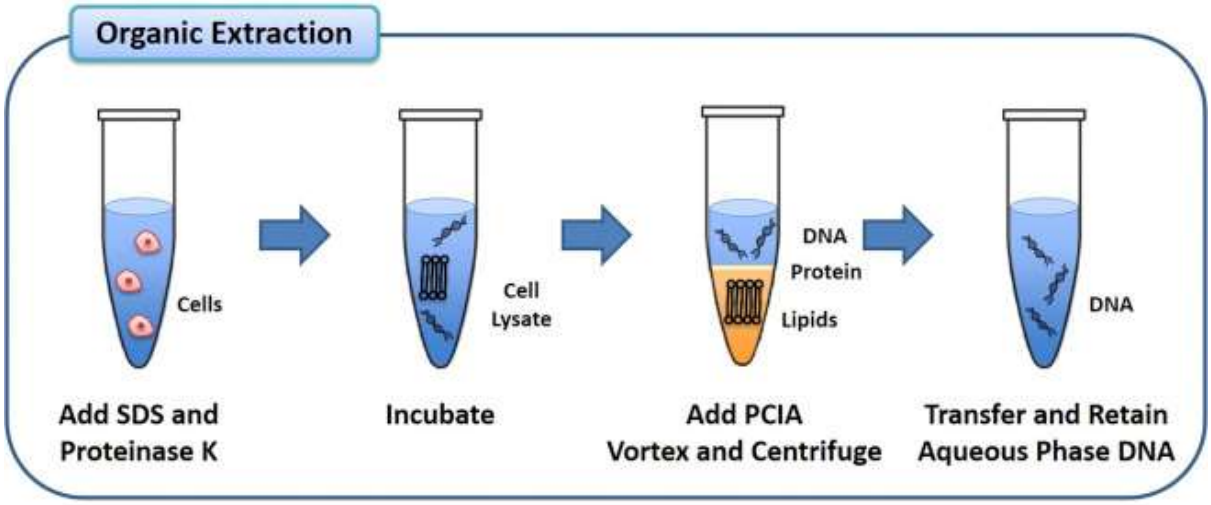

Figure 1.3*:* The general workflow of phenol-chloroform extraction (Image taken from McKiernan & Danielson, 2017:379). PCIA: phenol/chloroform/isoamyl alcohol.

Phenol–chloroform extraction uses sodium dodecyl sulphate (SDS) and proteinase K to enzymatically digest proteins and other cellular components. Following this, a mixture of phenol/chloroform/isoamyl alcohol (PCIA) (25:24:1) is then added, which causes the lipids and proteins to move into the organic phase, leaving DNA in the aqueous phase. Centrifugation allows for the two phases to separate completely. Following this, the aqueous phase containing the DNA can be removed and used in further analyses. The DNA can also be recovered and concentrated from the aqueous phase by ethanol precipitation or by using a centrifugal filter unit (McKiernan & Danielson, 2017).

The DNA extraction method that will be evaluated in this minor dissertation is the modified phenol-chloroform extraction method with ethanol precipitation described by Pagan *et al.* (2012) and used by the Australian Federal Police (AFP). This specific protocol was chosen due to its use by the AFP for forensic human identification for highly degraded bone and teeth (Pagan *et al*., 2012).

The QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany), which is currently used by SAPS FSL to extract DNA from teeth, is an example of a silica-based solid phase extraction method (Figure 1.4). DNA selectively absorbs to silica on a support, such as small glass beads, in the presence of high concentrations of chaotropic salts. The bound DNA is then washed with ethanol and finally eluted by means of an elution buffer (Boom *et al.,* 1990).

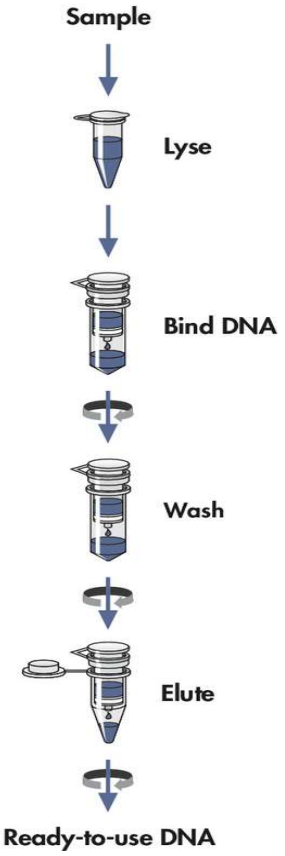

Figure 1.4: The QIAamp® DNA Investigator Kit workflow (Image taken from QIAGEN, 2019:9).

The organic phenol-chloroform method was previously regarded as the gold standard in forensics for DNA extraction; however, it has disadvantages as it makes use of hazardous chemicals, requires the use of extraction fans and other safety measures, and cannot be adapted for automation (Iyavoo, Hadi & Goodwin, 2013). The chemicals used in this extraction method are hazardous to health as they can result in acute oral, dermal and inhalation toxicity, as well as skin corrosion, and possible carcinogenesis and mutagenesis (Life Technologies, 2014). An advantage is that it is less expensive, and along with efficacy, the cost of a DNA extraction workflow is relevant for forensic laboratories. Conversely, silica-based methods, such as the QIAamp® kit are more expensive, but they are adapted to automation and are less hazardous.

The organic phenol-chloroform extraction method has been used in a number of studies (Davoren *et al*., 2007; Marjanović *et al*., 2007; Pagan *et al*., 2012; Ferrerira *et al*., 2013; Iyavoo, Hadi & Goodwin, 2013; Marjanović *et al.*, 2015; Kuś, Ossowski & Zielińska, 2016; Rubio *et al*., 2018) and has been shown to have varying success in recovering DNA from bones and teeth with mean DNA concentrations up to 271.607 ng/µl and up to 100 % DNA profiling

success. The organic phenol-chloroform methodology used in these studies varies greatly with different amounts of various sample types, different demineralisation steps and different phenol-chloroform extraction methods (standard *vs* modified). As there is no consensus on which methodology is the best for maximising intact DNA recovery, the protocol described by Pagan *et al*. (2012) was chosen as this study's baseline protocol as it is in use operationally and is easily reproducible (Pagan *et al*., 2012).

Very few of the studies investigating DNA recovery using the phenol-chloroform method have focused on human teeth as a sample type. Furthermore, many of these studies have used casework samples and studied teeth retrospectively. Therefore, there is a lack of prospective studies with teeth from a controlled environment, and where lots of teeth are available, and more importantly, available from the same person. There remains a need to investigate the performance of the phenol-chloroform method on human teeth in a controlled environment, and additionally, to evaluate whether it would be beneficial to implement this method in a local forensic setting.

1.5 Rationale

There is a growing burden of unidentified human remains in South Africa. More specifically, cases where human remains are skeletonised, decomposed, dismembered, or severely burned, as these are challenging to identify. In these cases, identification is often only possible by recovering DNA from hard tissues, such as bones and teeth (Sweet, Hildebrand & Phillips, 1999). The QIAamp® DNA Investigator Kit is often used in forensic laboratories globally, as well as in South Africa, for DNA recovery for identification purposes. However, in a local forensic setting, the DNA extracted from human teeth is often of inadequate quantity and quality for generating a DNA profile. While this method offers a health and safety advantage, the fact that it does not always produce enough DNA for identification purposes offers motivation for alternative methods to be investigated, provided the necessary safety measures are in place.

Some studies have demonstrated that the phenol-chloroform extraction method showed favourable results when used on hard tissues (Ferreira *et al*., 2013; Iyavoo, Hadi & Goodwin, 2013; Kuś, Ossowski & Zielińska, 2016; Rubio *et al.*, 2018) and it was previously regarded as the gold standard in forensics for DNA extraction (Iyavoo, Hadi & Goodwin, 2013). However, these studies have had varying success rates and there is no consensus regarding the methodology used. Therefore, there is a need for further investigation and optimisation of the performance of the phenol-chloroform method on hard tissues.

Furthermore, there is a gap in the literature regarding which tooth morphotype is best for DNA recovery in forensic human identification, as molars are often recommended, but this has not been empirically proven. In cases where multiple teeth are available, it would be useful for the forensic professional to have definitive guidelines on which tooth would be best for DNA recovery.

Lastly, there is a need for the improvement of the DNA identification of unidentified individuals to ease the burden on government resources and provide social justice. It is also anticipated that this improvement will assist in the overall resolution of cases, as well as providing families with closure and the opportunity to mourn their loved ones.

1.6 Research aims and objectives

1.6.1 Primary aim

The primary aim was to assess DNA recovery from human teeth using the organic phenol-chloroform DNA extraction method and to compare results with those generated with the QIAamp® DNA Investigator Kit.

To this end, the objectives were to:

- Establish and optimise a phenol-chloroform DNA extraction workflow on human teeth in the local laboratory setting.
- Assess the quantity and quality of DNA recovered from human teeth using the organic phenol-chloroform extraction method. To answer the following question:
    - Does the phenol-chloroform method recover enough intact DNA that is suitable for DNA profiling?
- Compare DNA recovery from human teeth using the phenol-chloroform method with that from the same teeth where DNA was extracted using the QIAamp® DNA Investigator Kit. To answer the following question:
    - Is the DNA recovered using the optimised phenol-chloroform method better than the QIAamp® DNA Investigator Kit in terms of quantity, quality and profiling?
- Evaluate DNA recovery from teeth of a forensic case using the phenol-chloroform method. To answer the following question:
    - How does the phenol-chloroform method perform in a local forensic case?

1.6.2 Secondary aim

The generation of the data for the primary aim provided the opportunity for a preliminary analysis of whether DNA recovery from human teeth using the organic phenol-chloroform extraction method is impacted by these factors: tooth morphotype, dental disease, or dental treatment.

To this end, the objectives were to:

- Compare the quantity and quality of DNA recovered between different tooth types (canines, incisors, premolars and molars), between teeth from the two jawbones

(mandibles and maxillae), and between teeth with intact roots and teeth without intact roots. To answer the following question:

    - Do (i) tooth type, (ii) jawbone and (iii) intact roots affect DNA recovery from human teeth using the phenol-chloroform method?

- Compare the quantity and quality of DNA recovered from diseased teeth (*e.g.* caries) and non-diseased teeth. To answer the following question:
    - Does dental disease affect DNA recovery from human teeth using the phenol-chloroform method?
- Compare the quantity and quality of DNA recovered from teeth with dental treatment (*e.g.* fillings) and teeth without dental treatment. To answer the following question:
    - Does dental treatment affect DNA recovery from human teeth using the phenol-chloroform method?

Chapter 2: Materials and Methods

2.1 Samples

This study made use of 52 previously ground tooth powder samples representing all tooth types from three deceased individuals (Appendix B.1: Table B.1). These samples were used in a study in our research group that assessed DNA yield from different tooth types using the QIAamp® DNA Investigator Kit (Zachou, 2019). The teeth came from deceased individuals who were donated for research under the National Health Act (No.61 of 2003) to the University of Cape Town, where after, Professor Graham Louw (Head of the Division of Clinical Anatomy and Biological Anthropology) gave permission for the teeth from these individuals to be used in this study. These teeth will be referred to as 'control teeth'.

In addition, two fresh human teeth were donated from wisdom tooth removals for optimisation purposes. These teeth will be referred to as 'optimisation teeth'. The optimised phenol-chloroform method was then applied to six teeth from the unidentified remains of a marine wash-up case (Appendix B.3: Table B.3). They consisted of a canine, central incisor, first premolar, first molar, second molar and third molar. These teeth will be referred to as 'forensic teeth'. Ethics approval was obtained for this research project from the Faculty Health Sciences Human Research Ethics Committee (HREC REF: 222/2019).

2.1.1 Sample preparation

The optimisation and control teeth had been previously cleaned, and the forensic teeth were cleaned, using a sterilisation protocol for the instruments and workspace as developed by Longden-Thurgood (2017). Instruments and workspace surfaces were sterilised between the sampling of individual teeth using this protocol, and personal protective equipment was used to decrease possible contamination. The outer surfaces of teeth were decontaminated as described by Longden-Thurgood (2017) by using bleach as a primary decontamination agent.

A non-targeted sampling technique was used as described by Pinchi *et al*. (2011). In brief, once each tooth was decontaminated, the outer enamel layer was removed using a rotary sanding tool (Dremel, Mount Prospect, USA), and subjected to grinding into a fine powder. Teeth were placed individually into ice-cold grinding jars and onto the TissueLyser II (QIAGEN, Hilden, Germany) at an oscillation frequency of 30 Hz for 1 minute.

The tooth powder from the optimisation and control teeth were used for the study by Zachou (2019) and then stored in 1.5 ml microcentrifuge tubes at -20 °C. The powder was then used for this project to facilitate direct comparisons between the two DNA extraction methods. The tooth powder generated from the forensic teeth was used for this study and stored in 1.5 ml microcentrifuge tubes at -20 °C.

2.2 DNA extraction and optimisation

The tooth powder from the two optimisation teeth was used for all optimisation experiments. Initially, extractions were performed using the modified phenol-chloroform method according to Pagan *et al.* (2012). In brief this included an overnight demineralisation in 5.25 ml of extraction buffer (Tris-HCl (10 mM), EDTA (0.1 mM), NaCl (100 mM), SDS (2 % w/v), proteinase K (5 mg) and solid DTT (30 mg)), followed by two additions of PCIA (25:24:1) and an ethanol precipitation. The supernatant was then removed, and the remaining pellet was resuspended in 50 µl of TE buffer. An elution volume of 50 µl was used by Zachou (2019) while processing the tooth powder using the QIAamp® DNA Investigator Kit. The following modifications were made to the protocol: the samples were incubated at 56 °C overnight in an oven and agitated manually every 20 minutes for the first hour. These results formed the baseline data to which the data from the optimisation experiments were compared. The following variables were then adjusted in an attempt to improve DNA recovery:

- The protocol specifies that only 500 µl of supernatant be transferred following the initial lysis step. This was adjusted to four aliquots of supernatant (each 500 µl) that were taken through the protocol separately with the precipitated DNA being pooled in the final step.
- Phenol was equilibrated from pH 6.7 to pH 8.0 using Tris-HCl buffer.
- The weight of tooth powder was increased from 0.1 g to 0.5 g. These samples were processed using phenol with pH 8.0. Zachou (2019) made use of 0.1 g of tooth powder following the QIAamp® DNA Investigator Kit on the same set of teeth.

The quantity and quality of DNA recovered was determined by quantitative PCR (qPCR) (section 2.3) and DNA profiling (section 2.4), and the results were compared to the baseline data after each variable was adjusted (Appendix B.2: Table B.2). It was determined that using phenol of pH 8.0 and a starting material of 0.5 g (dependent on sample availability) produced

better DNA yields and quality compared to the baseline protocol (Appendix B.2: Table B.2). Thus, this method was used for all subsequent DNA extractions on samples.

However, some of the tooth powder samples used in this study had a limited amount remaining after use in the QIAamp® extraction. Therefore, the amount of starting material used for phenol-chloroform extraction of samples varied depending on sample availability with a maximum of 0.5 g as per the optimised method. A DNA extraction blank was included and taken through the DNA assessment workflow. Additionally, positive and negative controls were included in each experiment.

2.3 Assessment of DNA recovery

The quantity and quality of DNA samples were assessed using qPCR. All DNA samples were quantified with the Quantifiler™ Trio DNA Quantification Kit (Applied Biosystems, Foster City, USA) according to the manufacturer's user guide (Quantifiler™ HP and Trio DNA Quantification Kits user guide, p. 27-30). Thermal cycling was performed according to the manufacturer's user guide on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, USA). Data was collected using HID Real-Time PCR Analysis software v1.2 (Applied Biosystems, Foster City, USA).

The Quantifiler™ Trio assay amplified a small (80 bp) and a large (214 bp) human autosomal target, as well as a region of 75 bp on the human Y chromosome. A mean DNA concentration (ng/µl) was calculated using the concentrations of the small and large targets as determined by qPCR. The ratio of the concentration of the small and the large autosomal target was used to calculate the degradation index (DI) as described by Vernarecci *et al.* (2015). The DI values were then divided into four degradation categories according to Vernarecci *et al*. (2015): 0-1.5 (non-degraded), 1.5-4 (mildly degraded), 4-10 (degraded), > 10 (severely degraded). Where the DNA was too degraded for the large marker to amplify and an index could not be calculated, they were denoted as 'fail'. An internal PCR control (IPC) of synthetic DNA was used in each reaction to identify samples containing PCR inhibitors. IPC values higher than 28 were an indication of the presence of PCR inhibitors (Fondevila *et al*., 2008).

The recommendation for the amount of template DNA for DNA profiling using the PowerPlex® ESI 16 System (Promega, Madison, USA) is 0.5 ng (with a maximum volume of 17 µl); therefore, in this study a DNA concentration of 0.5 ng in 17 µl (*i.e.* 0.029 ng/µl) was regarded as theoretically sufficient for DNA profiling. Furthermore, if DNA was non-degraded or mildly

degraded (DI < 4) it was regarded as suitable quality for DNA profiling (Vernarecci *et al*., 2015).

2.4 Assessment of forensic applications - DNA profiling

DNA profiling was performed to evaluate if the recovered DNA could be used for forensic human identification. The PowerPlex® ESI 16 System (Promega, Madison, USA) was used to prepare a multiplex PCR according to the technical manual (PowerPlex® ESI 16 System technical manual, p. 6-7). For each sample, 0.5 ng of template DNA was added to the PCR, and in cases where the quantity criteria were not met, the maximum volume (17 µl) of template DNA was added. Thermal cycling was performed on a BioRad T100 thermal cycler (California, USA) according to the technical manual (PowerPlex® ESI 16 System technical manual, p. 8). The multiplex system amplified the following loci: Amelogenin, D3S1358, D19S433, D2S1338, D22S1045, D16S539, D18S51, D1S1656, D10S1248, D2S441, TH01, vWA, D21S11, D12S391, D8S1179, and FGA.

After PCR, each amplified sample was added to an Applied Biosystems MicroAmp® optical 96-well plate containing WEN Internal Lane Standard 500 and Hi-Di™ formamide (Promega, Madison, USA). The samples were run on an Applied Biosystems Genetic Analyser 3130*xl* according to the technical manual (PowerPlex® ESI 16 System technical manual, p. 27-29) with the following deviations: a POP-7™ polymer with a 50 cm array and a run voltage of 15 kV. The results of the capillary electrophoresis were analysed using the Applied Biosystems GeneMapper v4.1 software (Foster City, USA).

DNA profiling success was categorised according to the number of markers that were successfully amplified: 0-3 (fail), 3.5-11.5 (partial), 12-15 (full). A full DNA profile was regarded as usable for forensic human identification. Markers where only one allele of a heterozygous locus was amplified were counted as half (0.5). The maximum volume of template DNA and two extra PCR cycles were used for the forensic teeth to maximise profiling results.

2.5 Data analysis

A Spearman rank correlation test was done to determine whether the amount of input tooth powder significantly affected the mean DNA concentration. The DNA recovery and profiling success of the organic phenol-chloroform method used in this study was compared to that from the QIAamp® DNA Investigator Kit which was used to extract DNA from the same teeth. A Wilcoxon signed-rank test was used to test for a significant difference in DNA quantity (mean qPCR concentration in ng/µl), quality (DI) and profiling success (number of STR markers out of 15) between the two methods.

A Mann-Whitney U test was done to test for significant differences between tooth types and individuals (control and forensic). A Mann-Whitney U test was also used to determine if dental disease, dental treatment, intact roots and jawbone significantly influenced results. To test for the influence of dental disease, teeth were grouped as 'Diseased' and 'Non-diseased'. To test for the influence of dental treatment, teeth were grouped as 'Fillings' and 'No fillings'. To test for the influence of intact roots, teeth were grouped as 'Intact roots' and 'Broken roots'. To test for the influence of jawbone, teeth were grouped as 'Mandibles' and 'Maxillae'.

A two-way ANOVA was done to determine whether there is an interaction between tooth type and jawbone on DNA quantity (mean qPCR concentration in ng/µl), quality (DI) and profiling success (number of STR markers out of 15). As the data were not normally distributed, it was first transformed to make it log. A p-value of 0.05 was used to evaluate statistical significance for all statistical tests. Data from one of the samples was excluded from analyses due to a technical error in the initial DNA assessment, and there was insufficient tooth powder for re-processing. Data analyses was performed using Stata statistical software package v13.1. Graphs were created using GraphPad Prism v8.2.1.

Chapter 3: Results

3.1 Is the DNA recovered using the phenol-chloroform method better than the QIAamp® DNA Investigator Kit in terms of quantity, quality and DNA profiling success?

DNA was extracted from teeth using the phenol-chloroform method and compared to those which were generated using the QIAamp® kit. The results showed that the mean DNA yield was significantly higher using the phenol-chloroform method compared to the QIAamp® kit ($p = 0.0454$) (Figure 3.1a).

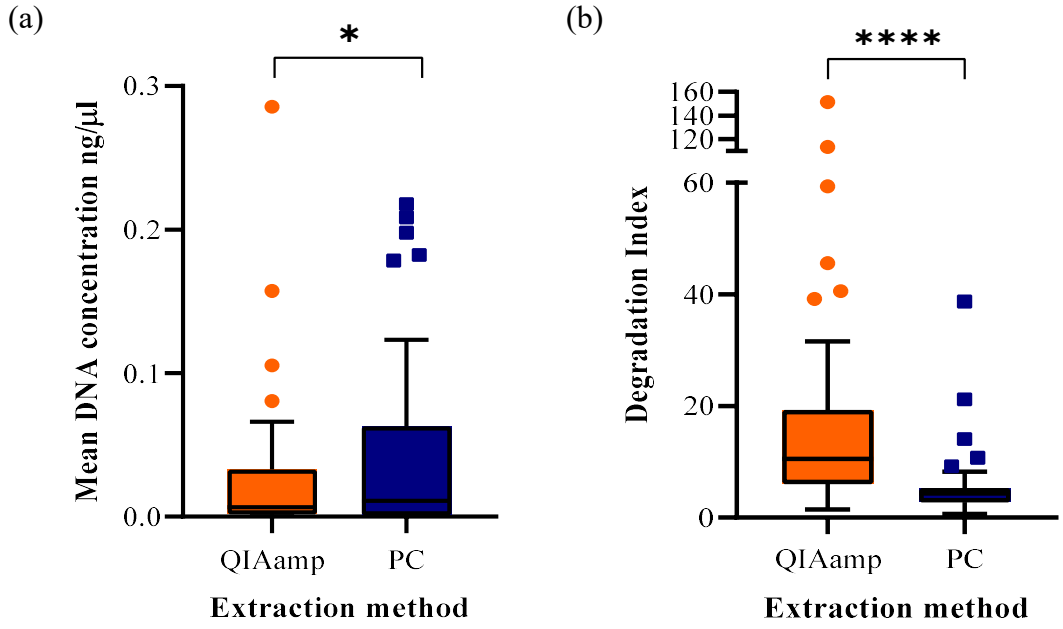

Figure 3.1: Box plots comparing (a) the mean DNA recovery and (b) the Degradation Index of the two extraction methods. Circles and squares indicate outliers; PC = Phenol-chloroform; * ($p < 0.05$) and **** ($p < 0.0001$).

There was also a significant difference between the degradation indices of the two methods ($p < 0.0001$), with the QIAamp® kit producing overall more degraded DNA samples (Figure 3.1 b). The DI values were calculated and categorised according to Vernarecci *et al.* (2015); however, for some of the samples the large autosomal target did not amplify and a DI could not be calculated (these were denoted as 'fail'). The QIAamp® kit had a higher number of samples that were severely degraded (Figure 3.2) and a higher number of samples where the large autosomal target failed to amplify compared to the phenol-chloroform method.

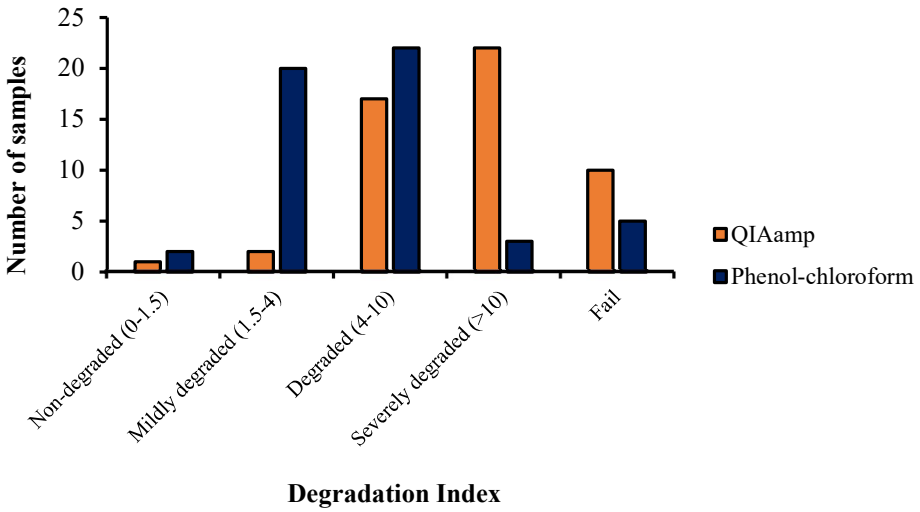

Figure 3.2: The number of samples in the degradation categories according to Vernarecci *et al.* (2015) for the two extraction methods.

Despite the differences in DNA yield and DNA degradation, there was no significant difference between the DNA profiling success of the two methods (p = 0.2803). While the phenol-chloroform method had a slightly higher number of full profiles (Figure 3.3b), this was not statistically significant. Overall, a forensically usable result *i.e.* a full DNA profile (12-15 markers) was generated for 41 % of the control teeth extracted with the phenol-chloroform method (Figure 3.3b) as opposed to 33 % with the QIAamp® kit (Figure 3.3a). However, the phenol-chloroform method had a higher failure rate, with 35 % of teeth extracted with the phenol-chloroform method generating a DNA profile of 0-3 markers (Figure 3.3b) compared to 28 % of teeth extracted with the QIAamp® kit (Figure 3.3a).

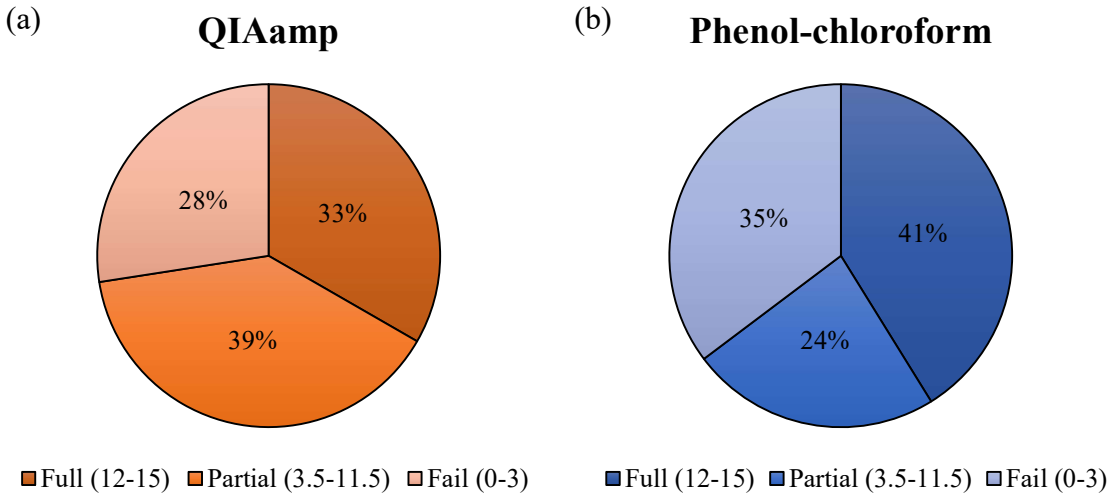

Figure 3.3: DNA profiling results for control teeth extracted using (a) the QIAamp® kit and (b) the phenol-chloroform method.

3.2 How much intact DNA suitable for DNA profiling was recovered from control and forensic teeth using the phenol-chloroform method?

The phenol-chloroform extraction method yielded a wide range of DNA concentrations between 0.000201 ng/µl and 0.217676 ng/µl (Appendix C: Table C.1) (Figure 3.1a). A Spearman rank correlation test showed that the amount of input tooth powder did not significantly affect the mean DNA concentration ($r = 0.0487$; $p = 0.8212$). However, the DNA concentration varied depending on individual (Figure 3.4) and tooth type (Figure 3.5a). The difference in mean DNA recovery was statistically significant between the three control individuals ($p < 0.05$ for each pairwise comparison). A full profile was generated for 43 % (n = 3/7) of the samples from Individual 1, whereas a full profile was only generated for 4 % (n = 2/25) of the samples from Individual 2. Furthermore, Individual 3 had the most profiling success with 89 % (n = 17/19) of samples generating a full profile.

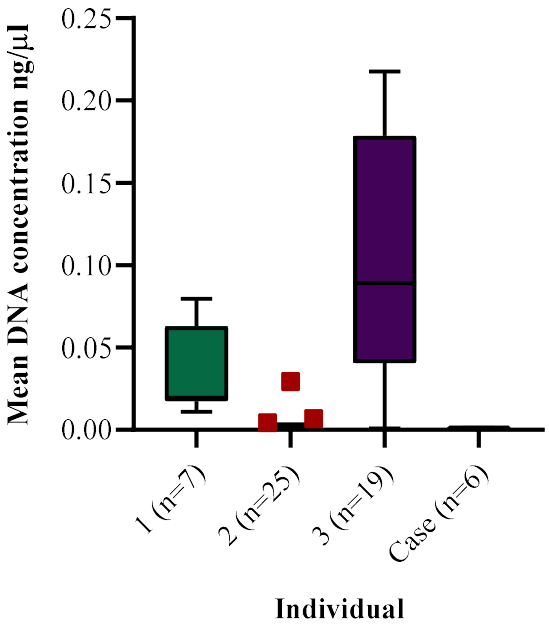

Figure 3.4: Box plot of the mean DNA recovery amongst the three individuals from the control teeth and the forensic teeth (Case) using the phenol-chloroform extraction method. Squares indicate outliers.

Six teeth from a forensic marine decomposition case were analysed using the optimised phenol-chloroform method to assess its efficacy in an authentic forensic context. They consisted of a canine, central incisor, first premolar, first molar, second molar and third molar. In comparison

with the control teeth, the forensic teeth samples yielded significantly lower DNA concentrations ($p = 0.0054$) with a range of between 0.000237 ng/µl and 0.001126 ng/µl (Appendix C.1: Table C.1) (Figure 3.4). No full DNA profiles were generated, but 50 % (n = 3/6) of teeth produced partial profiles.

3.3 Do tooth morphotype, dental disease and dental treatment affect DNA recovery using the phenol-chloroform method?

Premolars were the tooth type with the highest mean DNA concentration (Figure 3.5a); however, the difference between the mean DNA concentrations among tooth types was not statistically significant. Premolars were also the tooth type that provided the best profiling results with 54 % (7/13) of control teeth generating a full profile, and premolars were the tooth type that produced the lowest DI (mean DI = 3.332006 ± 1.878218) of control teeth. For forensic teeth, the first premolar had the highest mean DNA recovery (Figure 3.5b), but the first molar produced the best DNA profile (peaks at 8.5 markers).

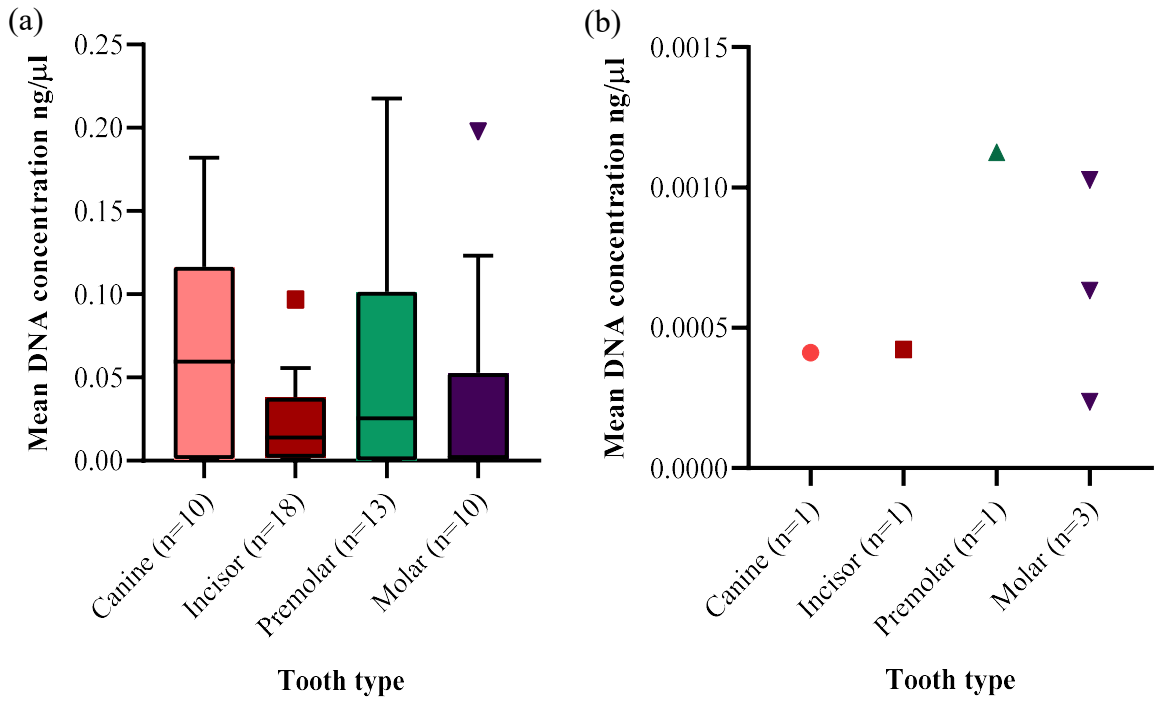

Figure 3.5: (a) Box plots of the mean DNA concentrations for different tooth types in control teeth and (b) a scatter plot of the mean DNA concentrations for different tooth types in forensic teeth. In the box plots squares and triangles indicate outliers.

The presence of intact roots alone did not significantly influence the mean DNA concentration, DI or the DNA profiling success of the control teeth. However, disease significantly influenced the mean DNA concentration ($p < 0.0001$) and DNA profiling success ($p < 0.0001$) (Figure 3.6), and jawbone was found to significantly influence DI ($p = 0.0249$) (Figure 3.7). Teeth with dental treatment (*e.g.* fillings) had substantially lower DNA yields, although this was not statistically significant ($p = 0.1884$). DNA profiling results from teeth with fillings were also very poor with 60 % producing failed profiles (0-3 markers) and 40 % producing partial profiles (3.5-11.5 markers). The forensic teeth did not have any dental disease (except for one superficial cavity) or dental treatments, and all the roots were intact.

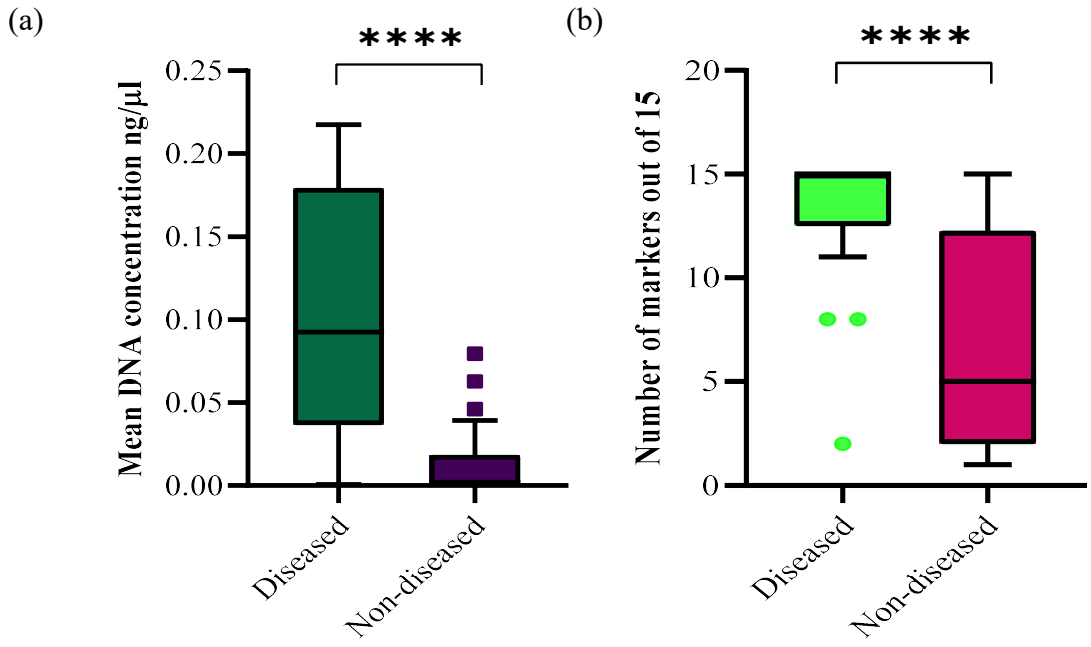

Figure 3.6: Box plots of (a) the mean DNA concentration and (b) the number of markers successfully amplified in DNA profiling between diseased teeth (n = 18) and teeth with no disease (n = 33). Circles and squares indicate outliers; **** ($p < 0.0001$).

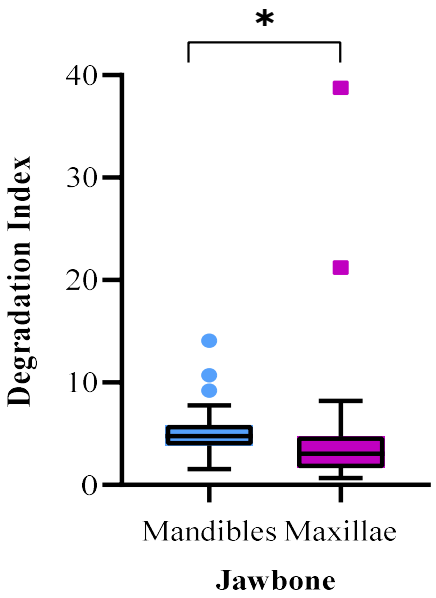

Figure 3.7: Box plot comparing the degradation indices between the teeth from the mandibles (n = 26) and from the maxillae (n = 25). Circles and squares indicate outliers; * ($p < 0.05$).

To assess the combined influence of tooth type and jawbone on the quantity and quality of DNA recovered, a two-way ANOVA was used following a log transformation of the data. Tooth type and jawbone were the variables included in the model to determine whether combined they might have a significant effect on DNA quantity and quality, even though individually they did not. This model showed that the combination of tooth type and jawbone accounted for 10.10 %, 17.48 % and 10.28 % of the variation in DNA quantity (mean qPCR concentration in ng/µl), quality (DI) and profiling success (number of STR markers out of 15), respectively. This model also showed that there was no statistically significant interaction between the effects of tooth type and jawbone on DNA quantity, quality or profiling success.

Due to the many variables that were observed to have an effect, the model could not be expanded to include more variables as the number of samples that would remain in each of those groups would be too few for statistical analyses. Therefore, a much larger sample size is needed to assess the effect of the combination of variables.

Chapter 4: Discussion and conclusion

The primary aim of this study was to evaluate the performance of the organic phenol-chloroform method on human teeth in terms of its potential for forensic human identification. This was done by first optimising the phenol-chloroform method and then implementing it in our local laboratory setting. The optimised method was then applied to a set of previously ground control teeth. These results were then compared to those obtained from the same teeth using the commercial QIAamp® DNA Investigator Kit currently being used by SAPS FSL. Lastly, the optimised method was applied to a forensic marine decomposition case to evaluate its performance in an authentic forensic setting. The secondary aim was to preliminarily assess the influence (if any) of tooth morphotype, dental disease and dental treatment on DNA recovery from human teeth using the phenol-chloroform method.

4.1 Is the DNA recovered using the phenol-chloroform method better than the QIAamp® DNA Investigator Kit in terms of quantity, quality and DNA profiling success?

The control teeth used in this study were used in a previous study by Zachou (2019) that examined the best tooth type using the QIAamp® DNA Investigator Kit, which is routinely adopted in forensic laboratories worldwide, including SAPS FSL. This was a strength of the study, as the same set of samples were available for both analyses, and this allowed for a direct comparison between the two extraction methods.

The methods were compared based on DNA quantity and quality as determined by qPCR, as well as, successful DNA profiling. Given that the elution volume was 50 µl for both the phenol-chloroform method and the QIAamp® kit, the concentration of DNA recovered from the two methods was directly comparable. The phenol-chloroform method produced significantly higher yields of DNA ($p = 0.0454$) (Figure 3.1a), more full DNA profiles (Figure 3.3), and DNA that was significantly less degraded ($p < 0.0001$) (Figure 3.1b) than the QIAamp® kit. The QIAamp® kit had a higher number of samples that were severely degraded, and a higher number of samples where the large autosomal target (214 bp) failed to amplify and a degradation index could not be calculated (Figure 3.2). This suggests an extremely high level of degradation. However, the QIAamp® kit generated less failed profiles than the phenol-chloroform method (Figure 3.3).

These results are in concordance with a study by Kuś, Ossowski & Zielińska (2016), which observed that both fresh (<5 years old) and old (~70 years old and completely skeletonised) bones and teeth extracted with the phenol-chloroform method had higher DNA concentrations than those extracted with the QIAamp® kit. They attributed the higher DNA concentrations to the five to eight times more starting material being used for the phenol-chloroform method (0.5 g – 0.8 g). The optimisation step of this study showed that more starting material improved results from the phenol-chloroform method; therefore, depending on sample availability, 0.5 g of starting material was used for the phenol-chloroform method, while only 0.1 g was used for the QIAamp® kit extraction as per the manufacturer's instructions. This would suggest that the phenol-chloroform method could be optimised even more by further increasing the amount of starting material. However, when the impact of input weight on DNA concentration was statistically assessed using the 51 control teeth, it was found that there was no significant impact on mean DNA concentration. Therefore, it should be considered that the two optimisation samples yielded more DNA when more sample was used purely by chance. Importantly, for some silica-based methods such as the QIAamp® DNA Investigator Kit, too much powder blocks the silica membrane (QIAGEN, 2006); therefore, increasing the amount of starting material would not necessarily be beneficial for this kit.

In contrast, a study by Iyavoo, Hadi & Goodwin (2013) showed that a standard phenol-chloroform extraction method produced a mean DNA concentration of 80 ng/µl when used on fresh pig bones. This was more than four other commercial silica-based methods and their study used a small amount of starting material across all methods (0.05 g). These results indicate that the phenol-chloroform method could produce good DNA concentrations from a much smaller amount of starting material of bone than previously suggested. This study did not assess DNA degradation or profiling success and therefore further comparisons could not be made.

Davoren *et al.* (2007) compared the International Commission on Missing Persons (ICMP) developed silica extraction method with a standard phenol-chloroform extraction method on the same set of femur samples exhumed from mass graves and divided the bone powder from each sample equally between the two methods (5.6 g – 9.8 g per sample for each method). The ICMP silica-based method had a higher DNA yield and better DNA profiling success. This method is based on Qiagen's QIAamp® Blood Maxi Kit protocol with the modification of a second EDTA demineralisation step. This additional demineralisation step may have contributed to the improved results of the ICMP method. Most DNA extraction protocols for

bones and teeth are based on an overnight demineralisation step involving powdered samples being placed in an EDTA-containing extraction buffer (Hagelberg & Clegg, 1991). The EDTA demineralises the bone/tooth (the amount of demineralisation is dependent on the EDTA concentration and the volume of extraction buffer) and inactivates deoxyribonucleases by sequestering magnesium and calcium ions (Loreille *et al*., 2007). Therefore, EDTA was also included in this study's extraction buffer; however, an additional demineralisation step would have taken up a substantial amount of time, and that is why only one was done in this study. In the future to try to further improve the method, two demineralisation steps could be assessed.

Previous studies comparing DNA recovery from the organic phenol-chloroform method and the QIAamp® DNA Investigator Kit have not primarily focused on human teeth as a sample type, and there is a gap in the literature regarding which method is better for DNA extraction from human teeth. The results of this study, albeit based on a relatively small sample size, demonstrated that the DNA yield was significantly better, and the DNA was significantly more intact, when the phenol-chloroform method was used on the control teeth. However, the impact of the phenol-chloroform method on DNA profiling remains unclear. It was thought that better DNA concentrations and quality would result in more full DNA profiles, but this was not observed.

The lack of significant difference between the DNA profiling results of the two methods could also be due to the small sample size, as small sample sizes are prone to false negative results. Therefore, further research with a larger sample size is necessary to confirm these observations. Additionally, this could be due to a sub-optimal DNA profiling procedure. Modifications to the DNA profiling step could also be implemented in the future to try to improve DNA profiling success.

Lastly, pairwise comparisons showed significant differences in mean DNA recovery between all three control individuals used in this study (Figure 3.4). This is in concordance with other studies that have also found there to be large differences in DNA yields from teeth between individuals (Gaytmenn & Sweet, 2003; Dobberstein *et al.*, 2008). As there were significant differences between control individuals, a limitation of the study is that there were only three individuals. However, this was also a strength of the study, as using lots of teeth from the same individuals allowed for the preliminary analysis of the impact of numerous variables on DNA recovery. In future research, the number of individuals should be increased to account for this inter-individual variation.

4.2 Does the phenol-chloroform method recover enough intact DNA that is suitable for DNA profiling?

The optimised phenol-chloroform method yielded a wide range of mean DNA concentrations between 0.000201 ng/µl and 0.217676 ng/µl (Figure 3.1a). While only 25 % of control samples met the combined quantity and quality criteria, we were able to get full DNA profiles for 41 % of samples. This suggests that these criteria are perhaps too conservative.

There are conflicting results as to what improves DNA recovery with regards to the phenol-chloroform methodology itself. In this study, the demineralisation step was kept as per the protocol followed (Pagan *et al*., 2012) to establish baseline measurements, which were the first to be generated for our laboratory. Table 4.1 is a summary of studies using the phenol-chloroform method to extract DNA from bones and teeth, and their differing methodology and results.

Table 4.1: A summary of the methodology and DNA recovery of studies that used the phenol-chloroform method to extract DNA from hard tissues.

Author	Year	n	Sample type	Sample condition	Extraction buffer for demineralisation step				Results	
					Volume (ml)	EDTA (mM)	Proteinase K (mg)	SDS (% w/v)	Mean DNA concentration (ng/µl)	Full DNA profiles [a]
This study	2020	51	Teeth	Control	5.25	0.1	5	2	0.000201 - 0.217676	41 %
Davoren *et al.*	2007	20	Bones	Severely degraded	20	50	20	0.5	0.00016 - 0.256	70 %
Marjanović *et al.*	2007	12	Bones (6) and teeth (6)	Severely degraded	2	500	1	0.5	0.008 - 0.6	100 % [b]
Pagan *et al.*	2012	16	Bones (11) and teeth (5)	Severely degraded	4 – 6	0.1	5	2	Bones: 0.032 - 0.034 Teeth: 0.017 - 0.021	0 %
Ferreira *et al.*	2013	20	Bone	Exhumed and highly degraded	10	10	6.8	2	0.2 - 1.0 (40 % of samples) < 0.2 (60 % of samples)	58 % (12.5-15 markers)
Iyavoo, Hadi & Goodwin	2013	NS	Pig bones	Fresh	NP	NP	NP	NP	80	-
Marjanović *et al.*	2015	10	Bones (1) and teeth (9)	Severely degraded	20	50	20	2	0.004 - 0.7	100 % [b]
Kuś, Ossowski & Zielińska,	2016	21	Bones and teeth	Fresh (< 5 years old) and old (~ 70 years old)	2.5	NP	0.4	-	Fresh: 0.021 Old: 3.4	100 %
Rubio *et al.*	2018	10	Teeth	Control	1.5	500	2 [c]	10	224.007 - 271.607	-

[a] Full DNA profile as defined by 12-15 successful markers
[b] After two extra PCR cycles and extended elongation step for samples with low DNA concentrations
[c] Additional two-hour proteinase K incubation
NP: not provided; NS: not specified.

This study's protocol was based on the methodology described by Pagan *et al.* (2012) and the extraction buffer components were kept the same. In this study, the overall DNA yields were higher and more full DNA profiles were produced, showing a possible improvement of the method. However, Pagan *et al.* (2012) used severely degraded samples which may explain their lower DNA concentrations. Furthermore, the lack of full DNA profiles could be due to Pagan *et al.* (2012) using higher thresholds to call their alleles. Given the lack of consensus on the demineralisation step of phenol-chloroform protocols, the protocol described by Pagan *et al.* was chosen as the baseline for this study, due to its reproducibility and internal validation within a forensic laboratory.

Davoren *et al.* (2007) had a similar DNA yield to this study and a higher percentage of DNA profiling success. They used a higher concentration of EDTA and amount of proteinase K in their demineralisation step, but a lower concentration of SDS (Table 4.1). Given that their DNA recovery was similar in concentration, their increased profiling success may have been due to more intact DNA being recovered.

In two separate studies which both achieved 100 % DNA profiling success, Marjanović *et al.* (2007) and Marjanović *et al.* (2015), showed that modifications to the demineralisation step of the phenol-chloroform method could increase DNA recovery from skeletal remains (bones and teeth). Both these studies used much higher EDTA concentrations in their demineralisation step than that used in this study; however, they used conflicting amounts of proteinase K and SDS, and different extraction buffer volumes (Table 4.1). Furthermore, Marjanović *et al.* (2007) and Marjanović *et al.* (2015), showed that adjusting the PCR conditions for samples with low DNA concentrations improved DNA profiling results.

The DNA concentrations recovered, and the percentage of full DNA profiles generated using the phenol-chloroform protocol in the study by Ferreira *et al*. (2013), were slightly higher than in this study (Table 4.1). Ferreira *et al*. (2013) used a higher concentration of EDTA, and this suggests that a higher concentration of EDTA is beneficial for improved DNA recovery.

The improved DNA yields seen in Rubio *et al.* (2018) might be due to differences in their demineralisation step: higher concentrations of EDTA and SDS, and a lower volume of extraction buffer (Table 4.1). This suggests that a smaller volume of extraction buffer does not negatively affect DNA recovery, and higher concentrations of EDTA and SDS may improve DNA recovery. However, the impact of this improved DNA recovery on DNA profiling is not known.

It should be noted that most of the studies (Table 4.1) used differing amounts of starting material per sample, which would have an additional impact on the quantity of DNA recovered per mg of input sample. Based on the above literature (Table 4.1), there is scope to improve the phenol-chloroform method and adjusting the demineralisation step could be one way of achieving this. What seems to be consistent is that this study used a much lower EDTA concentration than any of the other protocols; therefore, the next logical step would be to increase the EDTA concentration used in the demineralisation step. The amount by which to increase it would need to be determined by further optimisation. Additionally, this study and Pagan *et al.* (2012) used ethanol precipitation to recover DNA from the aqueous phase during phenol-chloroform extraction, while the other studies in Table 4.1 used centrifugal filter units. This may be another variable in the phenol-chloroform methodology that could be adjusted to further improve results. Furthermore, although the small sample size was a limitation of this study, Table 4.1 clearly illustrates that this study's sample size is still much larger than any other existing studies.

Marjanović *et al*. (2007) and Marjanović *et al.* (2015) also made adjustments to the PCR protocols of some samples with low DNA concentrations (Table 4.1), which resulted in full DNA profiles being produced. This suggests that adjustments could also be made post-extraction to improve DNA profiling success in both methods by adjusting PCR conditions, *i.e.* including extra PCR cycles or an extended elongation step. This went beyond the scope of this minor dissertation but could implemented in the future. Additionally, next generation sequencing (NGS), single nucleotide polymorphism (SNP) based assays, mitochondrial DNA (mtDNA) analysis and microhaplotypes, might be alternative approaches if DNA profiling cannot be improved.

4.3 How does the phenol-chloroform method perform in a local forensic case?

The phenol-chloroform method was tested and applied on teeth from a forensic marine decomposition case to evaluate its performance in an authentic forensic context. As anticipated the DNA yield was significantly lower than the control teeth ($p = 0.0054$) (Figure 3.4) with a range of between 0.000237 ng/µl and 0.001126 ng/µl. In view of the low DNA concentrations, the maximum amount of template DNA was used for DNA profiling and two extra PCR cycles

were added. Despite these adjustments, no full DNA profiles were generated, but 50 % of teeth produced partial profiles.

Both the control and forensic teeth were from deceased individuals and some post-mortem degradation of DNA was expected. The forensic teeth were from a marine wash-up case and exposed to additional environmental stressors, and this is likely why they have a lower yield. This demonstrates how different forensic scenarios are from control environments, emphasising the challenges of this discipline and the need for further optimisation and research.

This forensic case is from a seawater environment, and this highlights how immersion in seawater may affect DNA recovery from teeth and make it difficult to generate a full DNA profile for identification. On the other hand, given that there were significant differences between the results of teeth from the three control individuals used in the study, the differences observed between the forensic teeth and the control teeth may just be due to the teeth being from another individual, and not due to being immersed in sea water.

In South Africa, especially along the coast lines, the increasing burden of unidentified human remains retrieved from marine environments is problematic. While empirical data pertaining to the numbers of human remains from the ocean is lacking in South Africa, SRM recorded that 279 of the 3695 bodies received in 2015 were the result of water-related deaths (in-house data). When recovered they are frequently incomplete and badly decomposed, which poses challenges for medico-legal death investigations and for identification (Gruspier & Pollanen, 2000).

Some studies have examined the effects of immersion in water on DNA recovery from teeth. These studies have mostly been in simulated or semi-realistic environments and have had contradicting results (Alvarez García *et al*., 1996; Corte-Real *et al*., 2006; Hughes-Stamm, 2012; Drake, 2014). Alvarez García *et al*. (1996) showed that a 200 bp DNA fragment was amplifiable from the DNA recovered from human teeth after two weeks of immersion in fresh and sea water, and a 600 bp fragment was amplifiable after six months. In contrast, Drake (2014) found that there was not enough DNA present in human teeth to act as a template for PCR amplification after two weeks of immersion in a semi-protected marine setting. *In situ* pig teeth with an intact periodontal ligament showed significant DNA degradation after one week in sea water; however, within these teeth enough DNA remained after eight weeks of immersion to act as a template for amplification. These findings by Drake (2014) suggest that

DNA identification from teeth may be successful if the body has been in the water for eight weeks or less; however, the same results are unlikely from a loose tooth.

A study by Finaughty *et al.* (2019) found that forensically usable DNA could not be recovered from pig teeth immersed in a realistic South African marine environment. DNA yields from all experimental teeth were less than 1 picogram and intact DNA was smaller than 96 bp. However, the shortest period of immersion of teeth in this study was 17 days and it might be possible to recover DNA after a shorter immersion time. Studies looking at shorter immersion times would help to ascertain when forensically usable DNA is no longer attainable from teeth recovered from a marine environment.

Additionally, marine environments are likely to contain other factors such as unique organisms and compounds that may impact DNA preservation. Many microorganisms contain nucleases, which are capable of degrading DNA (Alaeddini, Walsh & Abbas, 2010); therefore, microbial contamination is regarded as one of the major factors affecting DNA degradation post-mortem (Burger *et al*., 1999). Finaughty *et al*. (2019) observed non-specific amplification in three DNA samples, which may have been from microorganisms present in the marine environment. It was found that a marine microbial species, *Oceanisphaera,* had the highest similarity to the unknown sequence; therefore, Finaughty *et al.* (2019) hypothesised that deoxyribonuclease activity of marine microbes may have played a part in the poor preservation of DNA. Finaughty *et al.* (2019) was also the first to suggest that the temperature of sea water might be associated with DNA preservation in an open marine environment where it was suggested that colder and more constant seawater temperatures acted to preserve DNA. However, further research is required to fully understand the influence of immersion in sea water on DNA recovery from human teeth.

The results from the forensic teeth used in this study emphasise how complicated forensic casework is, and that optimisation on control teeth does not always translate to an authentic forensic context. Based on these results, there is potential for the phenol-chloroform method to perform well in a local forensic setting; however, additional adjustments should be investigated to improve its performance when used in forensic casework.

4.4 Do tooth morphotype, dental disease and dental treatment affect DNA recovery using the phenol-chloroform method?

A strength of this study was that there were many teeth available from each of the three control individuals representing all tooth types, and this allowed for a preliminary investigation of the influence of variables such as tooth type, jawbone, intact roots, dental disease and dental treatment, on DNA quantity, quality and profiling success. There were significant differences in DNA recovery between individuals; therefore, in the future, to determine the best tooth morphotype with statistical significance, the sample size needs to be increased (with many teeth per individual).

4.4.1 Does tooth morphotype affect DNA recovery?

Molars were hypothesised as the preferred tooth type in the protocols published by Interpol in their Disaster Victim Identification (DVI) Guide 2009 (INTERPOL, 2009) and by the DNA Commission of the International Society of Forensic Genetics recommendations for DVI (Prinz *et al.*, 2007). Molars have been hypothesised to have a larger pulp chamber volume, and therefore, in theory would have more DNA available (Smith *et al.*, 1993; Higgins & Austin, 2013). Despite this trend in literature to tend to recommend molars, a thorough search of the literature revealed no empirical data to support this recommendation.

This study was able to investigate this hypothesis, and although DNA recovery varied among tooth types, the differences were not statistically significant. Additionally, this study found that premolars were the tooth type with the highest mean DNA concentration (Figure 3.5a), the best profiling results (54 % of premolars produced full profiles) and the lowest degradation index. Therefore, this study does not add to the hypothesis that molars are the best tooth type for DNA analysis. Similarly, the results of a study by De Leo, Turrina & Marigo (2000) that examined 51 teeth from an unspecified number of different individuals, showed a higher DNA yield from premolars compared with canines and molars.

The difference between the degradation indexes of teeth from mandibles and maxillae was significant, with maxillae having less DNA degradation (Figure 3.7). This would indicate that teeth from maxillae would be preferable for DNA analysis. However, a two-way ANOVA showed that the combined effect of tooth type and jawbone on variation in DNA quantity, quality and profiling success was only 10.10 %, 17.48 % and 10.28 %, respectively; suggesting that other factors that are not accounted for by this model, have a greater influence on results

(*e.g.* individual or environmental factors). Given the results of this study, the presence of intact roots did not significantly affect any of the results and is unlikely to be an additional reason. However, this study's sample size would not have been statistically significant if the model had taken all variables into account. In the future, the sample size should be increased to allow for a more comprehensive model, which can take the combination of all the variables into account.

4.4.2 Does dental disease affect DNA recovery?

Previous studies have found that dental diseases, *e.g.* caries, reduce the human DNA quantity in teeth (Higgins *et al*., 2011) and can ultimately lead to a complete loss of pulp or the tooth itself (Lee *et al*., 2006). However, teeth with pathology may still yield enough amplifiable DNA given careful consideration of the disease progression and the tissues involved (Higgins & Austin, 2013). In this study, teeth with disease had significantly higher DNA yields (Figure 3.6a) and significantly better DNA profiles than non-diseased teeth (Figure 3.6b). This reinforces the idea that the presence of disease should not immediately discount a tooth's suitability for DNA recovery.

A possible reason for the increase in DNA quantity in diseased teeth could be increased white blood cells (leukocytes) at the site of infection (Abramson & Melton, 2000). White blood cells are mainly involved in the body's immune response and their morphology differs from red blood cells in that they are nucleated and do not contain haemoglobin (Gordon-Smith, 2013). Given that they are nucleated they contain DNA, and this may contribute to the amount of DNA at a diseased site, resulting in the significantly higher DNA recovery and significantly better DNA profiling results than non-diseased teeth.

4.4.3 Does dental treatment affect DNA recovery?

Studies investigating DNA recovery from teeth have not examined how dental treatments, *e.g.* fillings, might impact DNA recovery. In this study, it was found that teeth with fillings had substantially lower DNA yields, although this was not statistically significant ($p = 0.1884$). DNA profiling results from teeth with fillings were also very poor with 60 % producing failed profiles (0-3 markers) and 40 % producing partial profiles (3.5-11.5 markers). Despite the lack of significance, the poor DNA recovery and DNA profiling results from teeth with fillings would suggest that teeth without fillings are recommended. This poor DNA recovery could be

caused by a ‘dilution effect’ from the material used for the fillings, or the material used for fillings could be acting as a PCR inhibitor.

4.5 Recommendations

These data combined suggest that premolars might be the best tooth type. Importantly, molars were not significantly better than other tooth types, which contrasts with recommendations for DVI made by both Interpol and the DNA Commission of the International Society of Forensic Genetics. Preliminary analysis of the data gathered in this study showed that teeth from the maxillae regardless of intact or broken roots and with disease can be sampled; however, those with fillings should be avoided. With further research, a guideline regarding the preferred tooth morphotype could be developed and be used to make informed decisions when sampling teeth from unidentified remains.

4.6 Conclusion

An optimised organic phenol-chloroform method was successfully implemented in the laboratory, it was then applied to control and forensic teeth and evaluated in terms of DNA quantity, quality, and DNA profiling success. The generation of this initial data allowed for the preliminary analysis of the impact of certain factors on DNA recovery and for a model to be generated that assessed the combined influence of tooth type and jawbone on DNA quantity, quality and profiling. However, the impact of all the factors combined could not be modelled statistically due to its multi-factorial nature, and thus, a larger sample size is needed in future research (which was not deemed necessary for the primary aim of this study).

The phenol-chloroform method showed significantly higher DNA yields and significantly less degradation of DNA, but no significant difference in DNA profile success, when compared to the QIAamp® kit. DNA quantities recovered using the phenol-chloroform method were overall quite low, with 41 % of control samples producing a full DNA profile. Furthermore, DNA quality was quite degraded, with 42 % of control samples meeting the criteria of a DI < 4 to be suitable for DNA profiling. There is scope for the phenol-chloroform methodology itself to be improved by either increasing the concentration of EDTA used in the demineralisation step or by further increasing the amount of starting material. To improve DNA profiling success, PCR protocols could be optimised by additional PCR cycles or extended elongation steps.

Alternatively, NGS, SNP-based assays, mtDNA analysis, or even microhaplotypes, could be additional downstream approaches to DNA profiling.

DNA metrics were particularly poor in the forensic casework example, suggesting that sea water may have had an additional influence on DNA yield and degradation. This influence is still not fully understood and further research is required given the high numbers of human remains that wash up along the South African coastline each year. The results from the forensic teeth demonstrate how different actual forensic scenarios are from control environments, emphasising the need for further optimisation and research of the phenol-chloroform method to improve its performance in forensic casework.

This study has provided a preliminary suggestion of the best tooth type, jawbone and tooth condition for DNA analysis for forensic human identification. Further studies with a larger sample size (more individuals and with many teeth per individual) would allow a more comprehensive model to be generated, which could incorporate more variables with statistical power to determine the most important factors that influence DNA recovery from teeth, and allow for a recommendation of the best combination of all variables. This would also allow for the necessary statistical analyses to definitively determine which is the more effective DNA extraction method. Overall, the results of this study provide motivation for further research into the use of the phenol-chloroform method for extraction of DNA from human teeth for identification purposes.

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Appendix A: Ethical approval

UNIVERSITY OF CAPE TOWN
Faculty of Health Sciences
Human Research Ethics Committee

Room E53-46 Old Main Building
Groote Schuur Hospital
Observatory 7925
Telephone [021] 406 6626
Email: shuretta.thomas@uct.ac.za
Website: www.health.uct.ac.za/fhs/research/humanethics/forms

24 July 2019

HREC REF: 222/2019

Ms Laura Heathfield
Forensic Medicine & Toxicology
Falmouth Building

Dear Ms Heathfield

PROJECT TITLE: OPTIMISATION AND INVESTIGATION OF DNA RECOVERY FROM DIFFERENT BIOLOGICAL SAMPLE TYPES FOR USE IN FORENSIC HUMAN IDENTIFICATION. (MPHIL CANDIDATE: MS T HAIKNEY)

Thank you for submitting your response to the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the HREC has **formally approved** the above-mentioned study.

Approval is granted for one year until 30 July 2020.

Please submit a progress form, using the standardised Annual Report Form if the study continues beyond the approval period. Please submit a Standard Closure form if the study is completed within the approval period.
(Forms can be found on our website: www.health.uct.ac.za/fhs/research/humanethics/forms)

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please note that for all studies approved by the HREC, the principal investigator **must** obtain appropriate institutional approval, where necessary, before the research may occur.

The HREC acknowledge that the student, Tarryn Haikey will also be involved in this study.

Please quote the HREC REF in all your correspondence.

Yours sincerely

PROFESSOR M BLOCKMAN
CHAIRPERSON, FHS HUMAN RESEARCH ETHICS COMMITTEE
Federal Wide Assurance Number: FWA00001637.

HREC 222/2019

Appendix B: Materials and methods

B.1 Samples

Table B.1: Description of control teeth.

Individual	Sample ID	Tooth type	Jawbone	Roots	Tooth condition
1	1	central incisor	mandible	1	Covered in a red dye
1	2	lateral incisor	mandible	1	Covered in a red dye
1	3	canine	mandible	1	Covered in a red dye
1	4	first premolar	mandible	1	Covered in a red dye
1	5	central incisor	mandible	1	Covered in a red dye
1	6	lateral incisor	mandible	1	Covered in a red dye
1	7	canine	mandible	1	Covered in a red dye
2	8	central incisor	maxilla	1	Normal
2	9	lateral incisor	maxilla	1	Normal
2	10	canine	maxilla	1	Normal with brown marks
2	11	first premolar	maxilla	1	Fillings and brown marks
2	12	second premolar	maxilla	1	Top of crown removed (internal tooth exposed)
2	13	first molar	maxilla	3	Fillings
2	14	second molar	maxilla	2	Fillings and brown marks
2	15	third molar	maxilla	3	Three broken roots and no crown
2	16	central incisor	maxilla	1	Normal
2	17	lateral incisor	maxilla	1	Normal with brown marks
2	18	canine	maxilla	1	Normal with black marks on distal side
2	19	first premolar	maxilla	1	Normal with brown marks
2	20	second premolar	maxilla	1	Normal with brown marks
2	21	first molar	maxilla	3	Fillings and one broken root
2	22	second molar	maxilla	3	Fillings
2	23	lateral incisor	mandible	1	Normal with brown marks
2	24	canine	mandible	1	Normal with brown marks
2	25	first premolar	mandible	1	Dental case (crown present)
2	26	first molar	mandible	2	Dental case (top of crown removed, and internal tooth exposed), fillings and diseased
2	27	second molar	mandible	2	Partial crown, brown marks, red colour, and diseased
2	28	third molar	mandible	2	Partial crown, brown marks and fillings

Individual	Sample ID	Tooth type	Jawbone	Roots	Tooth condition
2	29	central incisor	mandible	1	Normal with brown marks
2	30	lateral incisor	mandible	1	Normal with brown marks
2	31	canine	mandible	1	Normal with brown marks
2	32	first premolar	mandible	1	Normal with brown marks
2	33	second premolar	mandible	1	Broken roots and brown marks
3	34	central incisor	maxilla	1	Diseased, dental caries, red colour and eaten roots
3	35	lateral incisor	maxilla	1	Diseased, dental caries, red colour, eaten roots and small in size
3	36	canine	maxilla	1	Diseased, dental caries, red colour and eaten roots
3	37	first premolar	maxilla	1	Diseased and internal break from crown to pulp
3	38	second premolar	maxilla	2	Diseased and internal break from crown to pulp
3	39	second molar	maxilla	2	Diseased, dental caries, eaten roots and red in colour
3	40	central incisor	maxilla	1	Diseased, dental caries, eaten roots and red in colour
3	41	lateral incisor	maxilla	1	Diseased, dental caries, eaten roots and red in colour
3	42	canine	maxilla	1	Diseased, dental caries, eaten roots and red in colour
3	43	first premolar	maxilla	2	Diseased, dental caries, eaten roots, red in colour and cracked crown (internal break from crown to pulp)
3	44	first molar	maxilla	2	Diseased, dental caries, eaten roots, red in colour and cracked crown (internal break from crown to pulp)
3	45	central incisor	mandible	1	Normal with brown marks
3	46	lateral incisor	mandible	1	Normal with brown marks
3	47	canine	mandible	1	Diseased, dental caries, eaten roots and red in colour
3	48	first premolar	mandible	1	Diseased, dental caries, eaten roots, red in colour and internal break from crown to pulp
3	49	second premolar	mandible	1	Diseased, dental caries, eaten roots, red in colour and internal break from crown to pulp
3	50	lateral incisor	mandible	1	Normal with brown marks
3	51	canine	mandible	1	Diseased, dental caries, eaten roots and cracked crown
3	52	first premolar	mandible	1	Diseased, dental caries, eaten roots and cracked crown
Normal: no signs of dental disease or treatments and roots intact					

B.2 Optimisation teeth

Table B.2: Optimisation qPCR results.

Sample ID	Aliquots taken	pH of phenol	Input weight (g)	Large target (ng/µl)	Y target (ng/µl)	Small target (ng/µl)	IPC (C_T)	DI
Sample 1	✓	6.7	0.1	0.0237	0.0309	0.0432	27.5	1.8
Sample 2	×	6.7	0.1	0.0831	0.0796	0.1009	27.8	1.2
Sample 1	×	8.0	0.1	0.0477	0.0300	0.0429	27.9	0.9
Sample 1	×	8.0	0.5	0.3108	0.1956	0.2804	28.2	0.9
Sample 2	×	8.0	0.1	0.1032	0.0728	0.0945	28.1	0.9
Sample 2	×	8.0	0.5	0.4246	0.3589	0.5468	28.1	1.3
IPC: internal positive control; DI: degradation index; C_T: threshold cycle.								

B.3 Forensic teeth

Table B.3: Description of forensic teeth from marine wash-up case.

Sample ID	Tooth type	Jawbone	Tooth condition
1.1	canine	mandible	Normal
1.2	central incisor	maxilla	Normal
1.3	first premolar	mandible	Normal
1.4	first molar	maxilla	Normal
1.5	second molar	maxilla	Normal
1.6	third molar	mandible	Superficial cavity (no discolouration)
Normal: no signs of dental disease or treatments and roots intact			

Appendix C: Results

C.1 Control and forensic teeth

C.1.1 Quantity and quality

Table C.1: The quantity and quality data generated for all control and forensic teeth (as determined by qPCR and DNA profiling).

Individual	Sample ID	Input weight (g)	Large Autosomal concentration (ng/µl)	Small Autosomal concentration (ng/µl)	Mean DNA concentration (ng/µl)	DNA yield (ng/mg)	Degradation index	STR markers (out of 15)
Control teeth								
1	1	0.2353	0.007793	0.030861	0.019327	0.004107	3.960161	3
1	2	0.2628	0.006642	0.032195	0.019419	0.003695	4.847271	10.5
1	3	0.5	0.016475	0.062227	0.039351	0.003935	3.777168	13
1	4	0.5	0.014303	0.111331	0.062817	0.006282	7.783885	14
1	5	0.2130	0.005169	0.029696	0.017433	0.004092	5.745219	5.5
1	6	0.2657	0.004118	0.017901	0.011010	0.002072	4.347128	14
1	7	0.5	0.015574	0.143584	0.079579	0.007958	9.219703	10.5
2	8	0.5	0.000371	0.001677	0.001024	0.000102	4.520317	1.5
2	9	0.4535	0	0.000687	0.000687	0.000076	-	2.5
2	10	0.5	0.000102	0.000371	0.000237	0.000024	3.626650	0.5
2	11*	0.5	-	-	-	-	-	-
2	12	0.4007	0.000172	0.000596	0.000384	0.000048	3.467622	1
2	13	0.5	0.002642	0.056024	0.029333	0.002933	21.208480	0.5
2	14	0.5	0.000363	0.002986	0.001675	0.000168	8.228531	1
2	15	0.5	0.000655	0.002841	0.001748	0.000175	4.338577	12.5
2	16	0.4413	0.002125	0.011141	0.006633	0.000752	5.242096	10.5
2	17	0.3854	0.000098	0.003796	0.001947	0.000253	38.745540	6.5
2	18	0.5	0.000137	0.001108	0.000623	0.000062	8.115368	2
2	19	0.5	0.000134	0.000404	0.000269	0.000027	3.009483	0.5
2	20	0.5	0.000104	0.000297	0.000201	0.000020	2.849828	1
2	21	0.5	0	0.001847	0.001847	0.000185	-	3
2	22	0.5	0.000252	0.000365	0.000309	0.000031	1.448380	7.5
2	23	0.1261	0.000691	0.003400	0.002046	0.000811	4.918957	2
2	24	0.5	0.000154	0.002170	0.001162	0.000116	14.083860	4.5
2	25	0.2842	0.000099	0.000336	0.000218	0.000038	3.401607	2.5
2	26	0.3245	0.001478	0.007429	0.004454	0.000686	5.027569	7
2	27	0.5	0.000334	0.001630	0.000982	0.000098	4.875907	6
2	28	0.5	0.000127	0.001368	0.000748	0.000075	10.726180	3.5
2	29	0.3392	0.000863	0.005810	0.003337	0.000492	6.733613	9
2	30	0.1753	0.000535	0.002486	0.001511	0.000431	2.775651	2.5
2	31	0.5	0.000316	0.001516	0.000916	0.000092	4.798779	3

Individual	Sample ID	Input weight (g)	Large Autosomal concentration (ng/µl)	Small Autosomal concentration (ng/µl)	Mean DNA concentration (ng/µl)	DNA yield (ng/mg)	Degradation Index	STR markers (out of 15)
Control teeth (continued)								
2	32	0.3587	0.000472	0.002214	0.001343	0.000187	4.688508	3
2	33	0.2812	0	0.000501	0.000501	0.000089	-	2
3	34	0.3164	0.019877	0.061155	0.040516	0.006403	3.076643	13.5
3	35	0.0215	0	0.000656	0.000656	0.001526	-	1.5
3	36	0.5	0.131738	0.225117	0.178428	0.017843	1.708829	14.5
3	37	0.5	0.132303	0.303048	0.217676	0.021768	2.290564	15
3	38	0.5	0.163760	0.253126	0.208443	0.020844	1.545715	15
3	39	0.5	0.094778	0.151590	0.123184	0.012318	1.599425	15
3	40	0.4367	0.051796	0.142241	0.097019	0.011108	2.746164	15
3	41	0.2334	0.026715	0.084588	0.055652	0.011922	3.166350	15
3	42	0.5	0.094332	0.270093	0.182213	0.018221	2.863225	10.5
3	43	0.5	0.135330	0.092192	0.113761	0.011376	0.680777	15
3	44	0.5	0.148878	0.246678	0.197778	0.019778	1.656911	14.5
3	45	0.2520	0.010734	0.063763	0.037249	0.007391	5.940112	15
3	46	0.2095	0.035858	0.056463	0.046161	0.011017	1.574062	15
3	47	0.5	0.044624	0.126088	0.085356	0.008536	2.825573	12
3	48	0.5	0.031506	0.146867	0.089187	0.008919	4.661556	15
3	49	0.5	0.019998	0.030933	0.025466	0.002547	1.546824	15
3	50	0.1739	0.005874	0.028725	0.017210	0.004948	4.890436	15
3	51	0.5	0.035694	0.156354	0.096024	0.009602	4.380376	15
3	52	0.3897	0.024850	0.100834	0.062842	0.008063	4.057698	15
Forensic teeth								
4	1.1	0.5	0	0.000412	0.000412	0.000041	-	1
4	1.2	0.5	0.000424	0.000421	0.000423	0.000042	0.993026	2
4	1.3	0.5	0.000562	0.001690	0.001126	0.000113	3.008836	1.5
4	1.4	0.5	0.000546	0.000719	0.000633	0.000063	1.316050	8.5
4	1.5	0.5	0.000174	0.000300	0.000237	0.000024	1.726773	4.5
4	1.6	0.5	0.000956	0.001098	0.001027	0.000103	1.148347	7

* Data from this sample was excluded from analyses due to a technical error in the initial DNA assessment.

C.1.2 qPCR standard curve

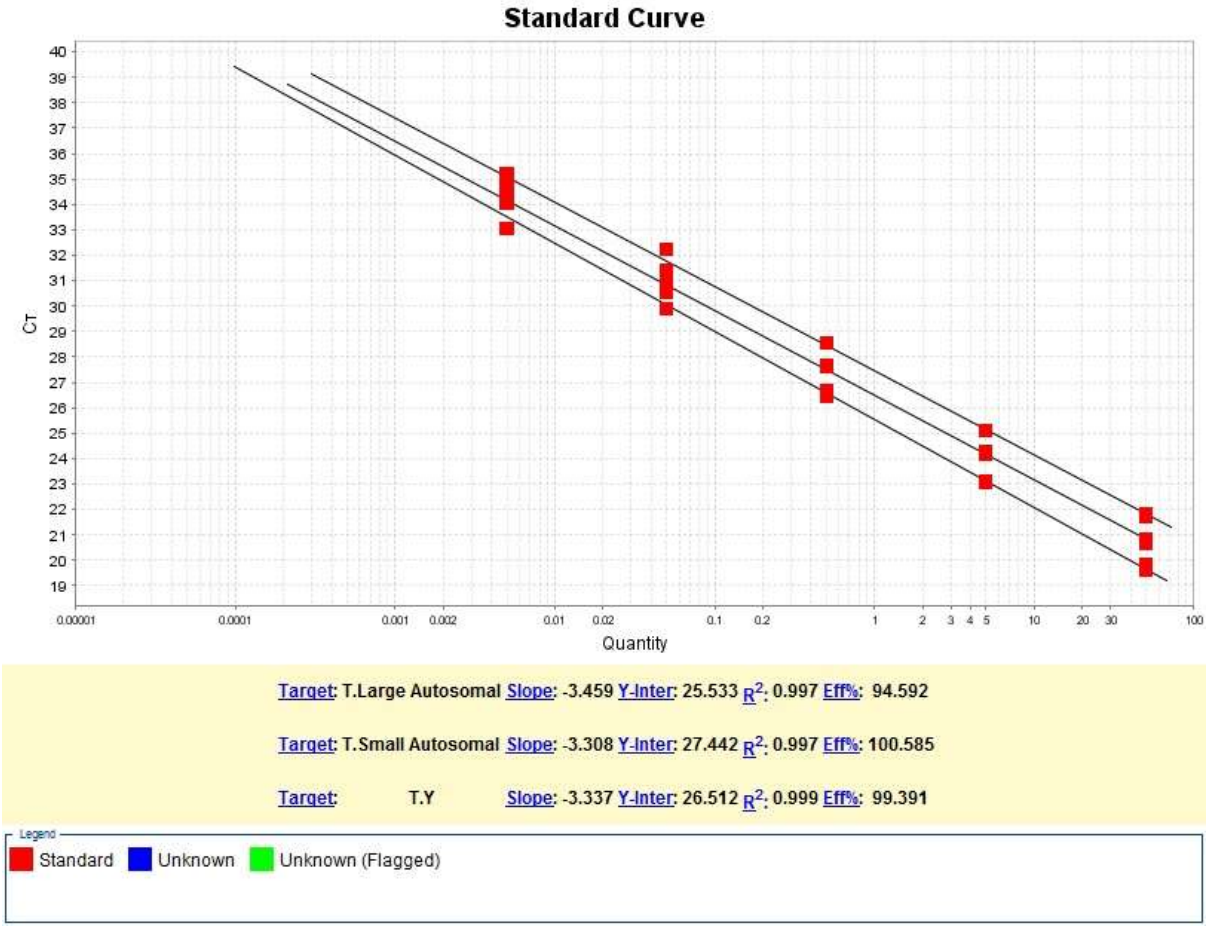
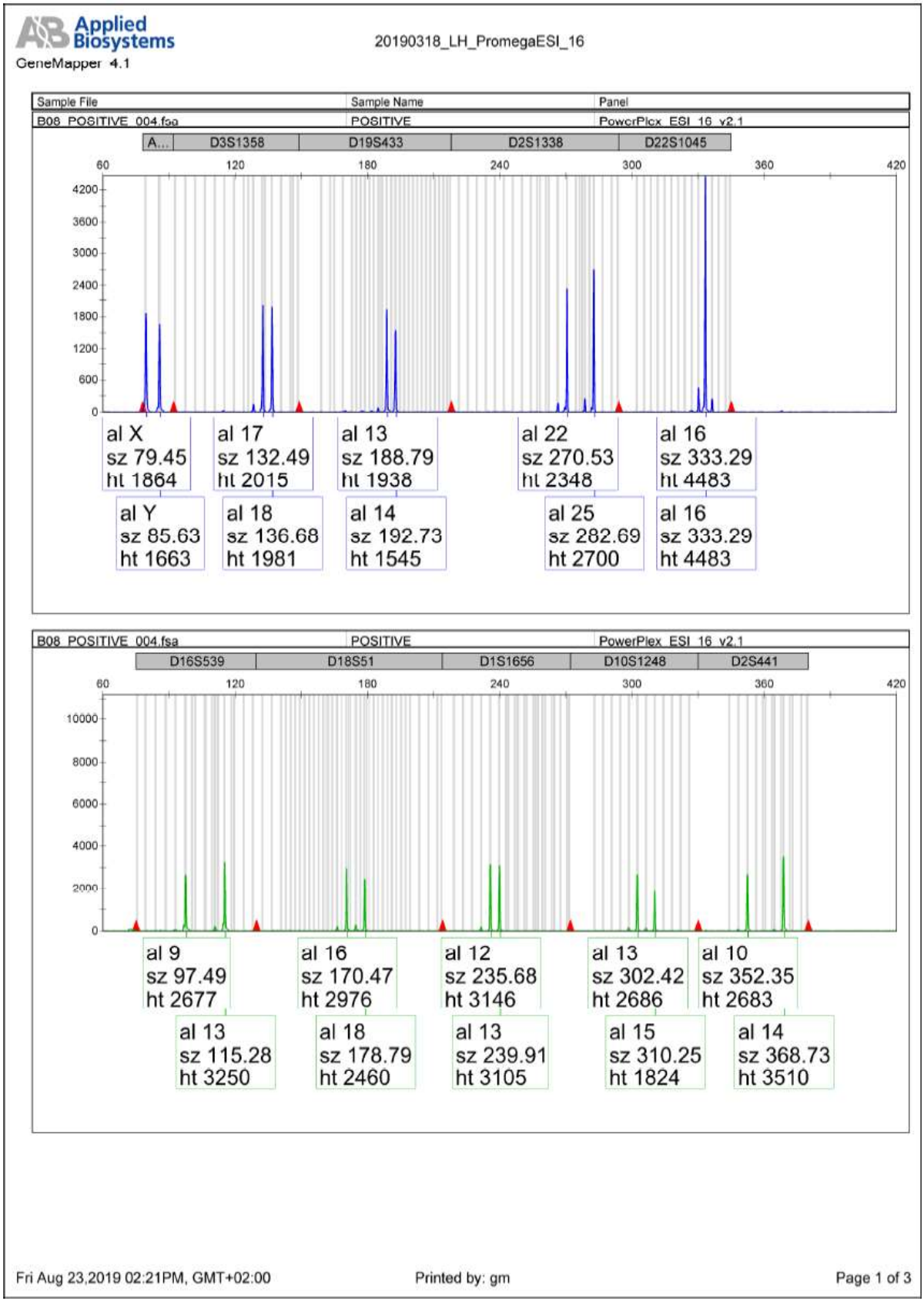


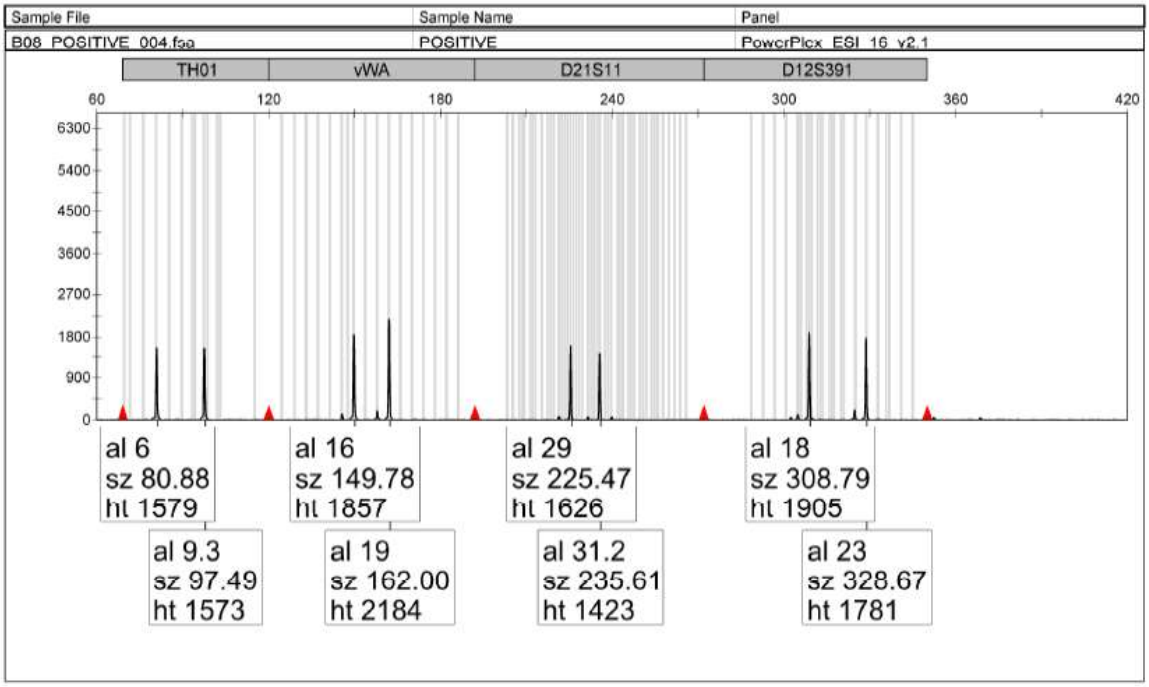
Figure C.1.2: The standard curve and quality metrics of the qPCR run for all control samples.

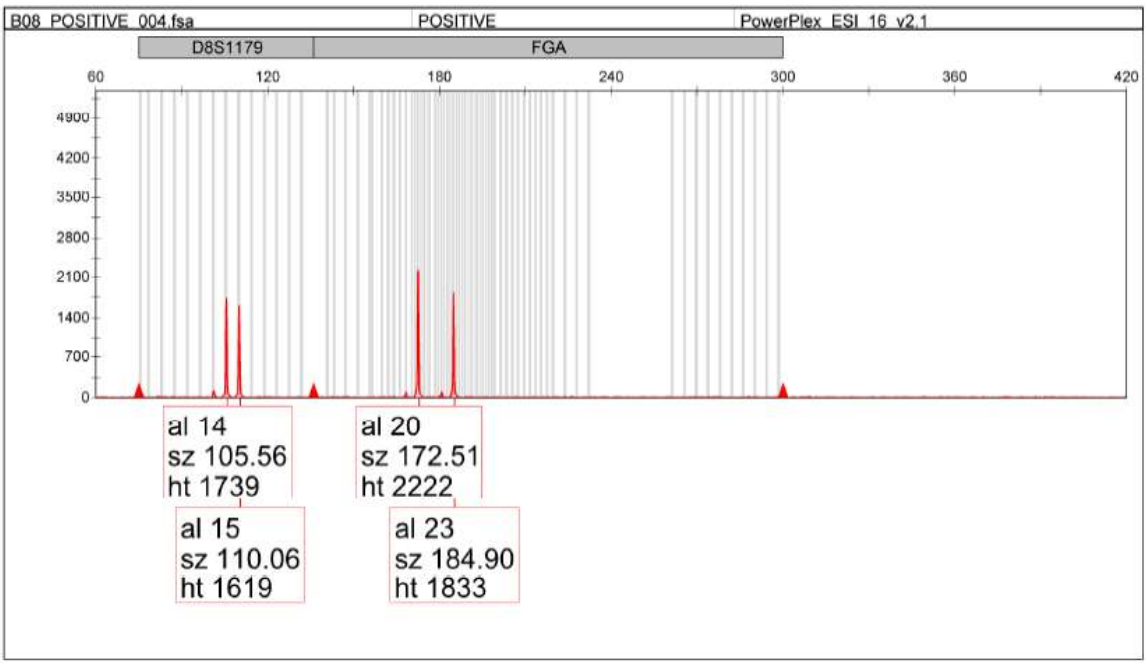
C.1.3 DNA profiling – positive and negative control



Applied Biosystems
GeneMapper 4.1

20190318_LH_PromegaESI_16





Fri Aug 23,2019 02:21PM, GMT+02:00 Printed by: gm Page 2 of 3

Applied Biosystems
GeneMapper 4.1

20190318_LH_PromegaESI_16

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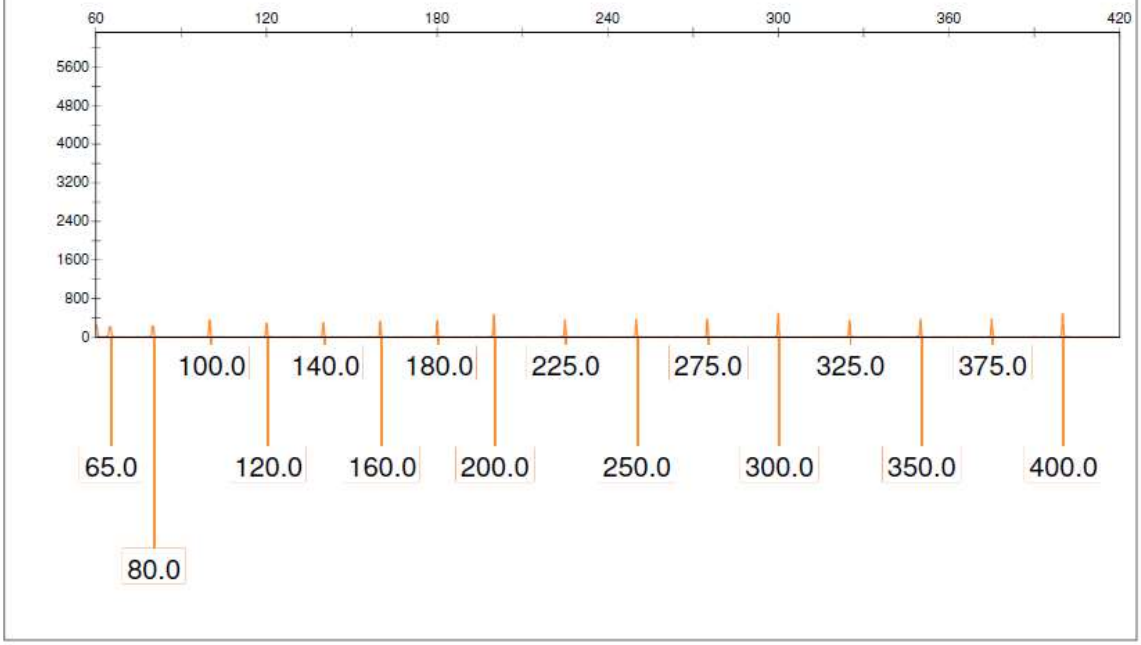

Applied Biosystems

GeneMapper 4.1

20190318_LH_PromegaESI_16

Sample File	Sample Name	Panel
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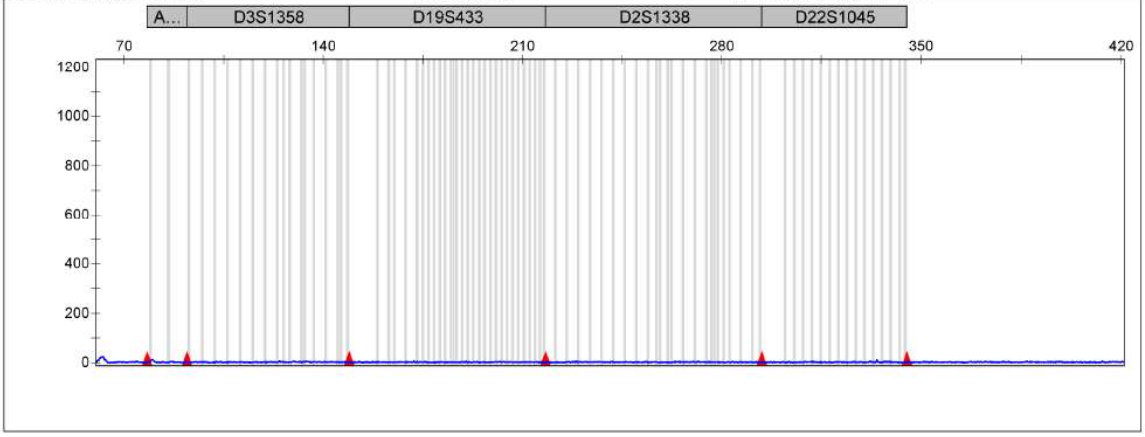

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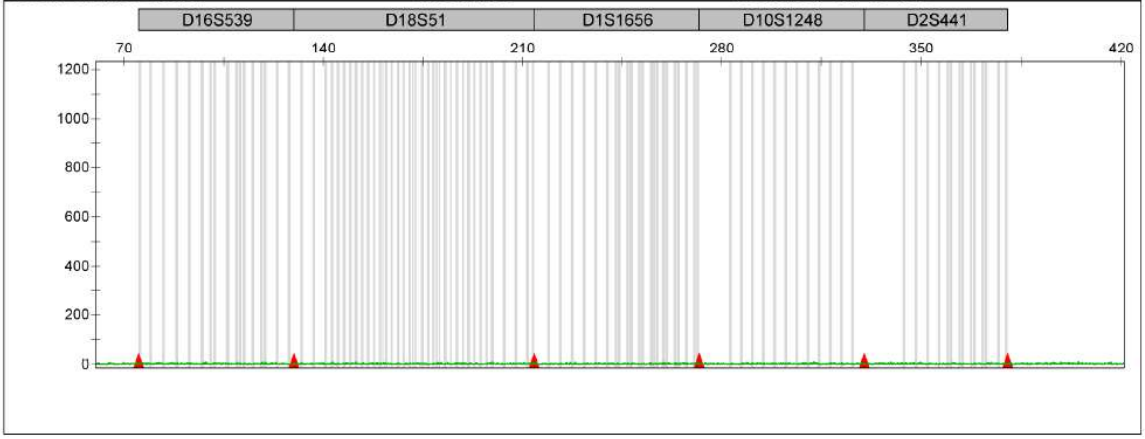

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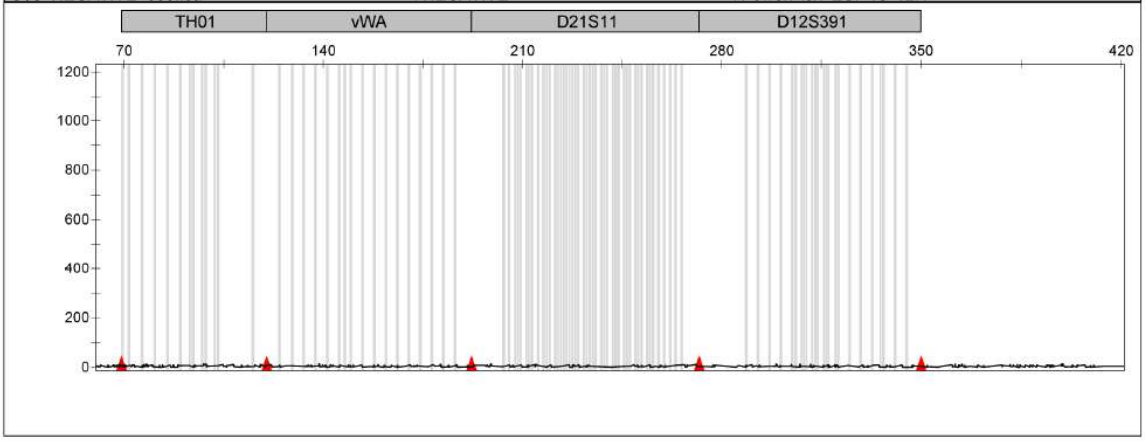

Applied Biosystems

GeneMapper 4.1

20190318_LH_PromegaESI_16

Sample File	Sample Name	Panel
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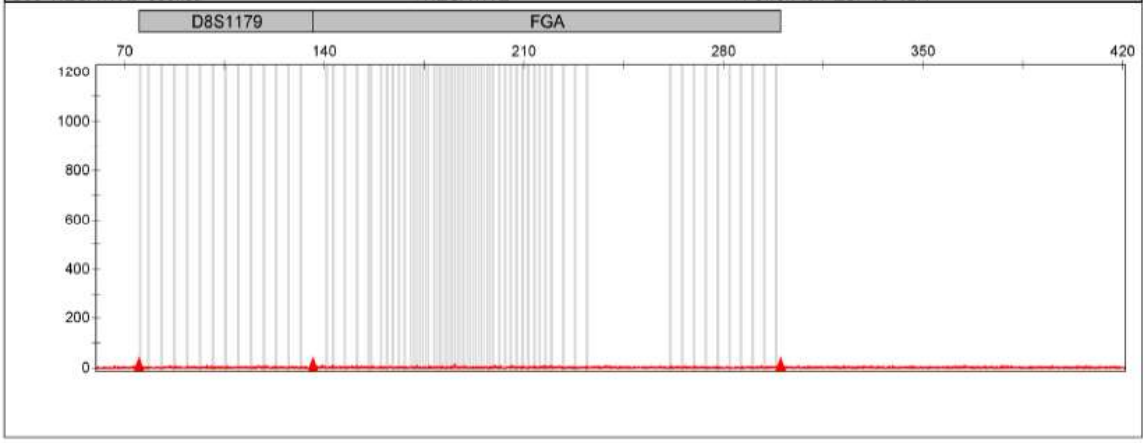

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