Human bone cells:

detection and STR profiling.

By

Thien Ngoc Le
BSc (*Honours*) in Biotechnology,
Master by Research in Genetics

Thesis
Submitted to Flinders University
for the degree of

Masters by Research

College of Science & Engineering
14th September 2022

TABLE OF CONTENTS

THESIS ABSTRACT

DNA encased in bone is a potential substrate for human identification in forensic casework if there is a need to associate human remains with reference samples. The source of DNA within bones is embedded in a calcium-based matrix that needs to be removed to allow subsequent DNA analyses. An initial step in DNA isolation may be to drill into the bone and create a fine powder. Decalcification is required to remove calcium from the powder and aid in the release of the cells containing DNA. The removal of calcium is performed in a solution containing a high molarity of EDTA. It is not possible to monitor how many cells, and therefore the amount of DNA, are present in the powder and then in the EDTA-rich solution. An outcome may be that some bone samples have no DNA, or insufficient DNA yields, for subsequent DNA profiling. The ability to record cells at the early stage of DNA isolation from bone, and therefore enhance DNA recovery, is the central part of this thesis. Cells were recorded as being present using a DNA binding dye (Diamond nucleic acid dye, or DD) and presented in the published paper '*A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue*' in the journal Forensic Science Medicine & Pathology.

Archived osteochondral tissues may be a potential template for short tandem repeat (STR) profiling as these samples have an abundance of nucleated cells, such as chondrocytes and osteocytes. DNA within these cells were rapidly visualised by use of a mini portable microscope after the application of the same DNA staining dye (DD). Stained cellular material appeared as fluorescent green images, and at the expected size and morphology of a bone cell. The number and location of the stained cells was recorded, along with areas within the bone matrix where there was an absence of cells. A standard DNA extraction method was performed to collect DNA from eleven bone sections (5 μm thickness). DNA yields were quantified by real-time polymerase chain reaction (qPCR). Ten of the eleven samples (91%) returned a low-level degradation status and subsequently generated complete STR profiles (based on the targeting of 15 STR loci). The remaining other sample reported a higher degradation metric than the others and subsequently recorded an incomplete STR profile. There was therefore a significant correlation between the number of DD-stained cells and the number of STR alleles amplified ($p < 0.05$) (publication I,

chapter 2). The inference is that staining sections of bone with a nucleic acid dye can determine the prevalence of bone cell nuclei and then significantly increase the chance of generating a DNA profile.

This thesis also investigated the use of a simple means to release bone cells from the bone matrix such that the cells could then be easily collected and used as a template for DNA profiling ('*DNA profiling from human bone cells in the absence of decalcification and DNA extraction*' was published in the Journal of Forensic Sciences). Nine stored tibial tissues (aged between 3 – 5 years between sample collection and analyses) were part of this study. Using these samples, approximately ~ 20 mg was removed and fragmented using a clean pestle, this was without the use of liquid nitrogen or any specific device. The fragmented remains were stained with DD to visualise the matrix-free cells. These cells were collected and subjected directly to qPCR. A standard DNA extraction process was performed in parallel to compare the two sets of results. Processing the nine samples by both methods resulted in informative STR profiles with minimal indication of inhibitors. By adding the stained cells directly to the qPCR and also as a template for a direct PCR approach, circumvented the decalcification step and DNA extraction process, resulted in STR profiles from tibia samples being generate within 8 hours (publication II, chapter 3).

DECLARATION

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Signed: Thien Ngoc Le
June 2022.

ACKNOWLEDGEMENT

09.03.2020. It began the trip to find myself. I learnt how to lose! I lost anger, greed, jealousy, and impatience. So I attained peace in my mind. Thanks to Adelaide, the land filled up with peace and nature. I found myself. I am really grateful to three persons: First is my principal supervisor, Professor Adrian Linacre, who gave me the ticket to arrive here and be his student. Thanks for his patience to supervise me asking myself in every circumstances in order to address the logic and feasibility. His unfathomed knowledge has assissted me to overcome the challenges and adapt Australia's culture. Words cannot express how grateful I am for your enthusiasm, but thanks for all your contributions to my study and life! I am eternally appreciated!

Secondly, this work is dedicated to my husband, Phuc. You believed in my dream and are always there to make me calm and happy. You also disregarded your interests and allowed me to be free to reach my dreams. I am specially grateful to you!

Third one is my little son, Khoa. Responsibility for a child is never easy, it requires patience and sympathy. I am an imperfect mother as I am not good at balancing the life, study and parenting. Time passed and mess still arounds. Now, vegemite is one of his favourites, he speaks Aussie accent and corrects my pronunciation. Thanks for his friendship and Mitcham community, we have muticulturally exchanged and fullfiled Australian lifestyles. Enormous thanks to our beloved uncle Jim Lesses, who is the kindest neighbour ever, but also seems to be our family member. Honest thanks to family of Elizabeth Day and Nukhba Mohsin Khan, who welcomed us with loves and dearest sharings.

To my mother, Hong Le, she endured pain, suffering and torture. Her love guides me, her blessing is always with me on my path. I am eternally grateful! Endless thanks to our Vietnamese families, where tears and smiles came, home is everything!

I would like to express my gratitute to all my co-supervisors, Dr. Dzenita Muratovic, Dr. Oliva Handt and Dr. Jullianne Henry, for their support and patience. Without their assisstance, no achievement obtained.

Last but not least, thanks to all my mates in Flinders University, particularly in Forensic Biology research group, who were been bothered but always helpful and honest. Sense of humor and modesty was learnt from them.

Honest thanks to the College of Science and Engineering, I received the fee waiver for the second year to complete this degree and had a chance to upgrade to PhD program. I really much appreciated that!

As with everything at the moment, “life is a juggle”. But I really have appreciated the kindness, care and compassion the community has shown to us, through these challenging years of COVID.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ATL	A tissue lysis buffer
bp	base pair
DD	Diamond™ Nucleic Acid Dye
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EG	EvaGreen™
FFPE	Formalin fixed and paraffin embedded
GG	GelGreen™
GR	GelRed™
H&E	Hematoxylin & Eosin
LCN	Low copy number
mtDNA	mitochondrial DNA
MSCs	mesenchymal stem cells
nDNA	nuclear DNA
ng	nanogram
NaOAc	Sodium acetate
PCR	Polymerase chain reaction
qPCR	quantitative real-time PCR
RS	RedSafe™
SDS	Sodium dodecyl sulphate
SG	SYBR® Green I
STR	Short Tandem Repeat
v/v	volume per volume
w/v	weight by volume

LIST OF ORIGINAL PUBLICATIONS

Publication I

Thien N. Le, D. Muratovic, O. Handt, J. Henry, A. Linacre, A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue, Forensic Science, Medicine and Pathology. 2021. doi: https://doi.org/10.1007/s12024-021-00428-3.

Publication II

Thien N. Le, Muratovic, D, Handt, O, Henry, J, Linacre, A. DNA profiling from human bone cells in the absence of decalcification and DNA extraction. J Forensic Sci. 2022; 00: 1– 7. doi: https://doi.org/10.1111/1556-4029.15033.

LIST OF AUTHOR CONTRIBUTORS

Author contribution includes:

Publication I – Thien N.Le, Dzenita Muratovic, O. Handt, J. Henry, A. Linacre. *A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue*, Forensic Science, Medicine and Pathology (2021).

	Conception and experimental design	Performed laboratory work	Results analysis and interpretation	Manuscript preparation	Manuscript editing
TNL	90%	90%	55%	60%	50%
DM	-	10%	10%	-	5%
OH	-	-	10%	10%	10%
JH	-	-	10%	10%	10%
AL	10%	-	15%	20%	25%

Publication II – Thien N. Le, Dzenita Muratovic, O. Handt, J. Henry, A. Linacre. *DNA profiling from human bone cells in the absence of decalcification and DNA extraction*, Journal of Forensic Science (2022).

	Conception and experimental design	Performed laboratory work	Results analysis and interpretation	Manuscript preparation	Manuscript editing
TNL	90%	90%	74%	55%	50%
DM	-	10%	-	10%	5%
OH	-	-	8%	10%	10%
JH	-	-	8%	10%	10%
AL	10%	-	10%	15%	25%

CONFERENCE AND PRESENTATION

(Name in bold denotes presenting author)

Thien N. Le, Dzenita Muratovic, O. Handt, J. Henry, A. Linacre, *A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue,* Oral presentation at 1st Annual College of Science and Engineering Higher Degree by Research (HDR) Symposium, Flinders University, Adelaide, Australia, November 2021.

CHAPTER 1: INTRODUCTION

1.1. Preface

Skeletal human remains may be the only source for human identification following events such as mass disasters, or historic cases, where human bodies have been extensively compromised. The source of the DNA is in the form of mature osteocytes, embedded within a mineralized bone matrix. Since the advent of DNA profiling, and in particular the application of mitochondrial DNA (mtDNA) in forensic science, methods have been reported on the isolation of DNA from bone samples [2-8]. There were, and remain, some challenges to the effective isolation of DNA from bone samples including: (1) bone is one of the hardest types of tissue among biological samples and requires pre-treatment steps prior to extraction; (2) the high amounts of calcium present in bone need to be removed as this is an inhibitor of downstream procedures; and (3) the number of cells containing DNA are few and deep within the bone matrix.

A standard protocol for DNA profiling from bones performed in forensic laboratories comprises seven steps including: (1) sample selection, (2) sample preparation, (3) decalcification, (4) DNA isolation, (5) DNA quantification, (6) DNA amplification, and (7) DNA profiling [4, 7, 9-11]. It can take up to two days to generate a profile when adhering to the protocol provided by commercial kits, particularly when there is an overnight incubation as part of the decalcification process [12, 13]. For compromised samples (human remains interred in soil or exposed to a hostile environment), complete demineralisation leading to increasing DNA yields is best achieved after 3 – 5 days incubation in an EDTA-rich buffer with frequent changes of this solution [6]. In general, it takes between 2-5 days to isolate DNA from bone material, depending on the quality of the bone sample [6, 12, 13]. The core aim of this project is to find an alternative process that increases the quality and quantity of DNA isolated from bone in a faster and more efficient way than current procedures.

This chapter introduces the basic background to human bone cells, including types of cells, their morphologies and location within the bone matrix. The chapter centres on the most abundant cell population which is used as a target cell in forensic identification from bones: osteocytes. Also outlined are previous and current studies of nuclear DNA profiling (nDNA) (short tandem repeats, or STR profiles) from human bones. This chapter outlines a standard and modified workflow commonly used in forensic laboratories from sample selection, decalcification, DNA extraction to DNA profile steps. The chapter also details the advantages and limitations of current processes

leading to the need of the research project: the detection of osteocytes and alternative STR profiling process from bone samples.

1.2. Histology of bone

1.2.1. Hierarchical structure of bone

Anatomically, a healthy adult skeletal system is made up of bones, ligaments, and cartilage. A complete human skeleton is comprised of 206 bones: 126 appendicular bones, 74 axial bones, and 6 auditory ossicles bones [14]. Bone consists of five hierarchical levels: macrostructure (whole bone), mesostructure, tissue, lamellar, and ultrastructure (Fig. 1) [15]. The top level is the macrostructure (or whole bone) which details the whole skeletal framework of the bone consisting of cortical bone (80% volume of the total skeleton) and trabecular bone (20% volume). The next level is the misostructure containing the structure that outlines the distribution of components within bone. The third is the microstructure level (tissue) and is inherent to the functional materials of bone. The fourth is the sub-microstructure (lamellar level) which is comprised of mineralized collagen fibril and minerals accumulated by bone formation cells – osteoblasts. The final level is the nanostructure, where occurs bone activities controlled by the chemical interactions between bone cells and bone matrix. Each level provides functional and structural support for the top levels.

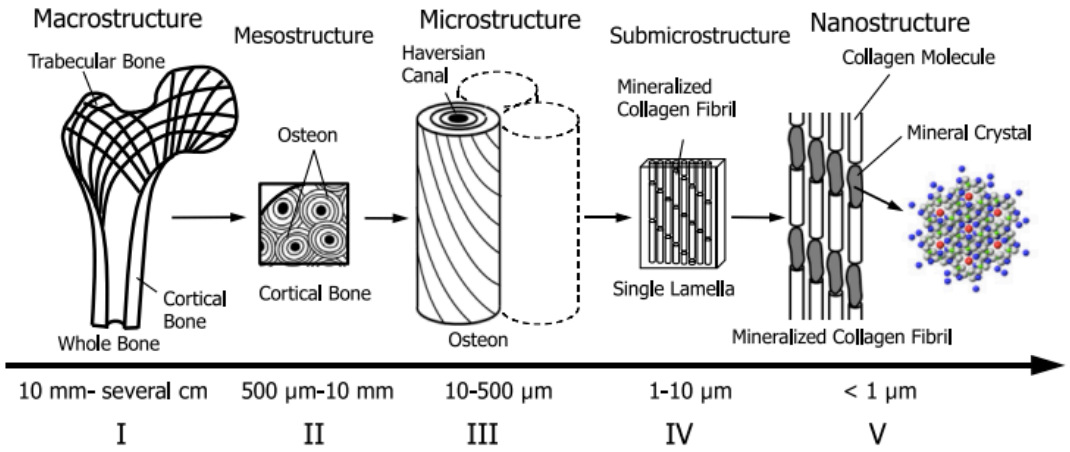


Figure 1: Bone hierachy. Five levels of hierarchical structure in cortical bone.
(I) Macrostructure (whole bone) including cortical bone and trabecular bone.
(II) Mesostructure. Cortical bone is one example.
(III) Microstructure, single osteon in cortical bone.
(IV) Sub-microstructure, single lamella level.
(V) Nanostructure consists of organic (collagen) and inorganic (minerals) materials.
The image is adapted from [15].

Cortical bone, also known as compact bone, is the outer core and consists mostly of thick and dense materials; this connects ligaments and tendons. Compact bone therefore mainly aids in mechanical functions such as locomotion and protection of soft tissues [16]. In compact bone, an entire functional unit is known as an osteon (Fig. 2). Osteons are associated with a central blood vessel, named the Haversian canal. These canals are structured as parallel tunnels which run down the length of the bone. Haversian canals contain blood vessels and nerve fibres. The canals are surrounded by central rings called lamellae, between lamellar rings are spaces called lacunae, which are houses of bone cells [17]. Lacunae are therefore structures within the bone matrix inside of which bone cells – osteocytes are located. These cells are the most abundant of the bone cells and are a rich source of DNA for subsequent analyses [18]. Osteocytes communicate via canaliculi with other cells and are central to functional activitives such as transfer of nutrients [17]. Trabecular bone, also known as spongy or cancellous bone, is responsible for bone metabolism such as regulation of calcium and minerals [19]. Bone metabolic disease (osteoporosis) is a consequence of the imbalance of minerals throughout the body. Osteoporosis can result in the loss of integrity of bone structure resulting in fragility and fracture. In contrast to compact bone, trabecular bone is thin and porous (Fig. 3, image A). Among trabecular zones is bone marrow containing red blood cells (Fig. 3, image B).

Osteoblasts, osteoclasts, osteocytes and bone lining cells are the four key cells of bone tissue [20, 21] (Fig. 4). Each of these cell types has a specific function, impacting on the activities of bone. Osteoblasts synthesise the bone matrix and are responsible for bone formation. Osteoclasts digest the bone matrix through the remodelling process. Osteocytes act as sensors of the remodelling process via transmitting signals through osteocytes network [22]. Bone lining cells are involved in bone formation and bone resorption by digesting collagen fibers [23]. The bone matrix consists 33% of unmineralized components (type I collagen and glycosaminoglycans) and 67% of minerals (calcium hydroxyapatite) that strengthen and solidify the bone.

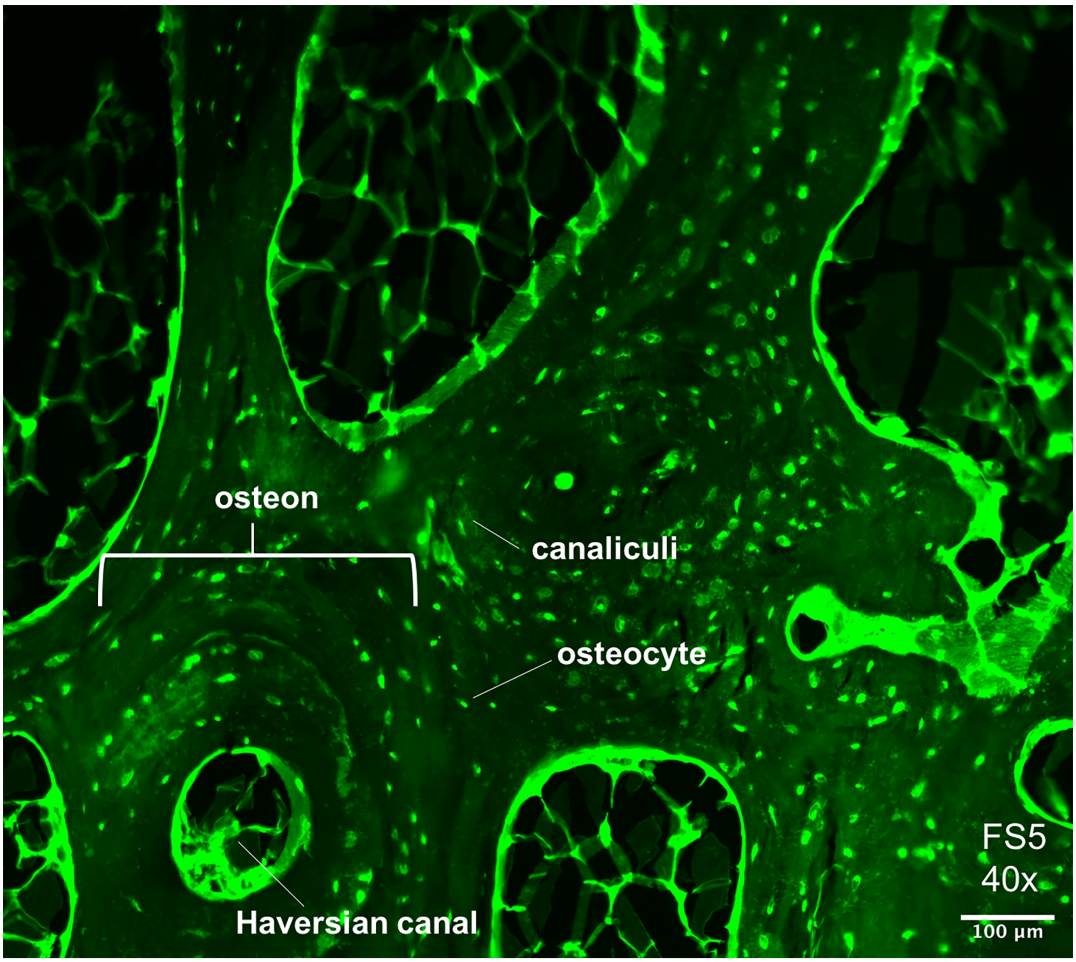


Figure 2: Osteon structure. Shown is a formalin-fixed and resin-embedded section of an osteochondral bone (sample FS5) after staining with 20x DD. Details of an osteon including osteocytes population, canaliculi and Haversian canal are shown. The image photographed at 40x magnification. The scale bar is 100 µm.

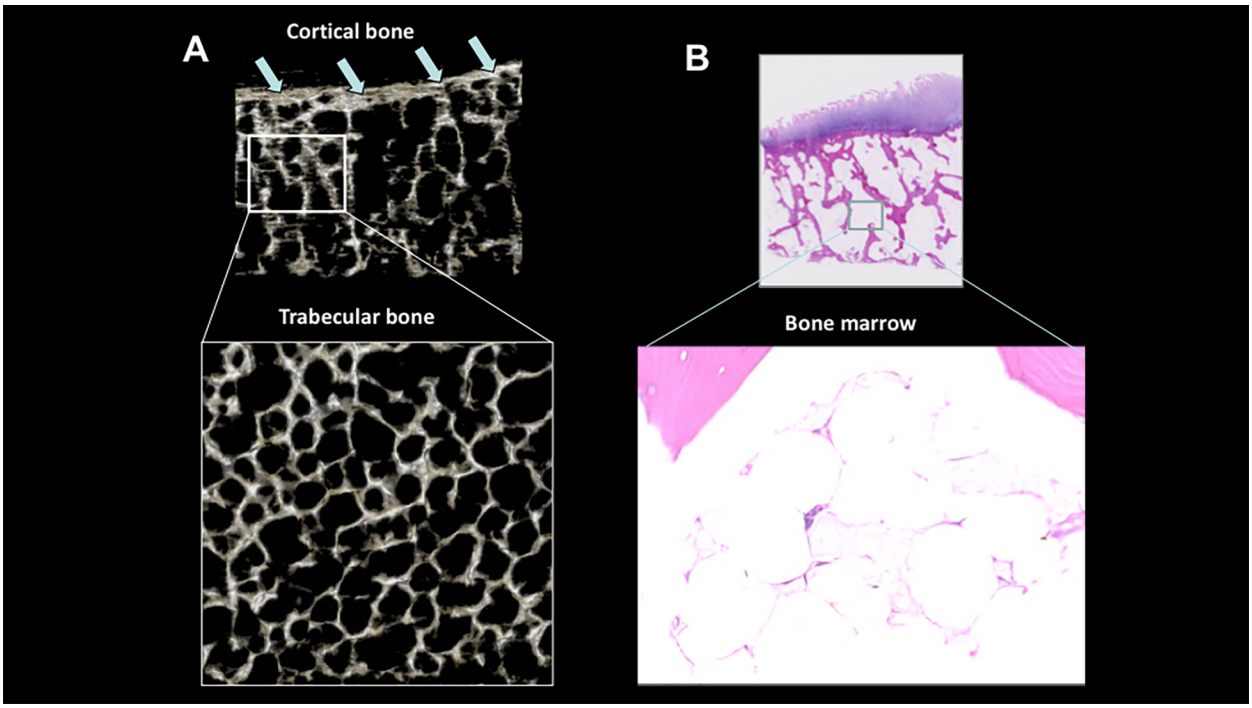


Figure 3: Location of cortical bone and trabecular bone. Bone sampled from a 72-year-old female taken during arthroscopy. Image A is a representative image of a bone sample scanned by micro-CT to illustrate bone structure, showing cortical bone material (indicated by four arrows) and below is a magnified part showing trabecular bone structure. Image B shows the trabecular structure stained with hematoxylin and eosin staining to illustrate the presence of bone marrow at the same area of bone as image A. Magnification is at 50× for the top images and 200× for the two bottom images.

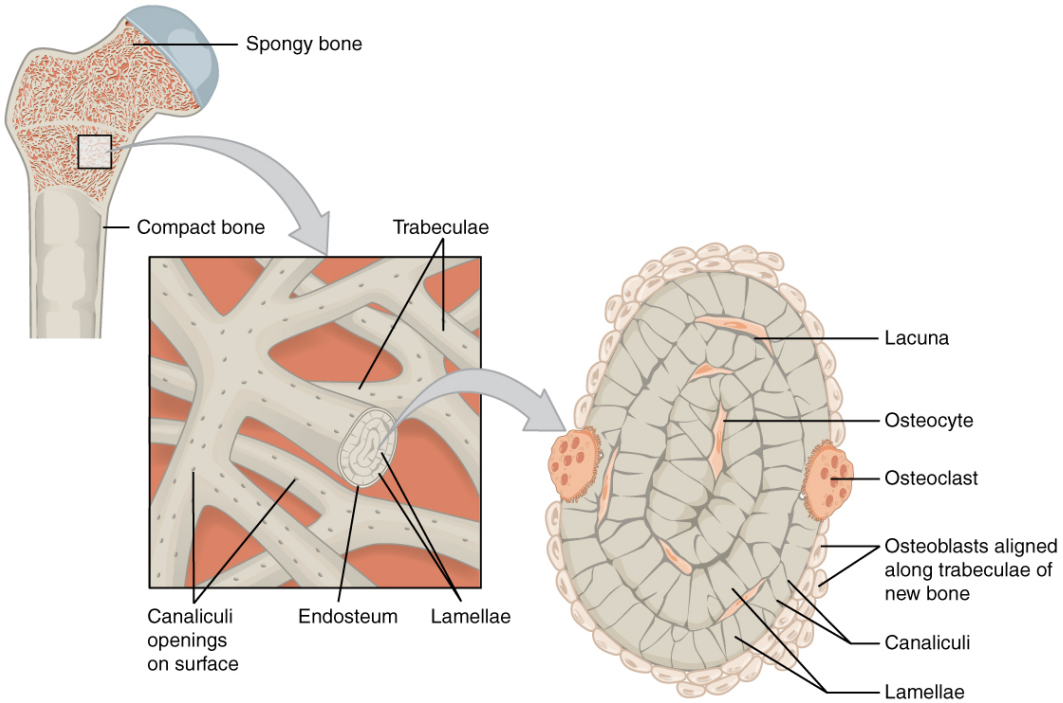


Figure 4: Trabecular bone structure. Four key bone cells consisting of osteoblasts, osteoclasts, osteocytes and bone lining cells. The image is abstracted from [24].

1.2.2. Types of bone cells

1.2.2.1. Osteoblasts

The word 'osteoblasts' comes from the Greek *osteo* for bone and *blastano* means germinate. They are single nucleated cells connected in groups that synthesize bone matrix. Osteoblasts are derived from pluripotent mesenchymal progenitor cells and comprise 4-6% of total bone cells [25]. These mononucleated cuboidal cells are found on bone surfaces. Bone formation involves two stages: (1st stage) organic matrix creation which provides flexible strength, and (2nd stage) minerals addition providing compressive strength of bone. First, osteoblasts secrete collagen proteins, mainly type I collagen, non-collagen proteins and proteoglycan that constitute the matrix. Second, the matrix is subsequently mineralized by deposition of minerals (hydroxyapatite).

A mature osteoblast with a single layered cell membrane has large nuclei and Golgi complexes. Osteoblasts undergo apoptosis or differentiates into osteocytes or bone lining cells. The changes in cell morphology and gene expression of osteoblasts mark the transitional stages during bone formation and the mineralization process [25]. Phosphate ions, osteocalcin, bone sialoprotein 2 and osteopontin are the products secreted by osteoblasts during transition [25]. At

the final stage of osteocyte differentiation, osteoblasts become mineralized embedded osteocytes with an interconnected network [25]. Table 1 shows the longevity of 4 types of bone cells. While osteoblasts can live 1 – 200 days, osteocytes can live up to 50 years [26].

Table 1: The longevity of resident bone cells. This table is adapted from [26].

The longevity of bone cell nuclei (approximate ranges)	
Osteoblasts	1-200 days
Osteocytes	**1-50 years**
Osteoclasts	1-25 days
Lining cells	1-10 Years

1.2.2.2. Osteoclasts

Osteoclasts comes from the Latin word '*oteon'* for 'bone' and *clastos* means 'broken'. As the name suggests, osteoclasts are cells that break down bone tissue. They are the largest cells (100 – 200 μm in diameter) compared to osteoblasts, osteocytes and bone lining cells. Osteoclasts, also known as multinucleated cells because they contain approximately 10 to 20 nuclei [26]. Activated osteoclasts originate from mononuclear monocyte-macrophage precursor cells. It is estimated that osteoclasts live only for 1 to 25 days and their average turnover is 10% per year depending on different regions of the skeleton [26].

Osteoclasts are located in shallow resorption bays (Howship's lacunae) on bone surfaces. The activity of osteoclasts is suppressed by calcitonin hormone and stimulated by parathyroid hormone. The cells are responsible for mature bone tissue being removed during the remodelling process; a process that lasts between 6-9 months in healthy adult humans [26]. Following this remodelling, new bone tissues are formed by teams of osteoblasts. During the remodelling process, osteoclasts secrete cathepsin K enzyme, hydrogen ions, gelatinase, acid phosphatase and matrix metalloproteinase resulting in digestion of the organic matrix. A new osteon is the final step of each bone remodelling cycle where there is a balance in the ratio between aging-change the old bone reabsorbed, and new bone formed.

1.2.2.3. Osteocytes

The term osteocytes comes from the Latin word '*osteo'* meaning bone and '*cyte'* meaning a cell. Osteocytes are mononucleated stellate shaped bone cells are derived from mesenchymal stem cells (MSCs). They are differentiated from osteoblasts and located within lacunae in mineralized matrix [25]. MSCs are 'fibroblastic-like' cells, which form clusters defined as a fibroblast-colony. The osteocyte populations comprise 90-95% of total bone cells (greater then 1000 times more abundant than osteoclasts and 10 times more than osteoblasts) and recorded as the long-lived cells, living up to 50 years [26]. Depending on bone type, the morphology of osteocytes differs from a rounded shape in trabecular bones to elongated shape in cortical bones [27].

Osteocytes are capable of forming a connection resulting in cell-to-cell communication between osteoblasts and lining cells (Fig. 5) [25]. The mechanosensory osteocytes have some special features including: signal transfer and ion exchange of bone formation or resorption to the superficial bone cells; systemic mineral homeostasis regulation; microenvironment modification; and mineral concentration maintenance [25]. The loss of osteocyte viability, known as apoptosis, results in the presence of empty lacunae in aged bone. These empty lacunae can be observed morphologically under a microscope (Fig. 6).

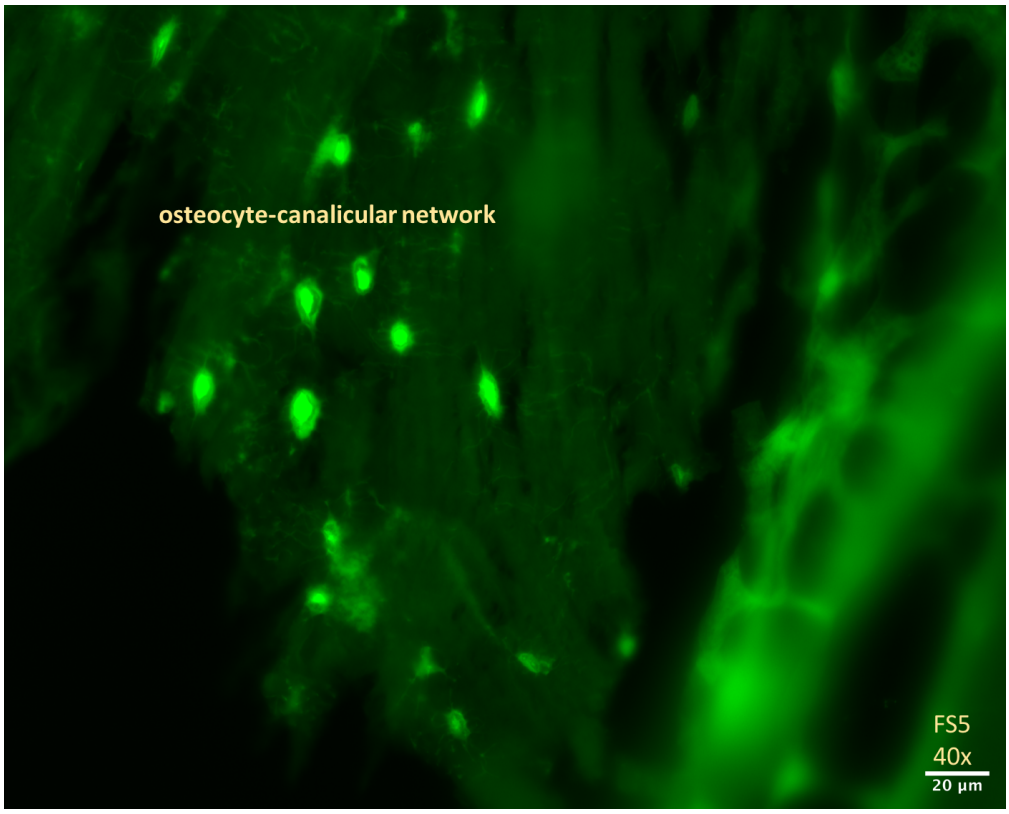


Figure 5: Osteocyte – canalicular network. The image illustrates a Diamond™ Nucleic Acid Dye stained bone section (5 µm thickness, sample FS5). Green dots represent osteocytes in lacunae communicating via canaliculi or the osteocyte-canalicular network. This was observed under a AX70 microscope with 40x magnification. Scale bar is 20 µm.

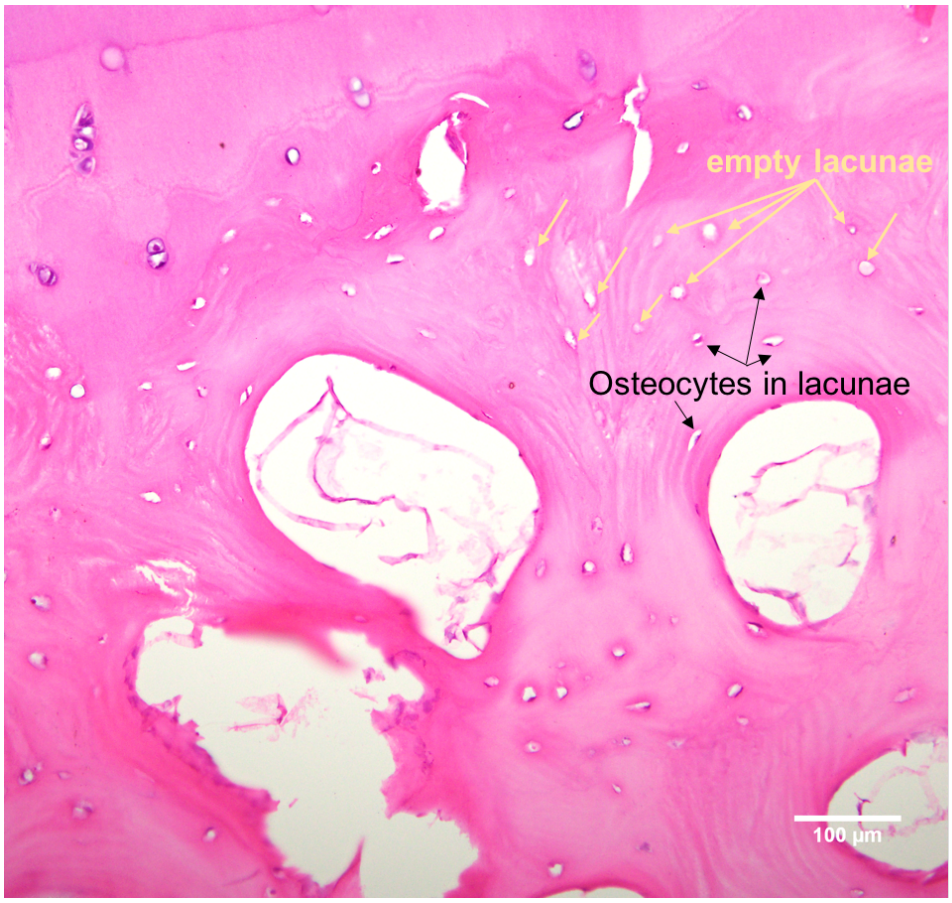


Figure 6: The presence of osteocytes. The picture shows a H&E stained bone section (5 µm thickness, sample FFPE – 815). Osteocytes in lacunae (black arrows) and empty lacunae (yellow arrows) are identified. This image was obseved under BX50 microscope with 20x magnification. Scale bar is 100 µm.

1.2.2.4. Bone lining cells

Bone lining cells originate from inactive osteoblasts which do not undergo apoptosis or differentiate to osteocytes [28]. Morphologically, they are slender, long and flat and located on the bone surface where they connect to other cells via gap junctions [29]. Instead of expressing intercellular adhesion molecule-1, like osteoblasts, such lining cells secrete osteocalcin that is responsible for bone remodelling and preparing the surface of the bone by removing nonmineralized collagen fibrils. They also control the passage of calcium and various hormones that activate the osteoclasts [28].

1.2.3. Bone Matrix Mineralization

Bone is composed of 50-70% mineral, mostly as hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$, 20-40% organic matrix, 5-10% water and less than 3% lipids [14]. Bone hydroxyapatite crystals consists predominantly of acid phosphate and calcium ions. Other components such as magnesium, potassium, bicarbonates, sodium, citrate, fluorite, zinc, barium are also found [14]. Calcium comprises approximately 40% of bone material. The non-collagenous proteins called calcium-and-phosphate-binding proteins play a vital role in regulating such hydroxyapatite crystals in the matrix maturation process [14]. During the maturation process, immature bone is replaced by mature bone, which is classified as two types: compact bone and trabecular bone [30].

In terms of interactions with bone cells, bone matrix proteins (collagen) releases adhesion molecules (integrins) that promote bone formation and bone repair. For example, during bone surface synthesis, osteoblasts make interactions with bone matrix via integrins, which bind to collagen and then impact on the bone surface organisation. This may lead to the bone remodelling, loss of bone mass and bone fracture [31]. Collagen changes therefore play a vital role in the structure and function of bone tissue.

Vitamin D promotes differentiation of osteoblasts, impacts on the apoptosis of osteocytes that results in the presence of empty lacunae (without cells) within bone (Fig. 7). The internal structure of bone is regulated by a number of hormones that affect the supply of calcium and phosphorus during the remodelling bone process [30]. The presence of calcium and phosphorous protects bone cells within these matrices from external influences such as temperature and humidity. This character is beneficial for DNA recovery from bone.

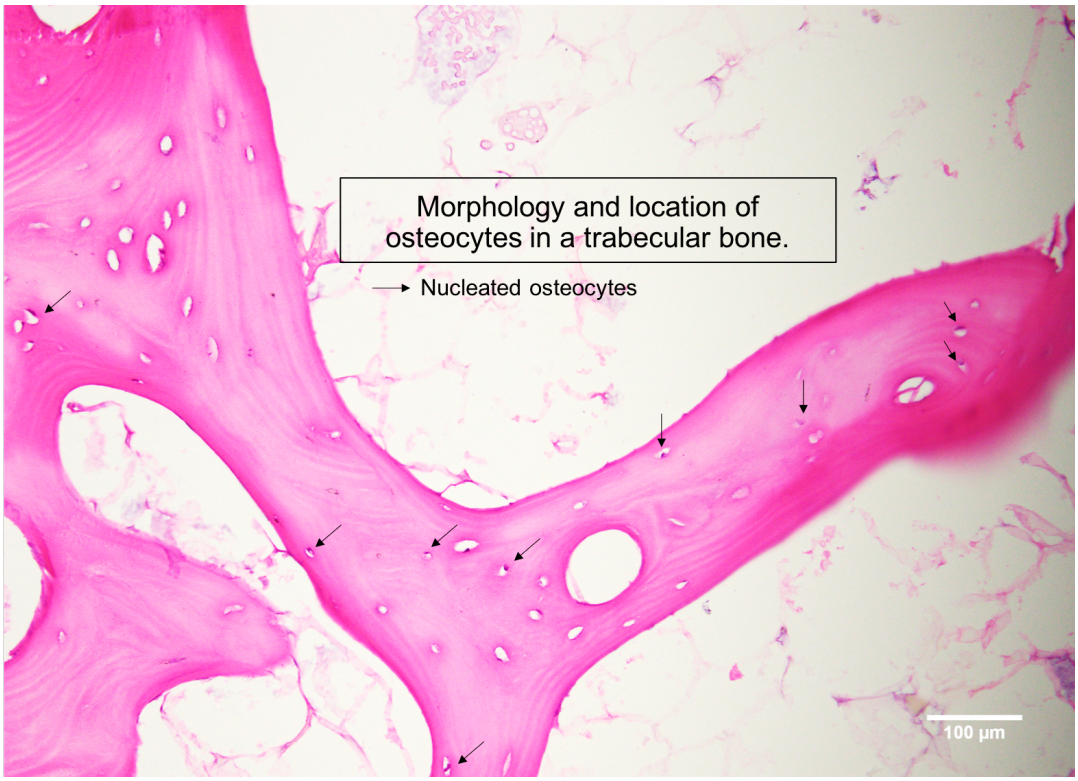


Figure 7: A H&E stained trabecular bone section (5 µm thickness) (sample FFPE – 815). Showing mineral matrix (pink coloured area), almond-like morphology and location of osteocytes. Black arrows indicate the presence of nucleated osteocytes. Image was visualised under a BX50 microscopy with 20x magnification. Scale bar is 100 µm.

1.2.4. Bone growth, modelling and remodelling process

Bone formation, also known as osteogenesis or ossification, starts from the sixth week of an embryo and continues throughout life in response to adaptations such as damaged bone [32]. This process occurs via two pathways: endochondral ossification and intramembranous ossification. In the endochondral pathway, bone grows in length, especially in the epiphyseal plate of long bones, where occurs the development of new cartilage. Here, the MSCs differentiate into chondroblasts, which secrete organic matrix of the bone (collagen and proteoglycans). Subsequently, the differentiation of the chondroblasts is performed into chondrocytes along with the secretion of growth factors and biochemicals. This process also initiates the deposition of minerals (such as calcium, phosphate) within bone in association with the formation of new cartilage during youth, which is replaced by bone. As a result, longitudial growth of the bone is produced. Figure 8 illustrates the chondrocytes located in cartilage plate and stained by a nucleic acid binding dye (Diamond Dye) (Fig. 8A) and Hematoxylin (Fig. 8B).

In contrast, the intramembranous ossification does not rely on the development of cartilage plate. Endochondral ossification focuses on the formation of long bones (such as femur) while the intramembranous ossification forms the flat bones (such as skull, clavicle and sternum). In the intramembranous ossification, a collection of MSCs surrounded by a membrane is formed. Subsequently, these MSCs differentiate into mature osteoblasts, which are bone forming cells and deposit the mineral bone matrix. The osteoblasts either die by apoptosis or become osteocytes embedding in the matrix. Briefly, the two processes of bone growth are also defined as bone modelling, where bone grows in length and in width. Bone development is completed and followed by a bone remodelling process to maintain normal healthy bone [32].

Bone remodelling is a continuous process that renews the skeleton and occurs throughout the life. In this event, the balance of bone formation and resorption changes depending on the age. For instance, in healthy individuals, bone formation dominates for the first 30 years to reach the peak of bone mass, and maintains for 20 years later until resorption exceeds formation at the aging process [33]. Bone remodelling involves five sections: quiescent, activation, resorption, formation and mineralisation (Fig. 9). At the quiescent stage, bone is inactivated until the occurence of some events such as microfracture, mechanical loads, or low calcium during pregnancy, which initiates the activation stage. Osteoclast precursors are recruited to bone surface to start the resorption phase. Osteoclasts resorb damaged bone to leave cavities, which are filled with new bone forming cells – osteoblasts while macrophages clear all the debris. The formation is then begun, osteoblasts secrete a collagenous matrix (called osteoid) and deposit the mineralisation with calcium and phosphorous ions. The final phase is mineralisation, where some osteoblasts either become osteocytes trapped within mineralised matrix or undergo apoptosis, or become bone lining cells located on bone surface [34]. In summary, osteoblasts and osteoclasts are the key bone cells involved in the remodelling process. The understanding of bone biology is also beneficial for bone related analysis in forensic context, such as the assessment of bone samples via observation of bone cells prior to molecular testing. The morphology of four types of bone cells and their location within bone are essential for determining the number of cells containing nuclei, which impact on subsequent DNA analysis.

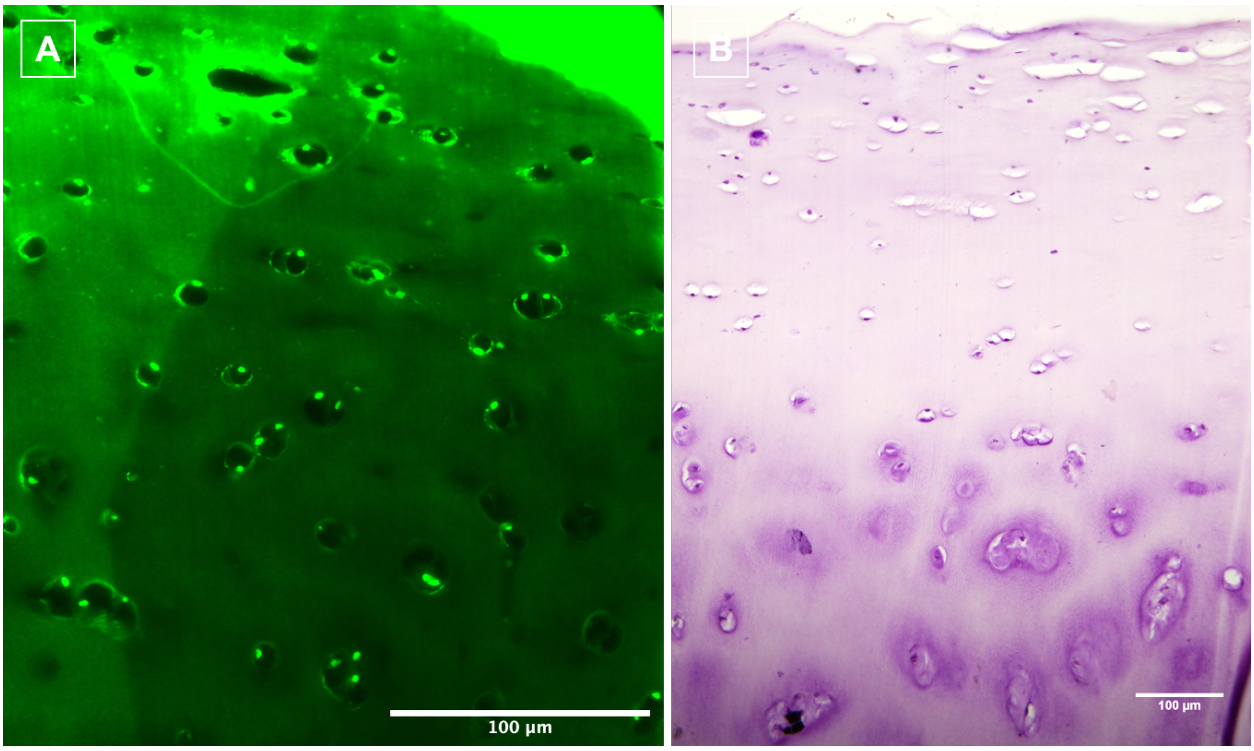


Figure 8: Morphology of chondrocytes. Showing chondrocytes located in the cartilage of a bone section (5 µm thickness, sample 366 – formalin fixed and paraffin embedded bone tissue).
A. chondrocytes are stained by a nucleic acid binding dye (Diamond Dye) and visualised under a fluorescent microscope (AX70). The green dots illustrated as the nuclei of the chondrocytes.
B. chondrocytes are observed by a microscope (BX50) after staining with Hematoxylin. The top shows the chondrocytes in oval shapes containing nuclei in purple. The below shows the chondrocytes columns. The magnification is 20x and scale bar is 100 µm.

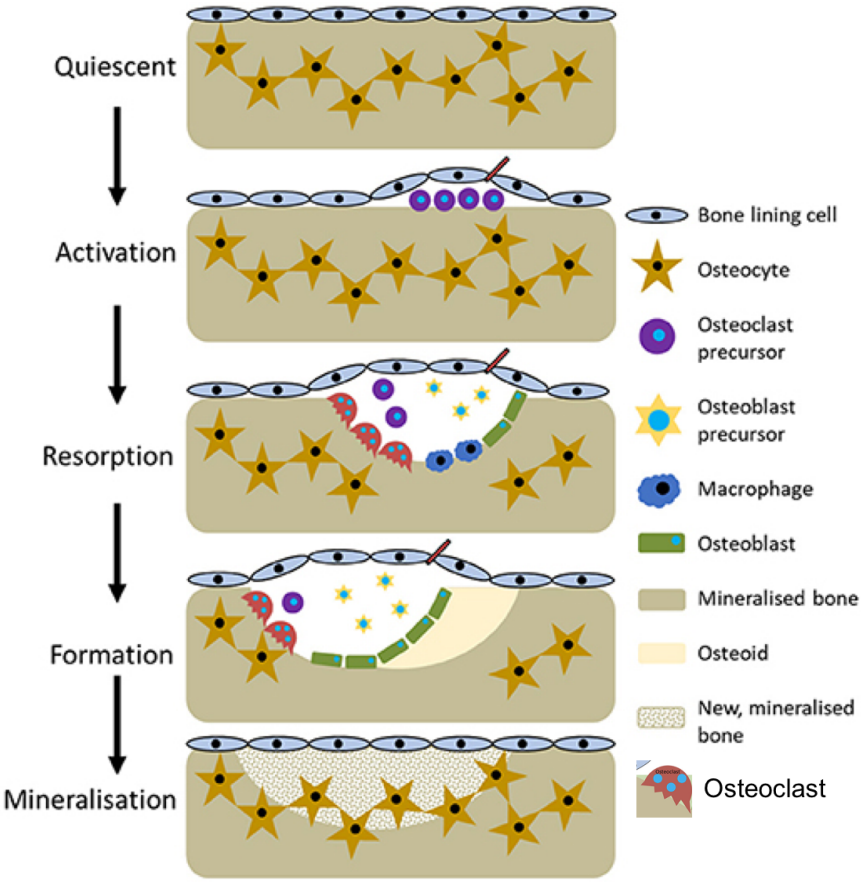


Figure 9: Diagram of the bone remodeling cycle. Showing the five phases of the bone remodeling cycle: quiescent, activation, resorption, formation, and mineralisation.
First phase – quiescent: bone is inactived.
Second phase – activation: some conditions such as mechanical load or low calcium initiate the activation. Osteoclast precursors are recruited to participate in this phase.
Third phase – resorption: osteoclasts degrade the bone matrix while macrophages clear away the debris to make cavities for new bone cells.
Fourth phase – formation: osteoblasts secrete the bone collagenous matrix (known as osteoid) and deposit the minerals.
Final phase – mineralisation: some osteoblasts differentiate into osteocytes trapped within bone mineral matrix, or undergo apoptosis, or become bone lining cells located on bone surface.
The image adapted from [34].

1.3. Workflow of STR profiling from bones

There are typically seven standard steps to recover DNA and type a STR profile from bones [4, 7, 9-11] in a forensic investigation. Firstly, a bone sample is selected based on the quality and the most likely source of DNA, for example femurs, ulna, humerus or other long bones are chosen rather than spongy bones (Fig. 10, step 1) [11, 35]. Then, cleaning and the removal of contaminants from the surfaces of sample are employed (Fig. 10, step 1). The selected and cleaned bone sample is cut into small pieces and ground into a fine powder often in the presence of liquid nitrogen (Fig. 10, step 2) [11]. Next, the amount of bone powder (0.2 – 2 g) from step 2 is decalcified using ethylene diamine tetra acetic acid (EDTA) by washing the bone powder for between 2 and 5 days (Fig. 10, step 3) [6, 12, 13]. After decalcification, DNA is isolated from the bone powder and stored at a low temperature (4 to -80^{0}C) prior to analysis of the isolated DNA (Fig.10, step 4 – 7).

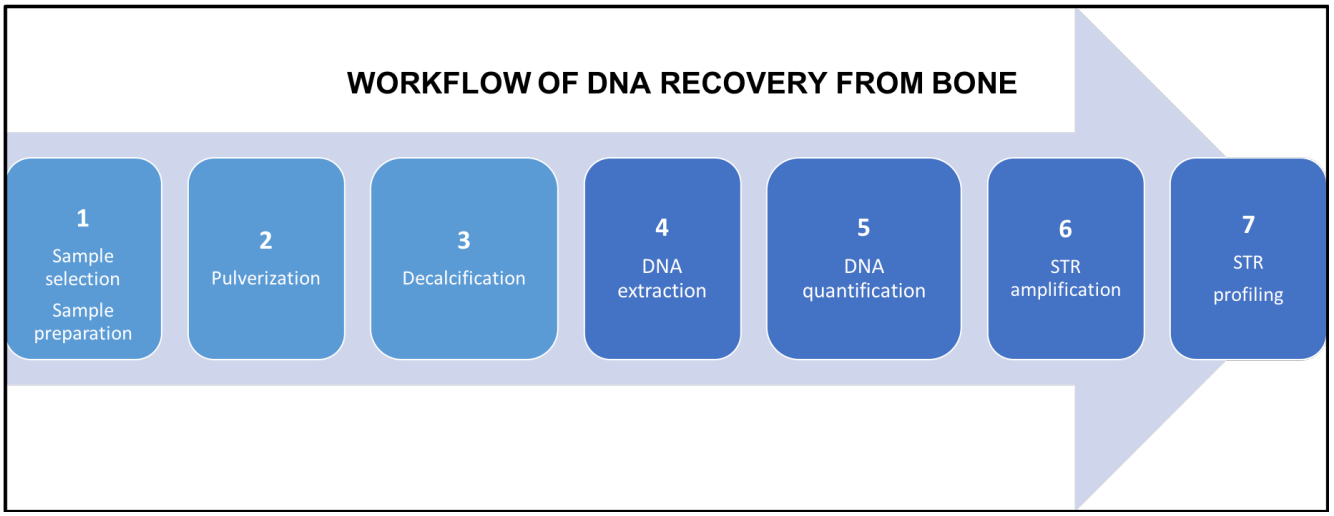


Figure 10: Standard process of STR profiling from human bones. It includes 7 steps from sample selection to STR profiling.

1.3.1. Sample selection and preparation (step 1)

Sample selection is a critical first step in forensic casework. Cortical or compact bone, such as petrous bone [36, 37] and the femur [38-40], have been reported as the optimal source of endogenous DNA but may not be available and therefore other skeletal elements may need to be targeted [38-40]. Osteochondral tissue contains articular cartilage, subchondral cortical and trabecular (cancellous) bone, and bone marrow. Osteochondral defects can cause degenerative changes and osteochondral tissue is regularly removed during medical intervention such as

arthroscopy. These samples have not yet been used in forensic human identification as a source of DNA. Another report showed the amount of DNA within small cancellous or spongy bones is much higher than dense cortical bones due to remnant soft tissue adherent to struts of cancellous bone [41]. Archived osteochondral sections can be a potential template for STR profiling [18] due to the abundance of nucleated cells such as chondrocytes and osteocytes (Fig. 11).

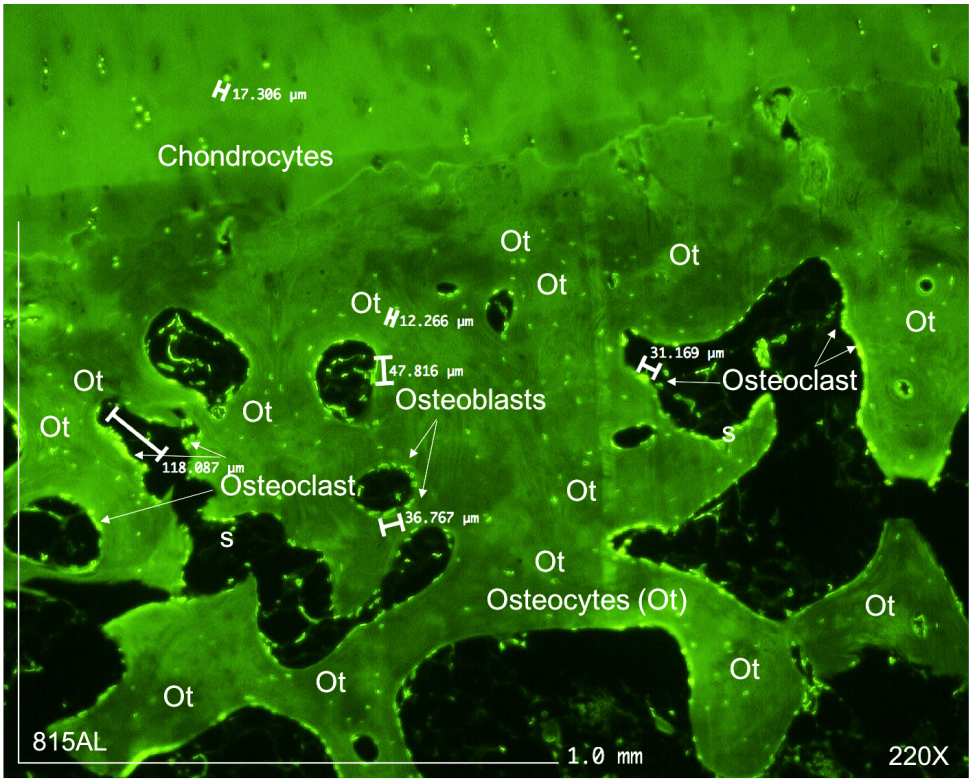


Figure 11: Morphology of an osteochondral section. Showing a variety of of bone cells types including: osteocytes (Ot), osteoblasts, osteoclasts and chondrocytes (sample FFPE – 815AL). The 5 µm section was stained by Diamond™ Nucleic Acid Dye. Magnification was at 220x and scale bar is 1 mm.

Preparation of bone specimens for DNA analyses typically starts by the removal of surface contaminants, such as soft tissues or soil, to minimize the possibility of contamination by an external DNA [38]. A tool such as a saw or a Dremel tool (Dremel, Racine, WI, USA) is then used to cut samples into small pieces of an approximate size (5 – 8 mm^3), and then the section is repeatedly cleaned with a series of washes of the following: 10% (v/v) bleach, then sterile water, then 100% (v/v) ethanol, followed by UV sterilization; UV can alter the DNA sequence of any residual DNA making it unanalysable. This may be followed by a dehydration step where the

sample is placed into an oven at ~36^{0}C or in a fume hood overnight at room temperature, before storage at – 20^{0}C until the next process.

1.3.2. Pulverization (step 2)

The bone cuttings can be frozen in liquid nitrogen and crushed in a SPEX 6750 Freezer/Mill (SPEXSamplePrep, Metuchen, New Jersey) to generate fine powder. The total amount of bone powder (0.2 – 2 g) is collected for subsequent decalcification [35, 38].

1.3.3. Decalcification (step 3)

Bone powder is routinely incubated in a decalcification buffer, with rotation, overnight or up to 5 days depending on the quality of samples and purpose of research [42]. Decalcification is a method to remove minerals, predominantly calcium, from calcified tissue such as bones and teeth [43]. For archived samples, this technique is carried out after the specimen has been formalin-fixed for preservation.

Additional decalcification depends on the nature and size of the specimen: for instance, compact bones contain a dense biological and inorganic matrix requiring a more thorough decalcification process than less dense bones [44]. A variety of decalcifying agents including strong mineral acids, weak organic acids or chelating agents have been used. Strong mineral acids, such as 10% (v/v) hydrochloric or nitric acid, are a rapid means for decalcification, but can cause a reduction in nuclear staining due to hydrolytic actions which in turn reduces the affinity of cellular structures for basic dyes [45]. Weak organic acids used include 10% (v/v) formic acid combined with formalin. EDTA, and other chelating agents, binds with ionised calcium located on the outer layer of the apatite crystal forming EDTA-Ca^{2+} complexes (Fig. 12) [46]. EDTA is the most commonly used chemical for decalcification when isolating DNA from bones and teeth.

$$Ca_{10}\,(PO_4)_6\,(OH)_2 \;+\; 8H^+ \;\leftrightarrow\; 10\,Ca^{2+} + \;6HPO_4^{2-} + \;2H_2\,O$$

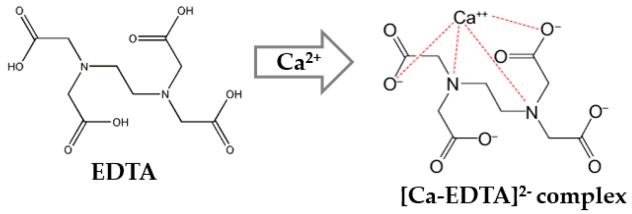


Figure 12: Chemical process for removal of hydroxyapatite (HA). HA is submitted to an ionisation in acids (above formation) or a chelation (below formation). This image is adapted from [1].

Lastly, it is crucial to determine the endpoint of decalcification in order to avoid damaging and compromising the specimen. Over-decalcification, particularly methods using strong acid, may lead to spoiling the cell nuclei [47]. Incomplete decalcification may adversely affect the process of sectioning a bone sample where a mechanical blade is used to cut through the bone material. One way to check on the process of decalcification is to use dual-energy X-ray absorptiometry (DXA); this examines the volume of bone mineral content (BMC) and bone mineral density (BMD) [47]. The X-rays can detect and record the presence of residual calcium deposits (Fig. 13).

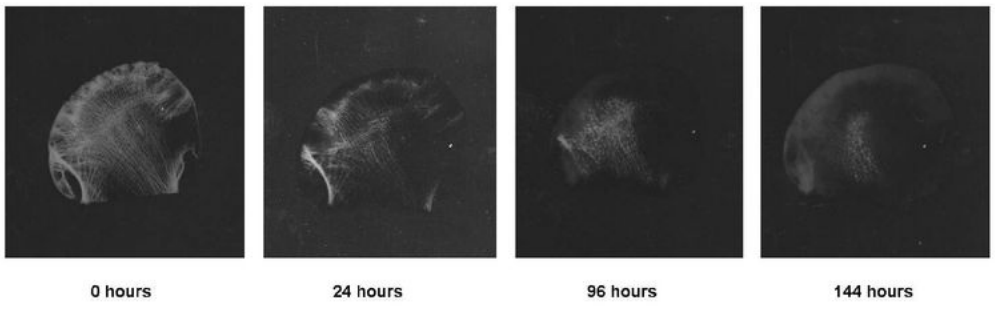


Figure 13: The process of decalcification. An X-ray series of a femoral head with formic acid/citrate decalcifier. The radiographs were produced using a Hewlett-Packard Faxitron® and allow the process to be accurately followed and the endpoint to be properly identified. Images adapted from Leica Biosystems.

1.3.4. DNA extraction (step 4)

This part of the thesis provides an overview of DNA extraction processes from human bone samples. A summary of the possible sources of contamination will be discussed. The type of inhibitors which have the potential to adversely affect success of DNA collection are discussed.

According to a study in 1960, the most abundant and long-lived cellular component of mammalian bones are osteocytes, which make up 95% of all bone cells, and live up to 50 years [48]. Osteocytes are embedded in the inner mineralized matrix and here the nucleic acids are protected from environmental exposures such as: bacterial degradation, intense heat, humid or contaminated conditions. This same matrix is also the main barrier in releasing the cells for DNA analysis and requires time-consuming processes for decalcification. The process of decalcification has therefore become a routine step in the isolation of DNA from bone.

The first reports on DNA analyses date to the recording of a hereditary material "nuclein" – later named deoxyribonucleic acid, by the Swiss physician Friedrich Miescher in 1869. Advances in the last century include the demonstration that DNA is the hereditary molecule, not protein as previously proposed [49]. The year 1953 marked the report on the double-helix structure of DNA by Watson, Crick and Wilkins [50]. Since then, methods for DNA isolation from the biological matrices have been developed with increasing reliability, speed, ease, and cost. Figure 14 illustrates the three DNA extraction methods have been performed in forensic laboratories. An overview of the methods of DNA isolation along with advantages and limitations is shown in Table 2.

Group 1: Organic extraction

The use of organic solvents phenol and chloroform was introduced in 1980s [51] and successfully recovered DNA from forensic materials such as bone specimens [5, 11, 38]. In principle, the proteins and non-nucleic acid cellular materials (such as lipids) is digested by lysis buffer containing sodium dodecyl sulphate (SDS), EDTA and proteinase K (Fig.14). A mixture of phenol:chloroform:isoamyl alcohol (25:24:1) is subsequently added to separate lipids and cellular debris into the organic phase. The polar (negatively charged) DNA molecules are retained in the aqueous phase. The purified DNA is obtained after a centrifugation and then transfered to a sterile tube for additional purification or concentration. By this way, the organic extraction recovers DNA for downstream DNA analysis such as amplification and DNA profiling. This method extraction

is reliable and efficient, but remains some drawbacks including a use of hazardous chemicals, time consuming process requires multiple tube transfers, and an increase of contamination risks [52].

A report detailed the use of a phenol-chlorophorm-isoamylalcohol organic extraction protocol to process more than 500 analyses, starting with 2 grams of bone tissue per sample. The protocol involved the following steps: (1) a forensic pathologist analysed the remains, (2) the remains that still connected with soft tissue were separated, (3) A tracking number was assigned for each specimen, (4) soft tissue removal was performed from bone surface, (5) DNA was extracted by organic extraction method. DNA was subsequently concentrated by Microcon 100 microconcentrators (Amicon, Inc., Beverly, MA, USA) and quantified by the QuantiBlot (Roche Molecular Systems, Alameda; Applied Biosystems, Foster City, CA, USA). The templates were amplified by the PowerPlex®16 multiplex STR system (Promega Corp., Madison, WI, USA) to generate STR profiles for bone samples. It was concluded that 50% of the bone samples generated more than 13 STR loci [38].

Another study that used phenol-chloroform to isolate DNA incorporated an initial staining of bone sections with hematoxylin and eosin to detect the presence of cell nuclei [5]. The samples were formalin fixed and paraffin embedded sections (5 μm thickness) from postmortem compact bones. Seven human remains found on the ground, five cadavers 12 to 16 hours postmortem, and eight exhumed remains were tested in the study. The morphology of bone cells (osteocytes) containing nuclei was visualised under a light microscope. As a result, five boiled samples showed no cells or material inside Havers channels within compact bone fragments. This indicated that boiling treatment at high temperature degraded osteocytes within lacunae. In contrast, all exhumed human remains presented the presence of red cells inside Haversian channels and osteocytes containing nuclei embedded in mineralised matrix, yielded higher DNA quantities than boiled samples. Morphologic analysis provided an additional support for forensic analysts in prediction of the chance to recover DNA from the samples containing preserved cells.

In an effort to increase the DNA yields and obtain complete STR profiles from human bones using the organic extraction method, a total demineralization procedure was performed for 14 challenging human bones (from 5 to 100 years post mortem) [53]. The five samples were powdered in a cryogenic impact grinder (CertiPrep 6750 Freezer Mill, Spex/Mill, Spex, Metuchen, NJ), while one sample was powdered in a sterilized Waring MC2 blender cup

(Warring:Torrington, CT) and 1.0/1.2-L laboratory blender mortar, and four samples were pulverized in both methods. In traditional method, bone powder (1 – 2 g) was incubated in 3 mL of a lysis buffer (10 mM Tris, pH 8; 100 mM NaCl; 50 mM EDTA, pH 8.0; 0.5% (v/v) SDS) and 100 µL of 20 mg/mL Proteinase K at 56^0C overnight. DNA was then extracted by an organic extraction and concentrated by a Centricon 100 centrifugal filter unit (Merck Millipore). In the total demineralization method, bone powder (0.6 – 1.21 g) was incubated in 9 – 18 mL of a buffer (0.5 M EDTA, 1% (w/v) lauryl-sarcosinate) and 200 µL of 20 mg/mL Proteinase K at 56^0C overnight. DNA was subsequently isolated by an organic extraction and concentrated by Centrifugal Filter Units (30 kDa, Amicon Ultra-15, Centricon+20, or Centriplus from Millipore) and Centricon 30 centrifugal filter unit (Millipore). Extracted DNA from both methods was quantified using a real-time PCR quantification and ultimately profiled with the PowerPlex 16 system (Promega Corporation, Madison, WI) or the Yfiler system (Applied Biosystems, Foster City, CA). Consequently, data showed DNA yields recovered by the total demineralization were higher (approximately 4.6 times) than the traditional method. Increasing concentration of EDTA and volume of extraction buffer promoted complete dissolution of the bone sample. The higher endogenous DNA quantity was subsequently approached. The disposal of undissolved bone materials was also minimised, resulting in extraction efficiency and optimal DNA recovery.

Group 2: Chelex®-based extraction

Chelating ion exchange resins was introduced to forensic laboratories in the early 1990s [54] and used for DNA isolation from bones and teeth [35]. The most common resin was Chelex® 100 from Bio-Rad Laboratories (Herculescules, CA, USA). The resin comprises a styrene divinylbenzene copolymer containing paired iminodiacetate ions, is added to a sample. Heat is then used to lyse the cells and release DNA. A protection of DNA molecules is provided due to the binding between the chelating ion exchange resin and 2+ charged metal ions such as magnesium (cofactor of nuclease). Chelex® resin and cellular debris are removed via centrifugation. DNA is ultimately transfered to a sterile tube for subsequent analysis (Fig. 14). The chelation-based extraction offers a rapid method, requires minimal sample transfer, and decreases contamination. However, there is no mechanism for removing inhibitions such as hematin in whole blood or humic acid in soil-contaminated samples [55].

A modified Chelex® 100 (Bio-Rad) extraction was performed to isolate DNA from degraded archaeological human bones and teeth [35]. Due to some PCR inhibitors not being removed effectively in a standard chelation-based extraction method, phenol:chloroform purification and isopropanol precipitation steps were included in this study in order to remove effeciently inhibitors from bone extracts and recover the best DNA yield. DNA was isolated from eight archaeological human bone samples (femur, humerous, and ulna) and two archaeological human teeth. Bone or tooth powder (100 mg) was either DNA extracted by a standard Chelex® method (without phenol:chloroform purification and isopropanol precipitation steps) or a modified Chelex method. DNA was then quantified by a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and a 7500 Real-Time PCR system (Applied Biosystems), and amplified using the AmpFlSTR® Identifiler® PCR Amplification Kit (Applied Biosystems, Warrington, UK). Additionally, the morphology of ultrastructure of the archaeological human bones was carried out to show the preserved bone cells at a microscopic level. This provided critical information for predicting the integrity of DNA molecules. The study concluded that amplicons of up to 250 bp in length were successfully amplified for modified protocol, whereas standard method resulted in unsuccessful amplification due to inadequate amounts of DNA concentration and the presence of PCR inhibitors. Transmission electron microscopy showed well-preserved bone materials via the microphotographs of intact osteons with well-organised bone matrix (collagen). Intact osteocytes located in lacuna within bone mineralised matrix, were also demonstrated, providing a potential source of preserved DNA.

Group 3: Solid phase extraction

A technique that can be easily automated is solid phase extraction method (Fig. 14). In contrast to Chelex®, the solid phase extraction technique relies on the affinity with the DNA molecules under highly chaotropic salt conditions. An example of such is guanidine hydrochloride. DNA is selectively bound to a solid phase, such as silica. The high salt buffer disrupts hydrophobic interactions between the silica bound DNA and proteins in the surrounding buffer leads to cellular proteins disruption and nuclease deactivation. These are separated by centrifugation or magnets. The outcome is an inhibitor-free DNA template which can be eluted from the solid phase (silica) by the immersion of pH – adjusted buffers [56, 57]. Contamination during the extraction process and PCR inhibitors is also minimised [56, 57]. The use of silica-binding membrane, such as silica

resin (QIAamp® spin columns from Qiagen) and paramagnetic resin beads (the DNA IQ kit from the Promega Corporation, or the PrepFiler system by Thermo Fisher Scientific), are two of the more recent advances [3, 9, 10, 56-59].

In 1998, silica-based spin columns (QIAquick, Qiagen) were carried out for isolating DNA from ancient bones (15 – 5000 years old) [9]. Sandpaper was used to polish the bone surface. Bone powder (0.5 – 5 g) was generated by drilling the surface, then incubated in 8 mL of a lysis buffer (0.5 M EDTA pH 8.0, 0.5% sodium dodecyl sulfate, and 100 µg/mL proteinase K) for 2 days (first day of incubation was at 55^0C and second day was at 37^0C). DNA was isolated using QIAquick silica columns and concentrated by Centricon 30 microconcentrators. Amplification was performed for human chromosome 17 (locus D17Z1). Amplicons with the size of 211 base pairs in length were separated by electrophoresis on a 2% NuSeive/2% agarose gel, which stained with ethidium bromide and photographed under UV illumination. Based on the observation of PCR products of DNA isolated from ancient bones on ethidium bromide–stained gel, the results demonstrated that QIAquick column-based method showed the strong amplification with free of pigmentation of PCR inhibitors. QIAquick column-based method therefore offered an ideal means for the recovery of PCR-amplifiable DNA from ancient bones.

To achieve adequate DNA yields for profiling skeletal samples, optimisations have been applied for extraction buffer. For example, adding reducing agents (50 mM dithiothreitol) and detergents (1% (v/v)Triton X-100) to the buffer to destroy proteins and cell membrane. One study in 2007 indicated that the presence of high concentrations of non-chaotropic salt, guanidinium thiocyanate (GuSCN), in post-decalcified samples improved DNA yields using the silica beads isolation protocol for ancient bones and teeth [10]. A protocol of DNA extraction from ancient bone and teeth was also introduced to isolate DNA from bone within two working days. Several advantages of the improved silica-based method applied in this study were highlighted. Bone powder (500 mg) initially incubated in 10 mL of extraction buffer (0.45 M EDTA and 0.25 mg proteinase K, pH 8.0) overnight (16 – 24 hours) in the dark at room temperature with slow rotation. Next day, an additional incubation (1 – 3 hours at 56^0C) was carried out to improve the digestion of bone powder and DNA release. The supernatant was collected by a centrifugation (5000xg for 2 mins) and then incubated in 10 mL of binding buffer (5 M GuSCN, 25 mM NaCl and 50 mM Tris, pH 7.0) with 100 µL of silica suspension at room temperature in the dark for 3 hours. Finally, extracted DNA was washed and eluted in 50 µL of elution buffer. In brief, the promissing process

of DNA extraction from ancient skeletal samples was provided with an optimised silica-binding protocol, while DNA release was improved and degradation of fragile ancient DNA was minimised.

In 2012, it was reported that 131 ng DNA/g of bone powder was collected from femurs using a Biorobot EZ1 device (Qiagen). Significantly, the demineralisation was a critical step prior to DNA isolation. The powder (0.5 g) was pulverized from 84 bones excavated from the mass graves in Slovenia dating from World War II [7], then decalcified for 3 days and subsequently extracted and purified using a Biorobot EZ1 device. The final volume of eluants was 50 µL. Extracted DNA was quantified using the Quantifier Human DNA Quantification kit (Applied Biosystems) and amplified with the three amplification kits (PowerPlex ESX 17 system (Promega), AmpF*l*STR NGM PCR Amplification Kit (Thermo) and Investigator ESSplex kit (Qiagen)). Full STR profiles were obtained from femurs from 86% of the bone samples using ESX 17, 75% using NGM and 82% using ESSplex kit. There were 16 partial profiles, which failed amplification at the longest loci (D21S11, D2S1338, FGA and D8S1179).

In the same year 2012, the use of a BioRobot EZ1 (Qiagen) and the EZ1 DNA Investigator kit, a simple and small volume (1.5 mL) method was introduced to yield high DNA concentration from challenging human bone samples and minimise the potential DNA contamination during the process [2]. The process began with 4 femurs and one hip section. A freezer/ mill grinder (SPEX 6750 Freezer/Mill) with liquid nitrogen was used to pulverize the bone after cleaning steps. Powdered bone samples (200 mg) were incubated in one of the digestion buffers including NLCL bone digest (NBD) buffer, or Buffer ATL (QIAGEN, Germantown, MD), or Buffer G2 (QIAGEN) with 1 µg/µL Proteinase K, 0.5 M EDTA, and 1.0 M DTT at 56^0C for 24 hours. DNA was isolated by a BioRobot EZ1 with an addition of 3M of sodium acetate (NaOAc), then quantified by QuantifilerTM Human DNA Quantification kit (Applied Biosystems, Emeryville, CA). The PowerPlex 16 System PCR amplification kit (Promega, Madison, WI) was used for amplification. The findings recorded that adding NaOAc to these buffers dramatically increased the recovery of DNA because sodium acetate adjusted the pH of solution when it exceeded pH 7.5. This also demonstrated that the binding of nucleic acids and silica membrane depends on the presence of chaotropic salts and a suitable pH. The combination of buffer ATL, Proteinase K, EDTA, DTT and NaOAc provided the best yields (1.313 ng average DNA) for the PowerPlex 16-loci profiles.

In 2016, a comparison of two silica-based methods using silica beads or silica membrane for DNA isolation from 8 human bone specimens was reported [57]. The samples included two bones of more than 500 years in a grave, 3 samples aged 50 – 90 years, and 3 fresh bones. It was concluded that the quality of input materials should be examined prior to choosing the most appropriate extraction method. The findings indicated the silica membrane (MiniElute® PCR Purification Kit, Qiagen) yielded more DNA than the silica beads method (the EZ1 ®Investigator Kit, Qiagen) [57]. Briefly, for silica membrane-based technique, bone powder (250 mg) was decalcified with 3.6 mL of 0.5M EDTA for 18 hours at room temperature. Then, cell lysis was carried out by adding 50 µL of 600 mAU/ml Proteinase K and an incubation at 56^0C for 2 hours. The cell lysate was subsequently incubated in 50 µL of 1% (v/v) SDS (Sigma Aldrich, Steinheim, Germany) at 65^0C for 5 minutes. After lysis, a centrifugation at 3300 RCF was performed to obtain the supernatant, which then transfered to a 50 ml Falcon tube containing 16 mL of PB buffer (Qiagen, Hilden, Germany) with 100 µL of sodium acetate buffer (pH 5.2; Sigma-Aldrich, Steinheim, Germany). From this step, DNA was recovered and purified using a silica membrane from the MinElute PCR Purification Kit (Qiagen, Hilden, Germany). For silica bead technology, bone powder (200 mg) was decalcified with 700 µL of 0.5M EDTA and incubated for up to 2 days at room temperature. Lysis was begun by adding 20 µL of 600 mAU/ml Proteinase K (Qiagen, Hilden, Germany) and an incubation at 56^0C for 4 hours. The supernatant was collected after a centrifugation at 6000 rpm for 4 mins, and subsequently DNA isolated with the cartridges from the EZ1 Investigator kit. Extracted DNA was quantified using the Investigator Quantiplex Kit (Qiagen, Hilden, Germany) and amplified using the AmpFLSTR NGM Select™ Kit (Applied Biosystems, Foster City, USA). As a result, the silica membrane extraction yielded higher DNA (3 – 12 times) than the silica bead method for all 8 bone samples. In terms of co-extraction of inhibitors, there was no presence of calcium recorded for both methods. The increased humic acid concentration was confirmed with the silica membrane method, whereas the silica bead method resulted no co-extraction of humic acid. One explanation is that big molecules such as humic acid and collagen cannot pass through the membrane and then eluted with DNA molecules.

One study in 2020 demonstrated that the optimised Qiagen EZ1 protocol is the best recovery of DNA from 50 – year – old remains with free of PCR inhibitors comparing to other two standard Qiagen EZ1 procedures [60]. Initially, eleven bones and teeth were cleaned with distilled water, 80% (v/v) ethanol and 5% (v/v) hypochlorite, then pulverized in liquid nitrogen into

powder. The three extraction procedures involved two standard Qiagen protocols (EZ1 2014, and EZ1 supplement 2016) and one EZ1 modified protocol were applied in this study. In the modified protocol, bone powder (150 – 300 mg) were decalcified in 0.5 M EDTA for 5 – 7 days at room temperature prior to access pre-lysis treatment. This modified method performed the longest decalcification aiming to dissolve effeciently bone powder and improve DNA release for subsequent analysis. In the Qiagen EZ1 2014 procedure, bone powder was decalcified with 0.5M EDTA for up to 2 days and then incubated in 20 µL Proteinase K at 56^0C for 3 hours. In the Qiagen EZ1 supplement 2016 protocol, bone powder was incubated in a cocktail of 225 µL of Lysis buffer G2, 250 µL of 0.5M EDTA pH 8.0, and 25 µL of Proteinase K at at 56^0C for 24 hours. The supernatant was obtained and transfered to EZ1 automated purification. Extracted DNA was eluted in 50 µL of elution buffer. DNA quantification was carried out using the Investigator Quantiplex® Pro RGQ kit (Qiagen, Hilden, Germany). All samples were amplified with the AmpF*l*STR® NGM SElect PCR Amplification Kit (Applied Biosystems) and Investigator® IDplex Plus kit (Qiagen). Despite the demineralization is a time consuming step, but it efficiently removes calcium, inactives Dnase and boosts the DNA extraction. The results showed that the modified protocol with the longest decalcification (5 – 7 days) achieved the best DNA recovery, which was 64% higher than EZ1 2014 protocol and 39% higher than EZ1 2016 supplement protocol. STR profiles obtained from the modified process also confirmed better quality than others. The profiles showed well-balanced peaks, low noise and no PCR artefacts.

Another study reported in 2020 that using whole bone chips (1 × 50 mg, 3 × 50 mg, and 1 × 150 mg chip(s)) overcame the drawbacks of current powder-based methods such as degraded or low template DNA, exogenous contamination, time-consuming and handling requirements [3]. The findings showed that samples directly extracted from bone chips yielded less DNA than traditional extraction methods. In this study, bone chips had calcium removed by a total decalcification for 20 hours. The supernatant was collected by a centrifugation at 2500 xg for 23 mins. DNA isolation was performed using the PrepFiler® BTA™ Forensic DNA Extraction Kit. Final volume of all eluants was 50 µL. DNA concentration was real-time quantified using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific) and amplified by the GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific). Overall, the higher DNA yield was obtained from the powdered samples (5.0 ± 1.6 ng/mg bone) than bone chips (3.0 ± 1.1 ng/mg bone) ($p>0.05$). Additionally, according to degradation index, DNA isolated from bone chips was

more degraded than powdered samples ($p<0.05$). These results can be explained that DNA degradation may came from the bone surfaces during sanding and cutting process while cells were well protected at the core of bones and then collected after pulverization. Another hypothesis was given that soil containing humic acid on the bone chips was not efficiently removed, resulting in the failed amplification.

In general, the findings showed silica-based technology has provided better recovery of DNA and more efficient removal of inhibitors than organic (phenol/chloroform) method [58, 61, 62]. The method offers not only successful amplification of inhibitor-free genomic DNA, biocompatibility, and rapid DNA recovery, but also is widely implemented in many laboratories [3, 9, 10, 56-59]. It is critical to notice that the pre-lysis treatment or total decalcification has been an essential improvement for yielding the best DNA recovery from human bone samples [6, 53].

Table 2 lists procedure for DNA isolation from human bones; these fall into three groups of extraction methods.

Table 2: Comparison of three DNA isolation approaches.

DNA isolation methods	Advantages	Limitation
Organic method (Phenol/Chloroform)	- Simple and rapid procedure [11, 38]. - Low cost [11]	- Intensive labour [5]. - Time-consuming with multiple centrifugations [5] - Loss of material [38] - Unable to automate - Use of hazardous organic chemicals [38]
Chelex®	- Rapid and simple extraction [35]. - No organic solvents required. - Avoids multiple tube transfers [35].	- Heating causes formation of single-stranded DNA, which is less stable in storage [63]
Silica beads Silica spin column Automation platform	- DNA recoveries for low-yield and degraded samples [57] - Cost-effective [64] - Simple, reliable and rapid procedure [57] - Minimal impurities [59] - Applicable to automation [64]	- More costly than organic method and Chelex® [64]

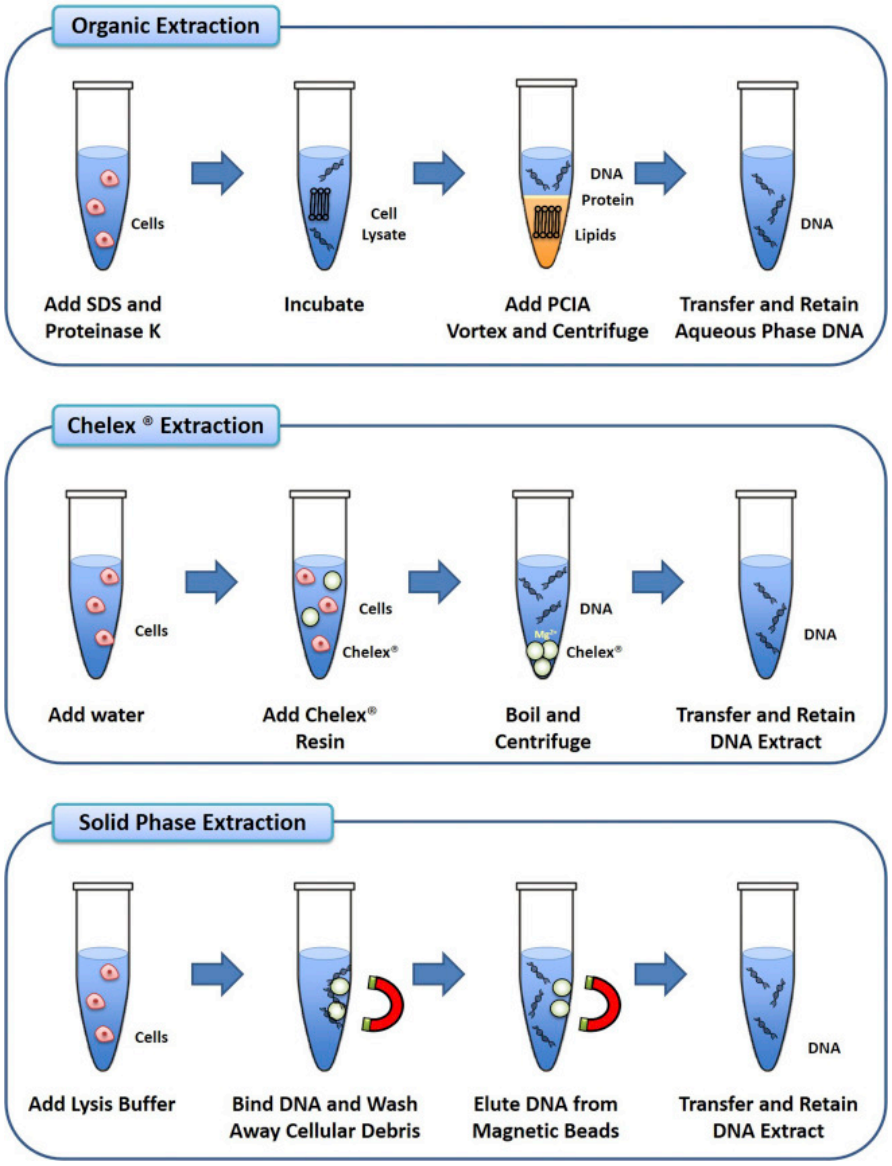


Figure 14: Three major methods of DNA extraction. The image abstracted from [55]. Organic extraction uses phenol:chloroform:isoamyl alcohol (PCIA) to separate DNA into the water phase while proteins and lipids are partitioned to the organic phase. The high-purity DNA is recovered.
Chelex® resin is added to a sample. A cell lysate is performed by heating. DNA is released and transfered to a new sterial tube. The method yields lower purity DNA and contains inhibitors.
Solid phase extraction uses silica-based paramagnetic resins to bind DNA molecules. A use of magnet is applied to isolate DNA from cellular debris. DNA is recovered and eluted through washes with low ionic strength buffers. Contamination and inhibitors are minimised. Purified DNA is ultimately obtained and stored in a new sterile tube.

1.3.5. DNA quantification (step 5)

The amount of DNA isolated can vary greatly due cell type, amount of cellular material present, and DNA loss due to degradation [65]. A sensitive quantitative real-time polymerase chain reaction (qPCR) is used to determine DNA yields, with this information subsequently used determined how much DNA should be added to achieve the optimal input amount of DNA for the PCR reaction (such as 0.5 or 1 ng which is pre-determined through validation studies). If too much DNA is added to the PCR it results in stochastic effects, such as increased stutter, split peaks, and the creation of off-scale data. In contrast, if the DNA template added to PCR is low or poor quality, stochastic effects such as peak imbalance, allelic or locus dropout may occur resulting in a partial STR profile [66].

There are four providers of five kits Investigator® Quantiplex® Pro Kit (QIAGEN, Hilden, Germany), Quantifiler® HP and Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, San Francisco, CA, USA), PowerQuant® System (Promega Corporation, Madison, WI, USA) and InnoQuant® HY (InnoGenomics Technologies, New Orleans, LA, USA) used for forensic application. Although Quantifiler® HP is used for forensic application, this is not captured in the discussion. The targets and internal positive control used are provided in Table 3. All kits generate results, tabulating: DNA yield, and indices of DNA degradation and amplification inhibition. Technology termed TaqMan® probes are common to this kits and are based on a fluorescent reporter at 5’ end of a primer and a non-fluorescent quencher at 3' end [67-70]. During the extension phase, *Taq* DNA polymerase enzyme cleaves the probes, resulting in a separation of the reporter and the quencher, and a resulting signal based on the fluorescence detected. DNA concentration is measured based on the amount of accumulated fluorescence signal of the sample and a calibration curve of standards [67-70].

The Investigator® Quantiplex® Pro Kit includes the longest human target (353 bp) [71], whereas others provide human targets which are between 207 and 294 bp [67-70]. These differences result in varying degradation ratios which are generated by a division of the small and the large target DNA concentration. The size of human-specific small targets and Y-chromosome targets are similar for all kits (Table 3). IPC is a synthetic DNA sequence that is amplified simultaneously with the human and male targets and acts an indicator of PCR inhibition [67]. Each

manufacter strongly recommends using the human DNA standards dilution series to generate standard curves for the reaction [67].

Table 3: Parameters and targets of the four qPCR kits.

Kits	Human autosomal targets		Male target	IPC	DI, degradation indices	Inhibition indices	Sensitivity	Ref.
	Large target	Small target						
Investigator® Quantiplex® Pro Kit	353 bp	91 bp	81 bp	434 bp	>10	>1	9.8 pg	[70]
Quantifiler® Trio DNA Quantification Kit	214 bp	80 bp	75 bp	130 bp	>10	>2	32 pg	[67]
PowerQuant® System	294 bp	84 bp	81 and 136 bp	435 bp	>	>0.3	2.4 pg	[69]
InnoQuant® HY	207 bp	80 bp	79 and 79 bp	172 bp	>20	>2	3 pg	[68]

Four qPCR kits listed in Table 3 were used for comparison of the quality and quantity of DNA isolated from 15 degraded samples [72]. Included in this study were 5 human bones, 5 decomposed tissues, and 5 formalin fixed and embalmed tissues. The outcome of the study was that the Investigator® Quantiplex® Pro provided the most accurate quantification results for the highly inhibited samples comparing to other three qPCR kits.

Another study extracted DNA from 11 skeletal samples (7 bones and 4 teeth buried for 1 – 50 years after death) [73]. DNA yields were quantified by the Investigator® Quantiplex® Pro Kit. The values of these samples were recorded as below the thresholds of degradation and inhibition, indicating that no degradation and external inhibitors had occurred.

Based on the advances of the Investigator® Quantiplex® Pro, this kit was employed to quantify the DNA template and to detect the degradation and inhibition within bones in this study.

1.3.6. STR amplification (step 6)

The optimal template DNA input is 500 picograms (pg) in current STR profiling kits [66, 74]. This correlates to only 80 diploid cells (~ 6 pg DNA/cell). To select the appropriate number of PCR cycles, some kits are optimised and validated to offer specific numbers ensuring well-balanced and high quality results are obtained. For example, there are two options of PCR cycles in a maximum input volume of 10 μL in the Identifiler® Plus Kit: 28 cycles for 1.0 ng DNA input, and 29 cycles for < 0.5 ng DNA input [66].

The concept of direct PCR was originally used in molecular microbiology in 1989 [75]. It was then introduced to forensic science for investigation of evidential or reference samples such as buccal cells, bloodstains, semen stains or cellular materials on fabrics [76-80]. Direct PCR means adding samples directly to the amplification reaction without prior extraction or purification. The aim is to optimise the collection of DNA template and avoid DNA loss during DNA extraction process. This is especially the case when starting with low template DNA (e.g. less than 100 pg) as any loss of DNA template may lead to allelic loss [80]. Furthermore, direct PCR offers the benefits of typing DNA faster and that in turn leads to speeding up the investigation process, and also reduces overall cost of the procedure. However, there are some concerns related to direct PCR such as the lack of any removal of inhibitors of the PCR process and no quantification step [81]. Due to advances in the buffer, to overcome inhibitors, and processivity of the polymerase, tolerance to PCR inhibitors has improved and the success rate of DNA profiling has increased [82-84]. The addition of internal PCR controls (IPC) in multiplexes also helps to indicate the presence of inhibitors [83]. The buffer is engineered to help lyse cells and release DNA from the sample, then proteins, lipids and other cellular debris will be damaged at the initial step of heating to 95^0C. Meanwhile, DNA polymerase activity is inhibited at ambient temperatures and activated after the initial denaturation steps. This therefore enables inhibitor resistance for increased robustness of the PCR workflow.

An application of semi-direct PCR was reported that used a supernatant in which 100 mg of bone powder was suspended [85]. Human bones were powdered using a freezer mill and then put in phosphate-buffered saline (PBS) buffer, followed by heating to 98^0C for three minutes. A total of 105 fresh human bones and 10 bones that were part of a case were used in this study, from which 94.3% generated partial or full profiles. Spongy bone significantly reported higher peaks

than compact bone ($p < 0.05$). A limitation of this study is DNA templates from the supernatant were not quantified. The lack of a DNA quantification step using qPCR meant that the amount of DNA template was not known, there was no information on the DNA integrity via degradation indices, and no inhibition indices were recorded. Despite these disadvantages, the findings did provide a rapid protocol for STR profiling from human bones.

1.3.7. STR profiling and data analysis (step 7)

An STR profile is generated by a multiplex PCR amplification of hypervariable regions of DNA [86, 87]. These regions of repetitive DNA are highly polymorphic due to the variations of sequence and numbers of repeated units, generating very distinctive profiles for human identification. The allele designation is defined based on the number of repeats present. A locus is a specific location on the chromosome and each length variant is an allele. An example is TPOX (thyroid peroxidase gene) found on chromosome 2 which has the repeat motif AATG. If this is found on one chromatid to have 6 repeats, then the allele is designed '6'.

One of the two primer sets that target each locus is fluorescently labelled. The AmpFlSTR® Identifiler® Plus PCR Amplification Kit (supplied by ThermoFisher Scientific) uses five dyes, with four fluorescent dyes (6-FAM™ - blue, VIC® - green, NED™ - yellow, and PET® - red) targeting 16 loci (15 tetranucleotide repeat loci and the Amelogenin gender-determining marker) simultaneously in single PCR tube (Fig. 15). The fifth dye, LIZ® dye, is used to label an internal lane size standard (GeneScan™ 500 LIZ®). It contains 16 single-stranded LIZ® labeled fragments aiding in the sizing of PCR amplicons. A standard employed for accurate genotyping is known as allelic ladder containing all the alleles reported for the 15 autosomal loci.

STR amplicons are generated by the PCR and then separated by capillary electrophoresis (CE). During electrophoresis, each of fluorescent dyes emits different wavelengths, which are detected by a charge-coupled device (CCD) camera using a laser near the anode. Smaller DNA fragments migrate more quickly than larger amplicons through a polymer matrix from the cathode to the anode of the capillary. The POP-4® Polymer is a common matrix for separation [66, 74]. It is specifically designed for forensic human identification (HID) applications.

An allele is recorded if a peak runs within the same bin as an allele within the allelic ladder. An internal size standard is included in each run to determine the length of PCR products. The size

standard is labeled with a different fluorescent dye to the STR loci and is designed for sizing DNA fragments in a wide range of nucleotides (20 – 600 nucleotides) [66].

The data are analysed using specific software with defined analytical thresholds. Designating true alleles from the baseline requires a threshold and 50 RFU is chosen as a standard threshold in 60% of forensic laboratories [88]. This though should be based on between 3 and 10 standard deviations above the baseline. At each locus, there should either be a single homozygous peak, showing one allele (two alleles running at the same position), or two peaks showing two alleles and thus indicating a heterozygote.

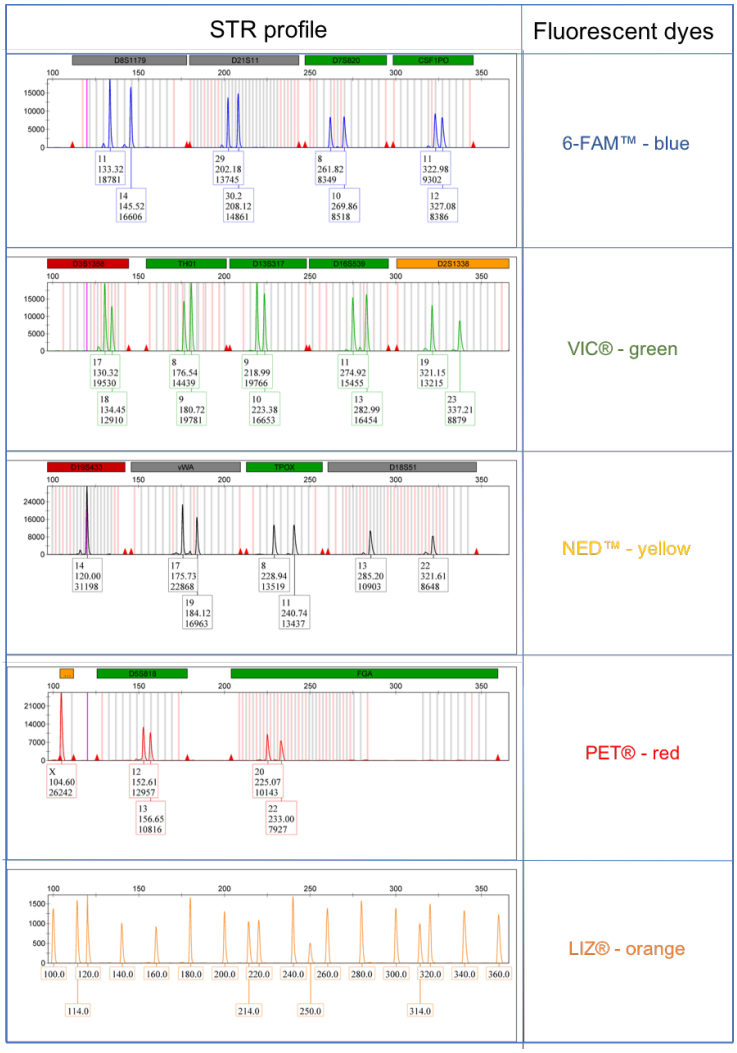


Figure 15: A STR profile of control sample. It was amplified by the AmpFlSTR® Identifiler® Plus PCR Amplification Kit. Five fluorescent dyes labelled for primers are shown.

1.4. Detection of bone cells

A dye that binds to biological material can act a rapid means to access the presence and location of cellular components such as proteins or DNA [89-94]. Detecting nucleated bone cells would be advantageous as it would allow a rapid assessments as to their number and location. A range of nuclei acid staining dyes are available depend on the imaging method [95], morphology, differentiation or RNA transcript profiling [96]. DAPI (4',6-diamidino-2-phenylindole) or methylene blue is commonly used for detecting nuclear fragmentation. Hematoxylin and eosin (H&E) staining enables an observation of nuclei and cytoplasm [95].

Four nucleic binding dyes (hematoxylin, methyl green, toluidine blue O and azure B) were used to quantify the genomic ß-actin DNA by the Taqman assay [97]. Eight frozen and eight archival formalin-fixed, paraffin-embedded tissues (FFPE) (breast, lung, colon and ovarian carcinoma) were part of the study. Manual dissection and laser microdissection were implemented in parallel to collect the cell populations from 5-µm sections for subsequent quantitative analysis. Hematoxylin staining provided the best discrimination between nuclei and cytoplasm whereas the other three dyes resulted in variable cytoplasmic background staining. An issues with H&E staining however is that it can lead to DNA degradation [1, 97-100].

There are three main classes of nucleic acid binding dyes (Fig. 16) depending on the linkage between dye and nucleic acid structure including: (1) intercalating dyes (SYBR® Green I (SG), ethidium bromide (EtBr), GelRed™ (GR) and GelGreen™ (GG)), (2) interal groove binding dye (DAPI), and (3) external groove binding dyes (Diamond™ Nucleic Acid Dye (DD) and Eva-Green™ (EG)). In order to increase success rates for detection of cells and STR profiling from crime samples in forensic science, the effects of six nucleic acid binding dyes (DD, GG, GR, RedSafe™ (RS), SG, EG) in the DNA workflow was evaluated in 2015 [90]. Initially, these dyes (20X, 10 µL) were added to the DNA template (Standard 2 (5 µL of 10 ng/µL) of Qubit® dsDNA HS Assay Kit (Life Technologies, Vic, Australia)) and isolated using the QIAamp® DNA Micro Kit (Qiagen, Vic, Australia). DNA extracted was eluted in 30 µL. DNA concentration was quantified using the Qubit® dsDNA HS Assay Kit and compared to the control without dyes. STR profiles were carried out using the AmpFLSTR® ProfilerPlus® Kit (Life Technologies). Consequently, for an evalution of the loss of dyes after DNA isolation, the Qubit readings showed that RS and EG were almost removed (99.6% and 99.4%, respectively), while DD and GR

recorded as 52.7% and 50.6%, respectively. However, DD was reported as no effect on the DNA signal whereas other dyes increased the DNA signal in the assay. It was critical to show DD was the dye had the lowest effect on DNA loss compared to other intercalating dyes [90]. This may be interpreted that the binding sites between DNA and chaotropic salts was limited due to the interaction of intercalating dyes with DNA molecules, resulting in reduced binding to silica membrane and increased DNA loss through extraction process. In contrast, DD is an external binder dye, allowing a removal from DNA molecules performed via buffer washes in isolation procedure.

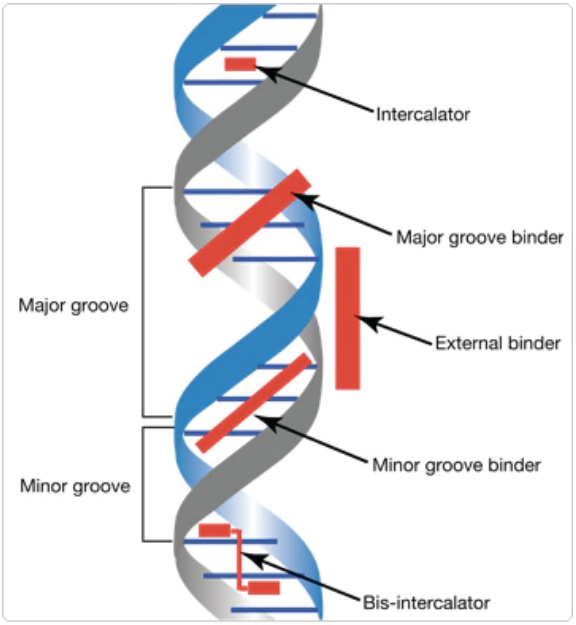


Figure 16: Schematic diagram of nucleic acid binding dyes. The different binding modes of dyes (and other ligands) to DNA are shown and abstracted from Thermo Fisher Scientific [101].

EtBr or SG has been known as a mutagenic and toxic dye, but still commonly used in molecular biology laboratories. The Short Communication presented a comparison of four nucleic acid staining dyes (GG, GR, RS and DD) used in electrophoresis gels according to their properties and application, aiming to provide alternative dyes that are reliable and less toxic than EtBr and SG [91]. Cell permeability and the sensitivity were addressed. In general, all dyes detailed in this study (except for DD) were intercalating dyes, which bind between base pairs of deoxyribonucleic acid (DNA). These dyes were diluted into a concentration of 1X. DNA template (mass known) was mixed with loading dye (6X) and pipetted into the wells of 2% agarose gel. A 100 bp ladder (Promega) was included. The run was performed for 45 minutes at 129 V. The gels were stained

with the dyes for 1 hour and visualised using a Bio-Rad Gel Doc EZ Imager (Bio-Red, Vic, Australia). Based on the data, low concentration of DNA (0.5 ng) was detected by the four dyes (GR, GG, SG and DD). At blue transillumination (460 nm excitation), DD was highly recommended as the sensitive dye to detect DNA at 0.5 ng and less toxic and mutagenic than SG.

DD was commercially designed as a sensitive fluorescent dye that binds to external groove of DNA molecules and can be used for detection and visualisation of DNA in gel electrophoresis (agarose or polyacryamide gel). In an attempt to broaden the application of DD, an use of DD was evaluated in real-time quantification PCR in comparison to other three fluorescent dyes (SG, EG and BRYT Green (BG)) [92]. A series of DD concentration involved 0.1X, 0.5X, 1X, 2X and 2.5X was added to DNA template (20 ng isolated from buccal swab) in each reaction. Amplifications were carried out in a Rotor-Gene Q (QIAGEN, Vic, Australia) using Fragment 2 primers, which produce 246-bp amplicons of cytochrome b – the mitochondrial gene. The data showed that SG and EG inhibit PCR at high concentrations, above 1X and 2.5X, respectively. The optimal concentration of DD dilution was 0.5X. At higher concentration (above 1.5X), DD gave complete to partial inhibition for qPCR. It was reported that DD, EG, RS, GR and GG are less toxic and mutagenic than SG and ethidium bromide. In terms of the efficiency and sensitivity of the reaction, the amount of DNA (0.28 pg ~ 20 copies of mtDNA template) was detected by DD. Overall, DD offered a reliable and less toxic approach for qPCR applications.

Latent DNA on different types of buccal swabs was visualised using DD in 2018 [94]. DNA template was isolated using the QIAamp mini DNA kit (QIAGEN, Vic, Australia). DNA concentration was quantified using a Quibit® 2.0 Fluorometer (Thermo Fisher Scientific, Vic, Australia). Extracted DNA (1 ng) was pipetted directly onto the swabs along with 1 µL of 20X DD. The images were visualised under a digital microscope. For direct STR profiling, the stained swab head was cut and placed into a PCR tube containing a cocktail of PCR master mix and primer of the AmpFlSTR® NGM Select™ kit. As a result, at 20x concentration, DD provided no inhibition for subsequent DNA analysis. Complete STR profiles were obtained from those swabs.

In brief, recent studies reported that DD is a potential dye to aid in the detection of DNA [89-94]. The application of DD to detect DNA was reported on cells deposited onto fabrics, hairs follicles, swabs, saliva or buccal cells [80, 89, 93, 102].

In this study, DD was used to stain cells within bone sections using fresh and archived samples. The first publication in August 2021 reported on the potential of DD to rapidly stain all bone-related cells (chondrocytes, osteoblasts, osteoclasts, osteocytes). The paper described an assessment of the number of cells within FFPE bone samples resulting in a potential success of DNA profiling [18]. The presence of nucleated cells as well as cell-free lacunae were recorded. The second publication in March 2022 described a process to visualize nuclei within fragmented bone samples which could then be added, as part of a supernatant, directly to a PCR to amplify short tandem repeat loci [103]. The described process offers a rapid and reliable approach for DNA profiling from bone within a day with no decalcification and DNA isolation.

1.5. Thesis aims

The central aim is to provide a simple and effective means to isolate high quality DNA from bone. Allied to this there are three aims that will be addressed.

An initial aim of this study is to examine bone micro-architecture that has previously been sectioned into thin slices and determine whether cells containing DNA can be visualised using a simple dye that binds to DNA, and if so whether the location and number of cells can be recorded accurately.

A second aim of this study is the determination of relationship between the number of nucleated cells and DNA yields to achieve an informative DNA profile that provides confidence in identifying the individual from which the bone came.

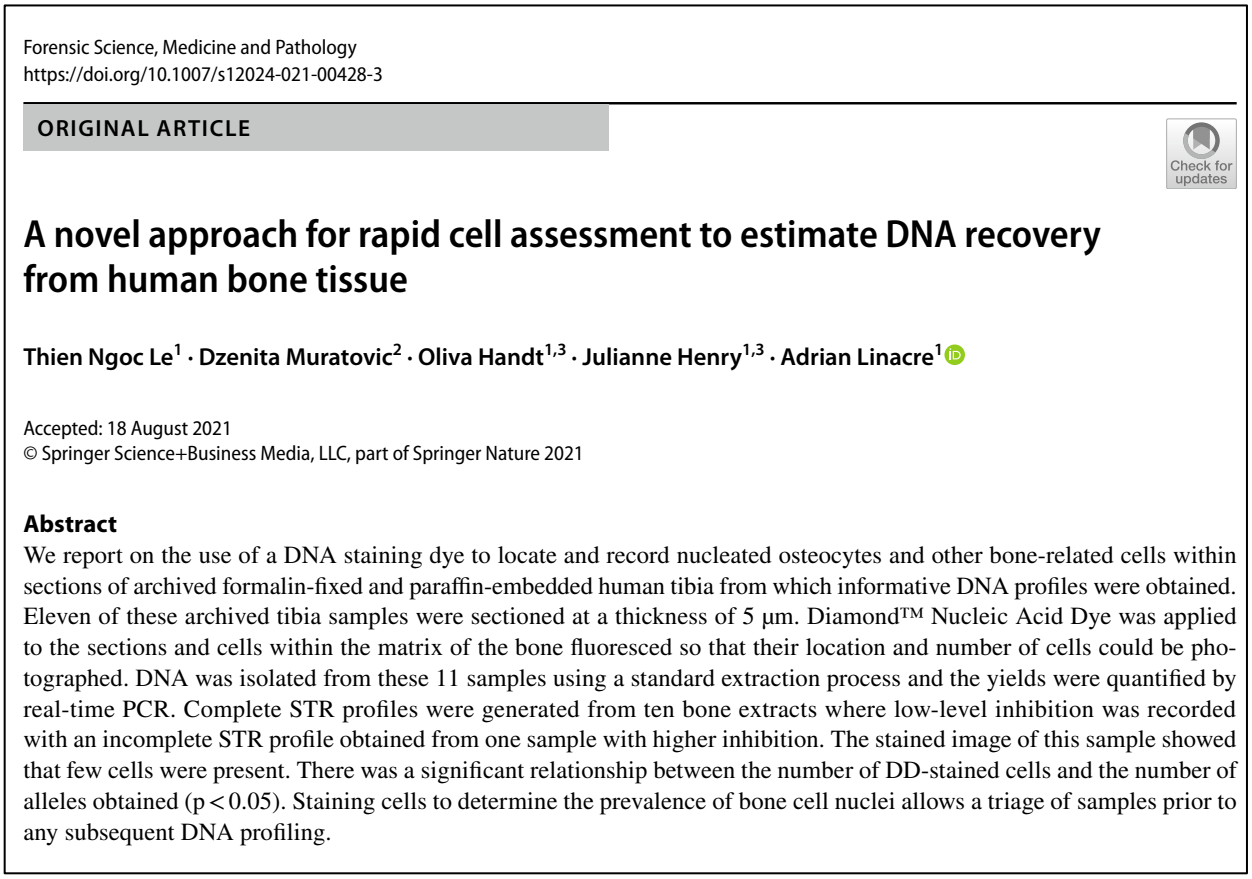
The final aim is a simplified approach to generate autosomal STR profiles from bone with no extraction and decalcification.

A comparison of the current methods used in forensic science and the simple processes described in this thesis is provided. This comparison will include: minimisation of contamination; presence or absence of known inhibitors to the PCR process; the relative time between initial examination and DNA profiling; the quality and quantity of the DNA using real-time PCR and comparison of the number of alleles generated; and ultimately to compare the advantages and drawbacks of this methodology to current practice regarding implementation into forensic practice.

CHAPTER 2: DETECTION AND STR TYPING OF BONE CELLS FROM ARCHIVED SAMPLES (PUBLICATION I)

2.1. Detection and STR typing of bone cells from archived samples (publication I)

Results from this study were published in The journal of Forensic Science, Medicine, and Pathology, 2021. doi: https://doi.org/10.1007/s12024-021-00428-3. The abstract of Publication I illustrated in Figure 17 below.



Forensic Science, Medicine and Pathology
https://doi.org/10.1007/s12024-021-00428-3

ORIGINAL ARTICLE

Check for updates

A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue

Thien Ngoc Le[1] · Dzenita Muratovic[2] · Oliva Handt[1,3] · Julianne Henry[1,3] · Adrian Linacre[1]

Accepted: 18 August 2021

Abstract
We report on the use of a DNA staining dye to locate and record nucleated osteocytes and other bone-related cells within sections of archived formalin-fixed and paraffin-embedded human tibia from which informative DNA profiles were obtained. Eleven of these archived tibia samples were sectioned at a thickness of 5 µm. Diamond™ Nucleic Acid Dye was applied to the sections and cells within the matrix of the bone fluoresced so that their location and number of cells could be photographed. DNA was isolated from these 11 samples using a standard extraction process and the yields were quantified by real-time PCR. Complete STR profiles were generated from ten bone extracts where low-level inhibition was recorded with an incomplete STR profile obtained from one sample with higher inhibition. The stained image of this sample showed that few cells were present. There was a significant relationship between the number of DD-stained cells and the number of alleles obtained ($p < 0.05$). Staining cells to determine the prevalence of bone cell nuclei allows a triage of samples prior to any subsequent DNA profiling.

Figure 17: The abstract of Publication I.

2.2. Statement of Authorship

By signing the Statement of Authorship, each author certified that their stated contribution to the publication (Publication I) is accurate (as detailed below), and that permission is granted for the publication to be included in the candidate's thesis.

Student Name: Thien Ngoc Le
Student ID: 2231326

CO-AUTHORSHIP APPROVALS FOR HDR THESIS EXAMINATION

PUBLICATION 1

This section is to be completed by the student and co-authors. If there are more than four co-authors (student plus 3 others), only the three co-authors with the most significant contributions are required to sign below.

Please note: A copy of this page will be provided to the Examiners.

Full Publication Details	Thien N Le, Dzenita Muratovic, Handt O, Henry J, Linacre A. A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue. Forensic Sci Med Pathol. 2021 2021/10/11. doi: 10.1007/s12024-021-00428-3.
Section of thesis where publication is referred to	Chapter 2

Student's contribution to the publication			
	90	%	Research design
	55	%	Data collection and analysis
	60	%	Writing and editing

Outline your (the student's) contribution to the publication:

TNL designed and performed the experiments.
DM provided the samples, assisted with experimental design and prac- tical advice.
OH assisted with the manuscript drafting and provided experimental advice.
JMH assisted with the manuscript drafting and provided experimental advice.
AL assisted with manuscript drafting, provided experimental advice, facilitated access the laboratory and funded this study.

APPROVALS

By signing the section below, you confirm that the details above are an accurate record of the students contribution to the work.

Name of Co-Author 1	Oliva Handt	Signed		Date	17/3/2022
Name of Co-Author 2	Jullianne Henry	Signed		Date	17/3/2022
Name of Co-Author 3	Adrian Linacre	Signed		Date	17/3/2022

2.3. Introduction

DNA extracted from formalin-fixed and paraffin-embedded (FFPE) tissue specimens is routinely used in molecular diagnostic analysis and histopathology [8, 104-107]. Fixation using formalin is current standard practice [108, 109] for molecular preservation as this material cross-links proteins (primarily lysine) through a $–CH_2$ linkage, with the effect of terminating cell metabolism [110-112]; the result is a prevention of self-digestion and inhibition of bacterial growth [42, 113]. A limitation with FFPE is that the process causes DNA-protein crosslinks, and also both intra and inter-strand DNA crosslinks, with the consequence of an electrophilic reaction with adenine and guanine [114]. Additionally, DNA depurination (loss of adenine or guanine bases) and DNA breaks are common products of oxidization of formalin to formic acid [115]. These consequences reduce down-stream PCR-based success [8, 116].

On occasion, archived bone material is the only source of ante-mortem material whereby either direct or indirect reference DNA profiles can be generated for the purpose of identifying human remains. It is commonplace to archive bone using the FFPE process. This raises issues as FFPE samples have several limitations such as: fragmentation of the DNA molecule resulting in short target sequences (50-300 bp in length) [115, 117]; poor STR amplification success due to the formation of cross-linking between DNA and proteins [118]; and any residual formalin inhibiting enzymatic reactions in subsequent analyses [119]. The silica membrane-based protocol (such as the QIAamp DNA FFPE Tissue kit) is a standard method to recover DNA from FFPE samples [116, 117]. Whilst the isolation of DNA from soft tissue (heart and liver), teeth, or bone powder is common [105, 116, 120-122], there have been no reports on the recovery of DNA from sectioned FFPE bone samples. This provides an alternative approach to specifically target, and then recover, nucleated osteocytes and bone-related cells from sectioned bone.

The location of nucleated cells can be achieved by using a dye, such as Diamond Nucleic staining Dye™ (DD), as this can stain DNA within the four different cell types found in bones: chondrocytes, osteoblasts, osteoclasts and osteocytes. The osteocyte population comprise 90-95% of total bone cells (10 times more abundant than osteoclasts and 1000 times more abundant than osteoblasts) and it has been reported that these cells can live up to 50 years [26].

The use of DD has been widely reported for identifying and locating nucleated cells in forensically relevant items [90, 123]. It has been found to effectively record the cells containing DNA in both

saliva and corneocytes [123]. This proof-of-concept study reports on the potential of DD to rapidly stain all bone-related cells and assess the number of cells within previously fixed and preserved FFPE bone samples to indicate the potential success of subsequent DNA profiling. As part of this study, there is an illustration of the presence of nucleated cells as well as cell-free lacunae.

2.4. Materials and methods

2.4.1. Sample preparation

Eleven archived FFPE subchondral bone of tibial plateaus (N=11) that had been preserved using a published method [124] were obtained from the Discipline of Orthopaedics and Trauma (Adelaide Medical School, The University of Adelaide). Before being used for this study, the bone samples had undergone the following steps to be archived: a cuboidal block of osteochondral tissue (articular cartilage + subchondral bone) of size 10 × 10 × 10 mm was dissected using a low-speed diamond wheel saw (Model 660, South Bay Technology). The samples were formalin-fixed for 24 – 48 hours and then slowly decalcified with 10% (v/v) EDTA for 6-8 weeks [124]. Subsequently, X-rays (Faxitron X-ray, Adelaide Medical School) were used to examine the end point of this decalcification. This was followed by tissue processing in an automated tissue processing machine (Leica, Biosystems) choosing a 6-hour long processing program. Then the samples were embedded in paraffin (Tissue-Tek®, Sakura, Adelaide Medical School, AU).

The outer surfaces of such archived bone samples and the microtome were cleaned with 100% (v/v) ethanol prior to processing. All equipment, tools and consumables were cleaned with 10% (v/v) bleach, sterile water and 100% (v/v) ethanol followed by UV for 15 mins to decrease the risk of DNA cross-contamination.

Sections were prepared at a thickness of 5 µm using a Microtome (Leica Biosystems, VIC, AU). The total volume (mm) of these samples was calculated according to their dimensions. Four or five of the 5 µm sections were dewaxed by placing them directly into a sterile 1.5 mL tube and washing with pre-warmed xylene for 5 mins. This was followed by centrifugation at 6000 rpm for 2 mins. The supernatant was removed by a pipette, followed by one rinse with 100% ethanol (v/v) of the pellet for 5 mins, an additional centrifugation at 6000 rpm for 2 mins, and removal of the supernatant by pipetting. The resultant cell pellets were air-dried at room temperature for 10 mins.

2.4.2. Morphological assessment and in-situ staining

Five µm thick sections were placed on the surface of a water batch (filled with 50°C distilled water) for 10 seconds and were then mounted onto HistoBond®+ adhesive microscope slides (Paul Marienfeld, Australia). These mounted sections were placed in a 70°C heater for 15 mins and then dewaxed with xylene for 5 mins, followed by a 100% (v/v) ethanol and diluted ethanol (95 %, 85%,70 %) (v/v) before water wash.

Dewaxed sections were stained with 10 µL of 20x DD (Promega Corporation, Madison, WI, USA) diluted in 75% ethanol (v/v). The samples were allowed to dry at room temperature for 10 seconds. The morphology of nucleated cells was visualized under a Dino-Lite EDGE AM15T-GFBW digital fluorescence microscope (AnMo Electronics Corporation, New Taipei City, Taiwan) at 220x magnification with a 480 nm LED light source and a 510 nm emission filter (Fig. 18). The sizes of stained cells were estimated using DinoXcope_OXS_2.0.2 software. In addition, the number of these sectioned cells was manually counted and recorded using Image J software, according to the green DD-stained nuclei. The image of the full thickness of osteochondral tissues was firstly divided into 16 frames at 20x magnification, then the number of nucleated cells from each frame were counted at 220x magnification and summarized into the total figure.

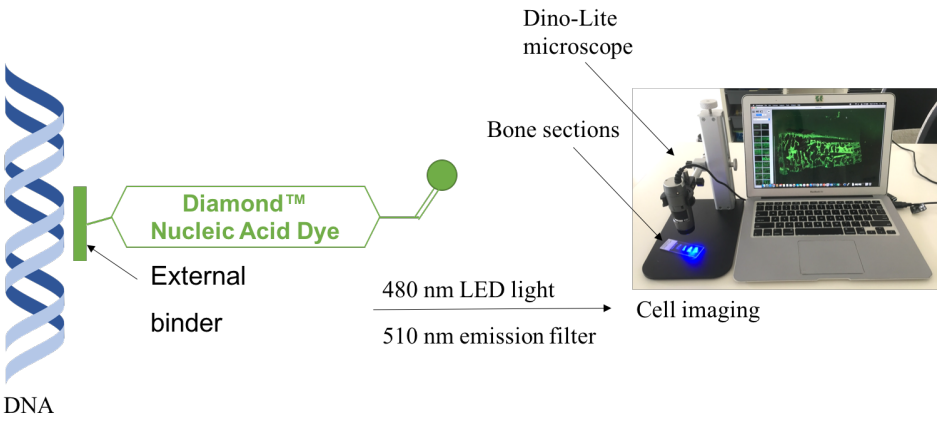


Figure 18: Diagrammatic set-up of the staining and recording of cells. An use of Diamond™ Nucleic Acid Dye and a mini fluorescence microscope is illustrated.

2.4.3. DNA isolation and quantification

DNA was isolated from eleven sectioned FFPE bone samples with the QIAamp DNA FFPE Tissue kit (QIAGEN, VIC, AU) as per the manufacturer's instructions, with the following modifications: the samples were incubated at 56 ^{o}C overnight; following incubation, all lysates were incubated at 90 ^{o}C for 30 mins to inactivate proteinase K and then finally elution was to a volume of 60 µL in EA buffer. All eluants were stored at -20^{0}C. A negative control (reagent blank) was included in all subsequent DNA analyses.

Real-time PCR quantification was performed using the Investigator® QuantiplexPro RGQ kit (QIAGEN, VIC, AU) on a Rotor-Gene Q (QIAGEN), following the manufacturer's cycling conditions. The quantity of human and male DNA, presence of PCR inhibitors, and mixture/degradation ratios were calculated from the Q-Rex software into the QIAGEN Quantification Assay Data Handling Tool (QIAGEN). A PCR negative control was included in the quantification step.

2.4.4. Short tandem repeat (STR) typing

The DNA extracts were amplified using the AmpF*L*STR™ Identifiler™ Plus PCR Amplification Kit (ThermoFisher Scientific, Waltham MA, USA) according to the manufacturer's protocol. Either DNA template (0.013 – 1 ng) or male DNA Control 007 (1 ng) or no template (nuclear-free water) was included in the amplification (29 cycles). The PCR products were separated and detected by 36 cm capillary electrophoresis on a 3500xL Genetic Analyzer (ThermoFisher Scientific). According to the National Criminal Identification DNA Database (NCIDD), a DNA profile is considered informative and uploadable to the NCIDD for comparison if it comprises ≥ 12 autosomal donor alleles. Resultant DNA profiles were analysed using GeneMapper ID-X v1.4 (ThermoFisher Scientific) with an analytical threshold of 50 relative fluorescent units (RFU), homozygous peaks (a single allele recorded ≥ 150 RFU), heterozygous peaks (RFU of relative alleles exceeded 70%), stutter peaks (≤ 15% of the parent peaks). Peaks lower than 15% of the parent peak were removed as stutter which can lead to the assignment of an additional contributor.

2.4.5. Data analysis

All statistical analyses were tested using R software (version 3.6.3), $p \leq 0.05$ was accepted for significant differences [125]. The number of cells and the RFU were transformed to a log10, and the Spearman correlation test was used in correlation analysis.

2.5. Results

2.5.1. Assessment of morphological cells

Osteochondral units are comprised of four different zones: articular cartilage, calcified cartilage, a thin layer of cortical bone-subchondral plate, and subchondral trabeculae that arise from the subchondral plate (Fig. 19A). Each of these formations exhibit specific tissue characteristics and cell types (Fig. 19A-D). Based on the locations and characteristics of these cells, using DinoXcope_OXS_2.0.2 software, the fluorescence from the nuclei appeared as green dots under 220 x magnification, and match that seen from other cell types stained with DD [123]. An abundance of chondrocytes and bone cells (osteoclasts, osteoblasts and osteocytes) could be observed from the tissue sections when stained with DD and observed at 220 x magnification (the maximum magnification of the fluorescence Dino-Lite microscope). The position and the presence of nuclei within each of the stained cells are illustrated (Fig. 19).

Osteocytes are the most numerous and long-lived bone cell and reside inside spaces called lacunae. When osteocyte cells undergo apoptosis, their nuclei are no longer present in the lacunae creating empty lacunae (Fig. 19). This phenomenon is often seen in aged bone [126]. In this study, we found an increase in the number of empty lacunae in the subchondral trabeculae of sample 10 (Fig. 20). Thus, the lack of staining, and hence the lack of any DNA, may be due to a larger number of empty lacunae. Note that these samples were collected from adult patients (Table 4).

The number of DD-stained cells per mm^2 of tissue was counted manually at 220X magnification. Fig. 21 depicts the estimation of cells per mm^2 of tissue for sample 2.

Fig. 22 shows the presence of cells in four regions of the bone material for all eleven samples. For ten of these samples (sample 1 – sample 9, and sample 11) numerous stained chondrocytes, osteocytes, a few of osteoblasts, and osteoclasts were observed. This contrasts with one sample (sample 10) where osteocytes appear to be absent presenting an example of an empty

lacunae. The absence of articular cartilage in sample 9 is the reason why no chondrocytes were recorded.

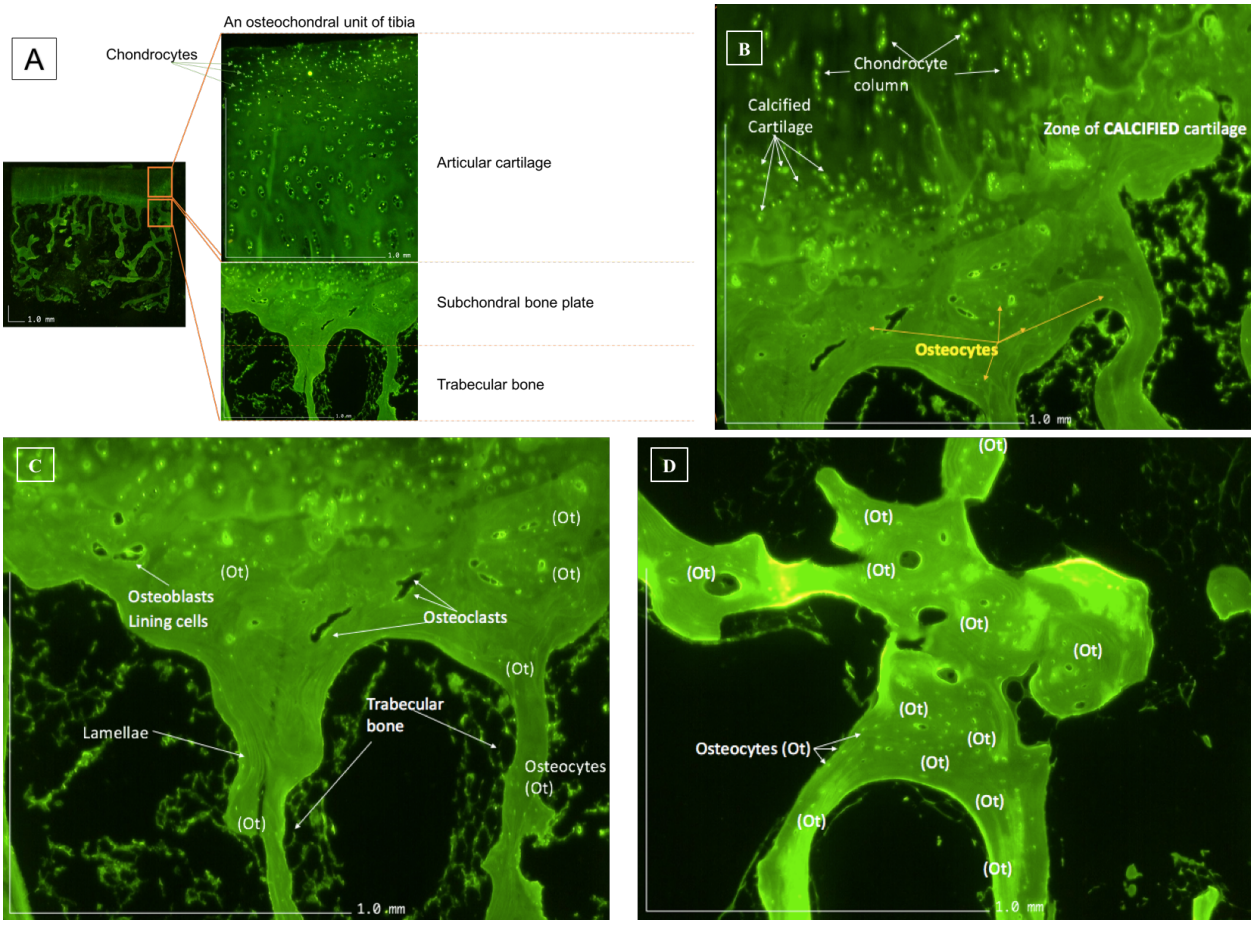


Figure 19: Microscopic images of sectioned FFPE bone (5 µm thickness). The section stained with 20x DD and viewed at 220x magnification. Image A shows the four different zones in an osteochondral unit (sample 7). Image B shows the location of osteocytes and chondrocytes (sample 7) in subchondral bone plate and within a zone of calcified cartilage. Image C shows four types of bone cells in trabecular bone: osteoclasts, osteoblasts, osteocytes (Ot) and bone lining cells (sample 7). D shows nucleated osteocytes (Ot) located in lacunae in trabecular bone (sample 3). The scale bar is 1 mm.

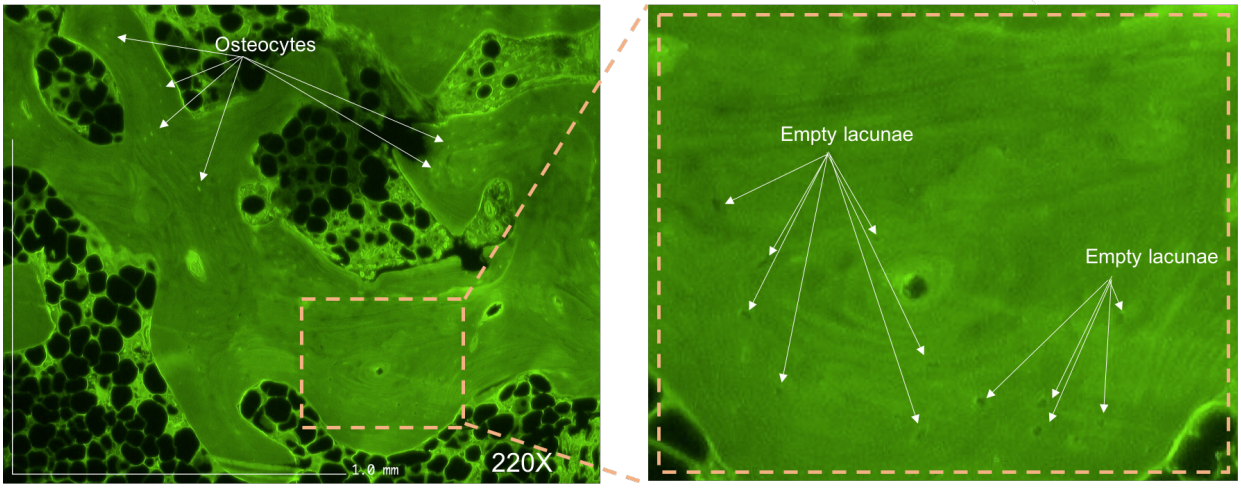


Figure 20: Morphology of a subchondral bone. A 5 µm section illustrates the presence of nucleated osteocytes and empty lacunae (without cell) (sample 10). These cells were photographed at 220x magnification after 10 seconds of staining with 20x DD. The scale bar is 1 mm.

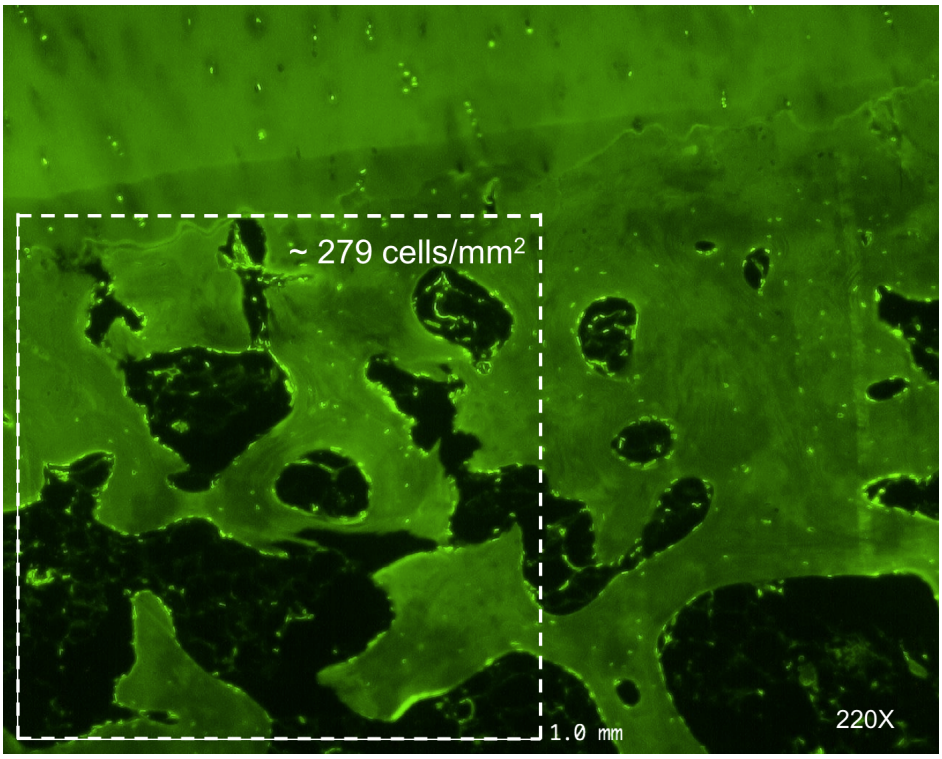


Figure 21: A record of numerous cells in trabecular bone. Sectioned DD-stained cells (sample 2) per mm^2 of tissue were counted manually at 220X magnification. The green dots reflect the nuclei of the cells.

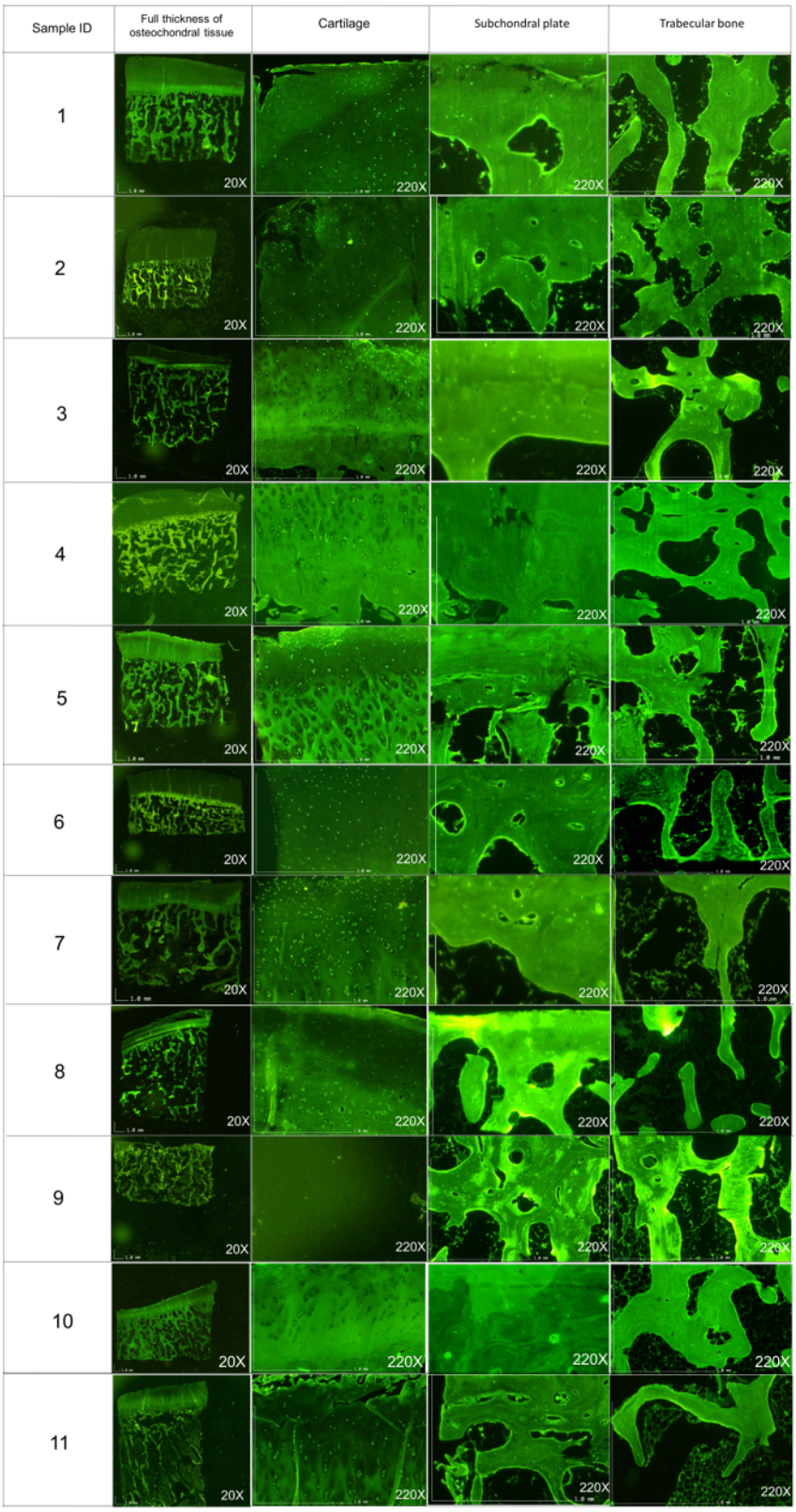


Figure 22: The morphological cells of 11 human subchondral bones. Images were photographed at 20x and 220x magnification after staining with 20x DD. The scale bar is 1 mm.

2.5.2. DNA yields from FFPE bone samples

The yield of DNA recovered from 5 µm sections of subchondral tibial bone is shown in Table 4. The extraction control showed no DNA detected (as expected) (data not shown). No inhibition of the real-time analyses was recorded for any of the 11 samples (data not shown). The three highest DNA yields (10.76; 6.29 and 6.04 ng) were recorded for samples 9, 8 and 7, respectively, sample 10 resulted in the lowest DNA yield (0.08 ng) (Table 4). Isolation of DNA from the other seven samples resulted in DNA yields from 1.51 to 3.77 ng.

Bone specimens with the input ranging between 127 – 356 sectioned cells/mm^2 of tissue generated informative profiles (≥ 12 autosomal alleles) from 10 of the 11 samples (91%), of these 10 samples, 6 had additional alleles (31-35 alleles) and 4 gave full profiles (30 alleles). One uninformative profile with 4 alleles recovered, sample 10 resulted in the lowest parameters including ~ 127 of the number of cells/mm^2 tissue and 0.08 ng of DNA yield.

Table 4: Information for the 11 tibial bone samples. Results for the number of sectioned cells/mm^2 tissue, total DNA yield, degradation index (DI) and number of recovered alleles are given.

Sample ID	Age	Bone area	Sectioned cells/ mm^2	DNA yield (ng)	DNA Concentration (ng/µL)	Degradation index (DI)	Number of alleles (30)
1	44	100 mm^2	~ 254	1.51	0.025	30.65	32*
2	61	64 mm^2	~ 279	1.69	0.028	18.72	31*
3	51	80 mm^2	~ 224	3.07	0.051	40.51	32*
4	74	72 mm^2	~ 205	3.77	0.063	28.32	34*
5	65	72 mm^2	~ 230	3.08	0.051	59.59	30
6	44	108 mm^2	~ 262	2.87	0.048	20.04	35*
7	54	81 mm^2	~ 253	6.04	0.101	47.32	30
8	86	88 mm^2	~ 240	6.29	0.105	82.09	30
9	59	100 mm^2	~ 356	10.76	0.179	18.21	30
10	86	130 mm^2	~ 127	0.08	0.001	1013.97	4
11	80	117 mm^2	~ 227	3.20	0.053	299.83	31*

Samples denoted with an * indicate that one major DNA profile was observed with all 30 alleles and traces of an additional minor profile was present at trace levels. These additional alleles were different between samples and varied between 1 and 5 additional alleles within the 15 STR locus kit.

Table 5: Statistical information of the variables (N = 11).

Group	Mean	Median	Range
Sectioned cells/mm^2	242	240	127 – 356
DNA yield (ng)	3.85	3.08	0.08 – 10.76
DI	150.84	40.51	18.21– 1013.97
Number of alleles	29	31	4-35
Total RFU	39,180	24,609	444 – 151,440

2.5.3. Degradation of DNA obtained from FFPE bone samples

The Quantiplex® Pro RGQ kit simultaneously examines the concentration of the targeted DNA and degradation by detecting a short (91 bp) and a long (353 bp) autosomal amplicon, resulting in an analysis of the degradation status of the template DNA (Degradation index, DI) [70]. The level of degradation of DNA obtained from Qiagen kit is shown in Table 4. These data indicate that the DNA isolated by Qiagen kit from nine samples recorded a DI below 100 (18.72 – 82.09) and one sample had a DI of nearly 300 (sample 11). DNA isolated from sample 10 had a substantively higher degradation status of 1013.97.

2.5.4. Autosomal STR analysis

All 11 samples were amplified with the Identifiler Plus kit. Positive control (DNA Control 007) gave a complete DNA profile (data not shown). Adding 0.013 – 1 ng of DNA as template, ten samples resulted in complete DNA profiles. However, the bone samples cannot be confirmed as there were no reference samples available to allow a comparison as all samples were deidentified and donated for research purposes following a medical procedure. Elimination comparisons to the author and other reseachers were done to ensure they did not contribute the DNA profiles obtained. In addition, none of the samples matched one another. The total RFU values of the profiles obtained are shown in Table 5. An example of a complete DNA profile (sample 9) is shown in Fig. 23. The donor of the sample was assumed to be heterozygous at all except one of the 15 STR loci (homozygous at TPOX). There is some imbalance between the alleles at the same locus and low RFU values were recorded for one locus (D18S51) hence the

different color above the locus. The reduction in peak height from smallest to largest alleles is most likely an indication of loss of template in the amplification.

No alleles were amplified from the negative control. Additional alleles were observed in sample 1 (TH01, D18S51), sample 3 (D21S11, D7S820, vWA), sample 4 (D21S11, D7S820, D2S1338), sample 6 (D8S1179, D21S11, D3S1358, TH01, D16S539, D2S1338) and sample 11 (D7S820) of the bone samples, even though all such be from the donor only.

Figure 23: An example of complete DNA profile from sectioned subchondral bone (sample 9).

2.6. Discussion

We report on a simple and cost-effective cell screening method for DNA typing from archived FFPE bone samples and aged bone specimens. DD staining indicated the location of DNA and by inference the presence of cell nuclei. This number of DD-stained nucleated cells correlated to the quality and quantity of DNA isolated from these sections.

An advantage with DD staining is that it is simple to apply and there are no wash steps, which contrasts traditional staining with hematoxylin and eosin. This means the staining procedure can occur within a matter of seconds, rather than 5 minutes. Hematoxylin and eosin are common histopathology stains which require a number of technical concerns including: de-hydration, rinse, accurate timing, monitor quality, uneven staining, blueing treatment, and renewing of reagents routinely that might be time-consuming and laborious [127]. To identify the morphology and characteristics of osteoblasts, immunochemical markers are required [128]. DNA dye such as DD in this study provided an alternative approach to simply stain all bone-related cells including osteoblasts.

Osteocytes are reported as having a long life-span, living as long as 50 years, unlike other short-lived bone cells including osteoclasts (1-25 days), osteoblasts (1-200 days) and lining cells (1-10 years) [119]. The DD-stained osteocytes were observed in both subchondral bone plate and trabecular bone, illustrating that they were the most abundant cell type. Further, this allows an evaluation of the number of cells in a bone section and an indication of the propensity to generate a DNA profile. There have been several studies of generating DNA profiles from bone materials from powder to powder-free methods [3, 9, 11, 129], none however have assessed whether the initial material has sufficient nucleated cells for downstream molecular analysis. The absence of DNA within empty lacunae indicated that the amount of bone powder alone if collected from these areas, is not an accurate indication of the chance of DNA isolation. Thus, DD staining can be an initial screening tool in determining whether there are sufficient cells present to warrant DNA isolation and down-stream STR analysis. It is also potential for museum specimens to identify the cells of interest for further analysis.

One advantage of sectioned bone is that DD requires penetration of the biological matrix, and here the stained nucleated cells could be visualized clearly in all 11 samples even when the sections were 5 μM in thickness. If the sections were thinner then there would be the risk of slicing

through cells and losing nuclear material, and if much thicker, then the ability of the dye to permeate the structure and allow cells to be recorded could be compromised.

The abundance of nucleated cells observed was in accordance with the number of alleles in DNA profiles. For example, 27 – 35 alleles were observed in ten DNA profiles and this also corresponded to recording the presence of an abundance of nucleated cells/mm^2 (205 – 356 cells/mm^2). This relationship was significant ($p < 0.05$). In contrast, numerous empty lacunae were photographed in sample 10 and that correlated with the lowest DNA yield recorded (0.08 ng). Recently, commercial kits have an optimum DNA template of 500 pg of DNA [66, 74], which correlates to only 80 diploid cells (~ 6 pg DNA/cell). In some studies, there was a wide range in the number of cells required for a complete DNA profile, such as 10 laser-captured blood mononuclear cells [130] and 30 sperm or 15 epithelial cells [131]. These findings were most likely based on using intact cells, whereas cells of interest in this study were sectioned at 5 µm thickness, which could slice through a cell resulting in only part of the genome being present. Hence, while the number of cells present in a good indication of the expected DNA yield, this may not always be the case.

A negative control (reagent blank) was included in all DNA extraction analyses, controlling the contamination during the process. The Quantiplex® Pro RGQ kit includes multiple primer sets to detect degradation in both autosomal and male targets. A degradation index (DI) provides a parameter of DNA integrity. In the data analysis software, a DI value ≥ 10 is defaulted as 'possibly degraded'. Across the samples, our data shows that all DNA obtained from FFPE bone samples are possibly degraded (DI range = 18.21– 1013.97). Hence, the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit was chosen to amplify 15 loci all with an amplicon size < 360 bp. Even though there was no significant relationship between DI and number of alleles obtained, all ten informative profiles had peak imbalance (see Fig. 24 as an example). The DI of sample 10 was exceptionally high (DI = 1013.97). In agreement with a very recent study, the combination of DNA yield and DI value provides a measure for the success of STR profiling [132]. Despite of given high DI (18.21 – 299.83), there was 90.9% (10 out of 11) samples had profiles with reportable allele calls.

Additional alleles were recorded in some loci of 5 samples. The negative result from the control sample indicates that the additional alleles were added prior to any DNA extraction step

where the archiving process many have been performed without DNA-free equipment and the wearing of standard PPE. Whilst not ideal, probabilistic genotyping software (such as STRmix®) [133], could be used in most instances to resolve the reference profile of interest from low-level contaminating peaks.

2.7. Conclusion

This report adapted the use of DD to the detection of nucleated osteocytes and other bone-related cells within sectioned bone. Additionally, counting the number of DD stained nucleated-cells and real-time PCR are indicators of the quality and quantity of DNA. The presence or absence of stained cells correlates well to the alleles obtained and degradation status of samples. Ten complete autosomal STR profiles of FFPE bone sections were generated from as little as 1 ng when isolated using a standard isolation protocol. In combination, DD staining and quantification can aid in assessing if the quality and quantity of DNA preserved in ante-mortem bone samples is sufficient for generating useful reference DNA profiles for use in human identification.

2.8. Highlights

- Diamond™ Nucleic Acid Dye (DD) offers a rapid, reliable and safe assessment of the nucleated osteocytes and bone-related cell populations in archived samples.
- The combination of DNA yields identified by QuantiplexPro RGR kit and degradation index is an indicator of the success of autosomal STR profiling from human bone samples.
- Complete STR profiles can be amplified from bone sections (5 μm thickness).

2.9. Conflict of interest

The authors declare no conflict of interest.

2.10. Acknowledgements

This research was funded by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund. A special thanks to Mr. Adnan Mulaibrahimovic and Ms. Pat Vilimas for their assistance to TNL to perform histological work. The authors are grateful for access to the Adelaide Health and Medical Sciences (The University of Adelaide).

2.11. Supplemental content

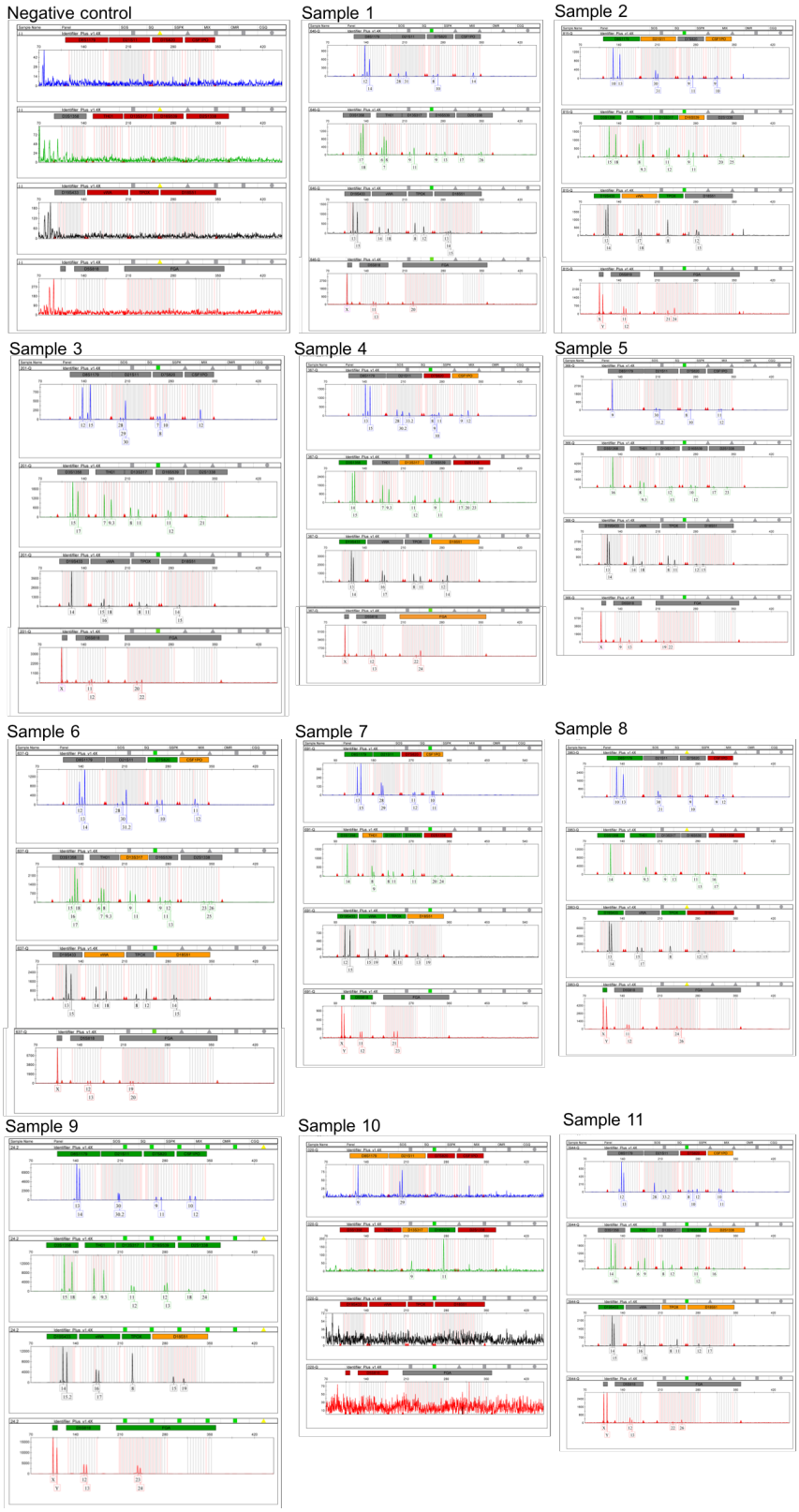


Figure 24: STR profiles of eleven bone samples and an extraction negative control.

CHAPTER 3: STR PROFILING FROM HUMAN BONE CELLS WITH NO DECALCIFICATION AND DNA EXTRACTION (PUBLICATION II)

3.1. STR profiling from human bone cells with no decalcification and DNA extraction (publication II)

Results from this study were published in the Journal of Forensic Sciences, 2022, doi: http://doi.org/10.1111/1556-4029.15033. The abstract shown in Figure 25 below.

Received: 12 January 2022 | Revised: 2 March 2022 | Accepted: 10 March 2022

DOI: 10.1111/1556-4029.15033

JOURNAL OF FORENSIC SCIENCES

TECHNICAL NOTE

Criminalistics

DNA profiling from human bone cells in the absence of decalcification and DNA extraction

Thien Ngoc Le MS[1] | Dzenita Muratovic PhD[2] | Oliva Handt PhD[1,3] | Julianne Henry PhD[1,3] | Adrian Linacre DPhil[1]

[1]College of Science and Engineering, Flinders University, Bedford Park, South Australia, Australia

[2]Discipline of Orthopaedics and Trauma, Adelaide Medical School, The University of Adelaide, Adelaide, South Australia, Australia

[3]Forensic Science SA, Adelaide, South Australia, Australia

Correspondence
Adrian Linacre, College of Science and Engineering, Flinders University, Bedford Park, SA 5042, Australia.
Email: adrian.linacre@flinders.edu.au

Funding information
This research was funded by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund.

Abstract

Bone cells are a suitable substrate for DNA analysis if required to identify the person from whom a sample was taken. Osteocytes, the most abundant cell type in bone, are embedded within mineralized bone matrix. To release DNA from osteocytes for subsequent analyses, either demineralization of the mineral matrix or an overnight incubation is routinely carried out. In this study, we report on a simplified and rapid approach to analyze preserved bone samples that omits this lengthy decalcification process. Nine tibial bone samples were processed to release matrix-free bone cells after fragmentation without the use of liquid nitrogen. Cell morphology was assessed by microscopy at 220× magnification following staining with Diamond™ Nucleic Acid Dye. Based on the presence of stained nuclei, samples were processed either using a DNA extraction process or by a semi-direct PCR process. The analysis of the quantity and quality of DNA isolated by both methods was carried out by real-time PCR and STR profiling to assess inhibition of PCR and DNA degradation. All samples resulted in informative STR profiles with minimal indication of inhibitors. These results demonstrate a potential approach of STR profiling from matrix-free bone cells within 8 hours without decalcification and DNA extraction.

Figure 25: The abstract of Publication II.

3.2. Statement of authorship

By signing the Statement of Authorship, each author certified that their stated contribution to the publication (Publication II) is accurate (as detailed below), and that permission is granted for the publication to be included in the candidate's thesis.

Student Name	Thien Ngoc Le
Student ID	2231326

CO-AUTHORSHIP APPROVALS FOR HDR THESIS EXAMINATION

PUBLICATION 2

This section is to be completed by the student and co-authors. If there are more than four co-authors (student plus 3 others), only the three co-authors with the most significant contributions are required to sign below.

Please note: A copy of this page will be provided to the Examiners.

Full Publication Details	Thien N. Le, D. Muratovic, O. Handt, J. Henry, A. Linacre, DNA profiling from human bone cells in the absence of decalcification and DNA extraction, Journal of Forensic Science (2022).
Section of thesis where publication is referred to	Chapter 3

Student's contribution to the publication			
	90	%	Research design
	74	%	Data collection and analysis
	55	%	Writing and editing

Outline your (the student's) contribution to the publication:

TNL designed and performed the experiments.
DM provided the samples, assisted with experimental design and prac- tical advice.
OH assisted with the manuscript drafting and provided experimental advice.
JMH assisted with the manuscript drafting and provided experimental advice.
AL assisted with manuscript drafting, provided experimental advice, facilitated access the laboratory and funded this study.

APPROVALS

By signing the section below, you confirm that the details above are an accurate record of the students contribution to the work.

Name of Co-Author 1	Oliva Handt	Signed		Date	17/3/2022
Name of Co-Author 2	Jullianne Henry	Signed		Date	17/3/2022
Name of Co-Author 3	Adrian Linacre	Signed		Date	17/3/2022

3.2. Introduction

Sample selection is a critical first step in forensic work. Cortical or compact bones, such as petrous bone [36, 37] and femur [38-40], have been reported as the optimal source of endogenous DNA but may not be available and therefore other skeletal elements may need to be targeted. A further report showed the amount of DNA within small cancellous, or spongy bones, is much higher than dense cortical bones [41]. For example, osteochondral tissue contains articular cartilage, subchondral cortical and trabecular (cancellous) bone, and bone marrow. Osteochondral defects can cause degenerative changes and osteochondral tissue is regularly removed during arthroscopy [124]. However, it has not yet been a sample of choice in forensic human identification. Thus, archived osteochondral sections can be a potential template for STR profiling [18] due to the abundance of nucleated cells such as chondrocytes and osteocytes.

The main source of DNA within bones are osteocytes; these are also the longest living cellular component of mammalian bones, which live up to 50 years [26, 134]. When a bone, or a portion of it, is removed from the body as part of a medical procedure, bone tissue with intact cells can be frozen, which allows storage and preservation for many years [135] and could be used as ante-mortem specimen if other suitable samples are not available for identification of a deceased. Osteocytes are embedded in a dense mineral matrix and can be released by a decalcification step [43]. EDTA binds with ionized calcium located on the outer layer of the apatite crystal within bone, forming EDTA-Ca complexes, and is commonly used in this decalcification process [2, 3, 40, 46, 136]. Either total, partial, or extended demineralization is a lengthy (days to weeks) and laborious process prior to DNA isolation [2, 12, 13, 137, 138]. In addition, the use of high molarity EDTA solutions raises the pH of the digestion buffer and has been reported to result in the loss of DNA, particularly when using a silica-based extraction method [2]. Additionally, any residual EDTA can chelate Mg^{++}, inhibiting enzymatic reactions in subsequent PCR analyses [139].

Silica membrane-based DNA extraction protocols (traditional methods) are commonly employed to recover DNA from bones [58, 140, 141]. These protocols use overnight incubation where bone powder is added to a lysis buffer containing proteinase K. Not only is DNA isolated from the matrix but also inhibitors such as high amounts of Ca^{++} are removed to minimize the effect on subsequent enzymatic analyses of DNA [58, 140, 141]. When using a DNA extraction kit, multiple tube transfer is one of the factors contributing to DNA loss. For example, some studies

identified up to a 20-80% reduction in DNA yield [142, 143]. To address this DNA loss, in this study aliquots of the supernatant containing fragmented bone particles were added directly to amplification reaction, circumventing the loss during standard DNA extraction processes. The described process offers a rapid and reliable approach for DNA profiling from bone within a day.

3.3. Materials and methods

3.3.1. Ethics approval

Nine subchondral tibia bone samples used in this study were collected with informed written consent from patients who had undergone knee arthroplasty surgery. Samples were approved for use in this study by the Human Research Ethics Committees of the Royal Adelaide Hospital and The University of Adelaide, South Australia (HS-2013-003). Age and sex details were provided. The ages of the donors for eight of the samples ranged between 58 and 81 years; the age was unknown for one donor. Reference DNA samples from the donors were not available.

3.3.2. Preparation of bone specimens

Archived bone core biopsies (8x8x8 mm in diameter) containing osteochondral tissue were accessed for the nine donors. These were provided by the Discipline of Orthopaedics and Trauma (Adelaide Medical School, University of Adelaide). Records show that the samples had been stored at -80 ^{o}C for between 3 and 5 years. The bone samples were designated as: A, C, D, E, F, J, K, O, and T. To minimize the risk of DNA cross-contamination, all equipment, tools and consumables were cleaned with 1% (v/v) bleach, sterile water and absolute ethanol. Bone specimens were further cut into small cubes (approximately 3 mm x 4 mm) using a sterile scalpel. The nine cubed bone samples were placed into separate sterile 1.5 mL tubes and washed twice in 800 µL sterile phosphate buffer solution (PBS) pH 7.0. The tubes containing the washed cubed bone samples were placed in a microfuge and centrifugation was performed at 6000 rpm for 15 minutes to remove residual bone marrow within the trabecular bone structure. The bone specimens were transferred to a new sterile 1.5 mL tube using sterile forceps and then washed twice using 800 µL of 70% (v/v) ethanol for 5 mins. The samples were air-dried at room temperature for 15 mins on a sterile petri dish. Bones were fragmented using a clean pestle. The bone fragments (10 - 20 mg) were transferred to a sterile 1.5 mL tube using a sterile pipette tip.

The workflow of the traditional method and matrix-free cells approach from bones is illustrated in Fig. 26. The traditional method included a DNA extraction, which was not required in the matrix-free cells approach.

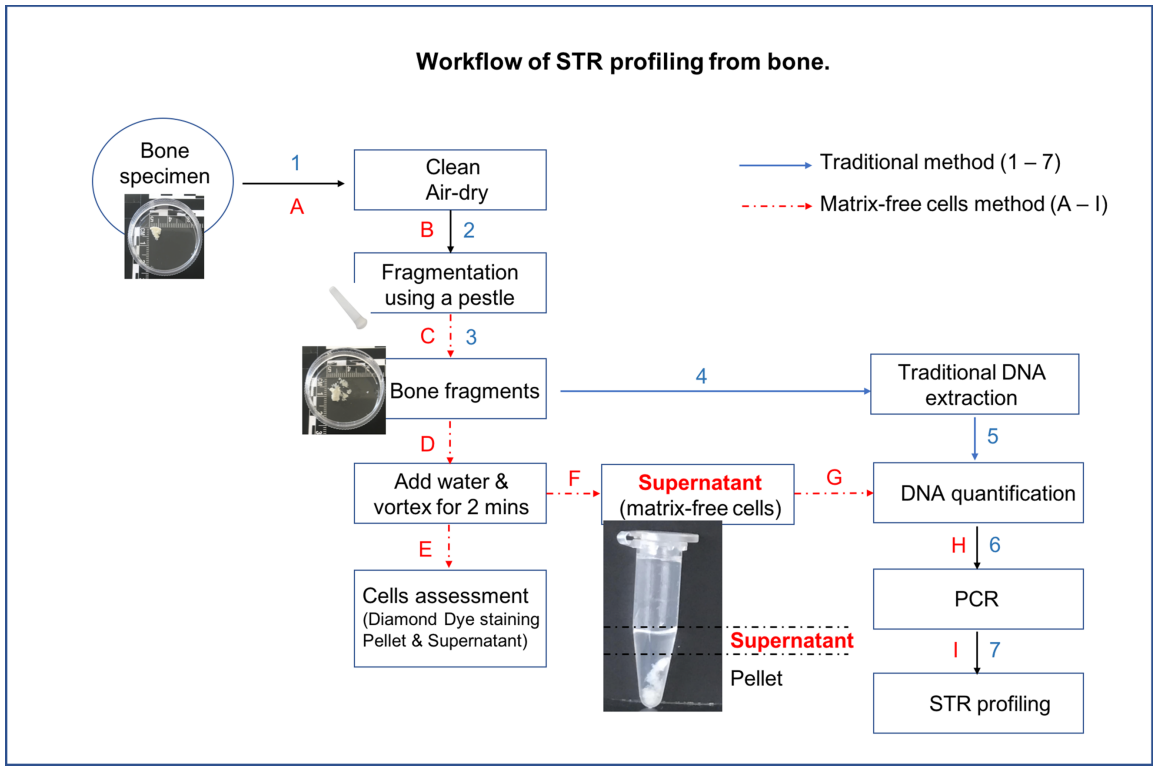


Figure 26: Workflow of STR typing from human bones. Bone specimens (~20 mg) were cleaned to remove contaminants, then air-dried and fragmented using a pestle (steps A to C, or 1 to 3). The bone specimens were treated in one of two ways, highlighted in blue or red. After fragmentation, the samples were processed using a standard/traditional DNA extraction kit (steps 3 to 7). The matrix-free cells approach adopted in this study placed the fragmented bone samples into a sterile tube, added sterile water and vortexed for 2 mins, a separation of the supernatant and pellets was presented after standing for 1-2 mins (step F). From this, the supernatant of suspended matrix-free cells (colored red) was used for DD staining to assess the number of nuclei present (steps C to E), or subjected to a real-time DNA quantification step (G), followed by PCR and STR profiling (steps H to I).

3.3.3. DNA extraction

Bone fragments (~20 mg) were extracted using the QIAamp® DNA Investigator Kit protocol (Qiagen, VIC, AU) as per the 'Isolation of Total DNA from Bones and Teeth' protocol. Overnight incubation in lysis buffer at 56 ºC was employed. The extracted DNA was eluted in 60 µL of ATE buffer. All eluants were stored at -20 ºC. A negative control (reagent blank) was included in all subsequent PCR analyses.

3.3.4. Visualization of matrix-free bone cells

Nuclei within the bone fragments were observed and using a DNA staining dye, Diamond™ Nucleic Acid Dye (DD). This dye has been reported to stain DNA within cells and allow visualization of cellular material relevant in forensic applications [89, 90, 93, 94, 123]. One recent study demonstrated the use of DD to visualize bone-related cells within formalin-fixed and paraffin-embedded (FFPE) specimens [144]. During this research, DD was used only for screening nuclei within fragmented bone samples which could then be added, as part of a supernatant, directly to a PCR to amplify short tandem repeat loci.

In order to collect matrix-free bone cells, after the initial fragmentation and centrifugation step, the bone fragments (10 - 20 mg) were put into separate 1.5 mL tubes containing 500 µL of sterile water. This solution was vortexed for 10 seconds and allowed to stand for 3-5 mins to separate into two distinct phases: 1) the supernatant containing the matrix-free bone cells and 2) the pellet containing mineral-embedded cells in residual sterile water. Five µL of either the supernatant or the pelleted bone material was pipetted onto individual glass slides and left to dry for 15 mins at room temperature. After drying, cells were stained with 5 µL of 20x Diamond™ Nucleic Acid Dye (DD) (Promega Corporation, Madison, WI, USA) diluted in 75% ethanol (v/v). Fluorescence was visualised after 10 seconds of DD staining using a Dino-Lite EDGE AM15T-GFBW digital fluorescence microscope (AnMo Electronics Corporation, New Taipei City, Taiwan) at maximum magnification (220x) with a 480 nm LED light source and a 510 nm emission filter (Fig. 27, 28). These matrix-free cells were observed to be floating freely and located in the supernatant (Fig. 28).

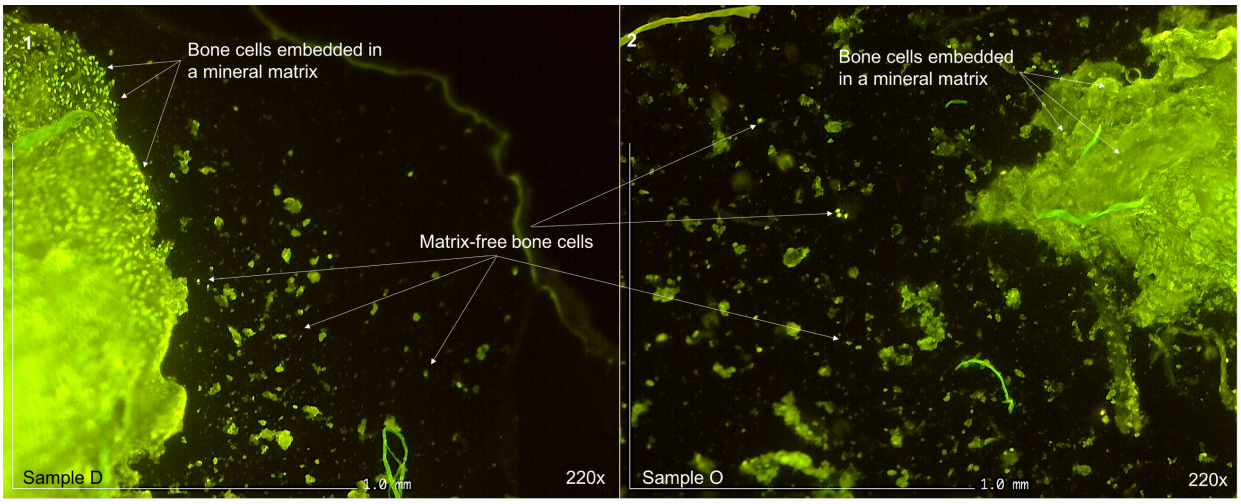


Figure 27: Detection of matrix-free bone cells. Residual liquid surrounding the pelleted bone material of two bone specimens stained with 20x DD. The observation is at 220x magnification. The scale bar is 1 mm.

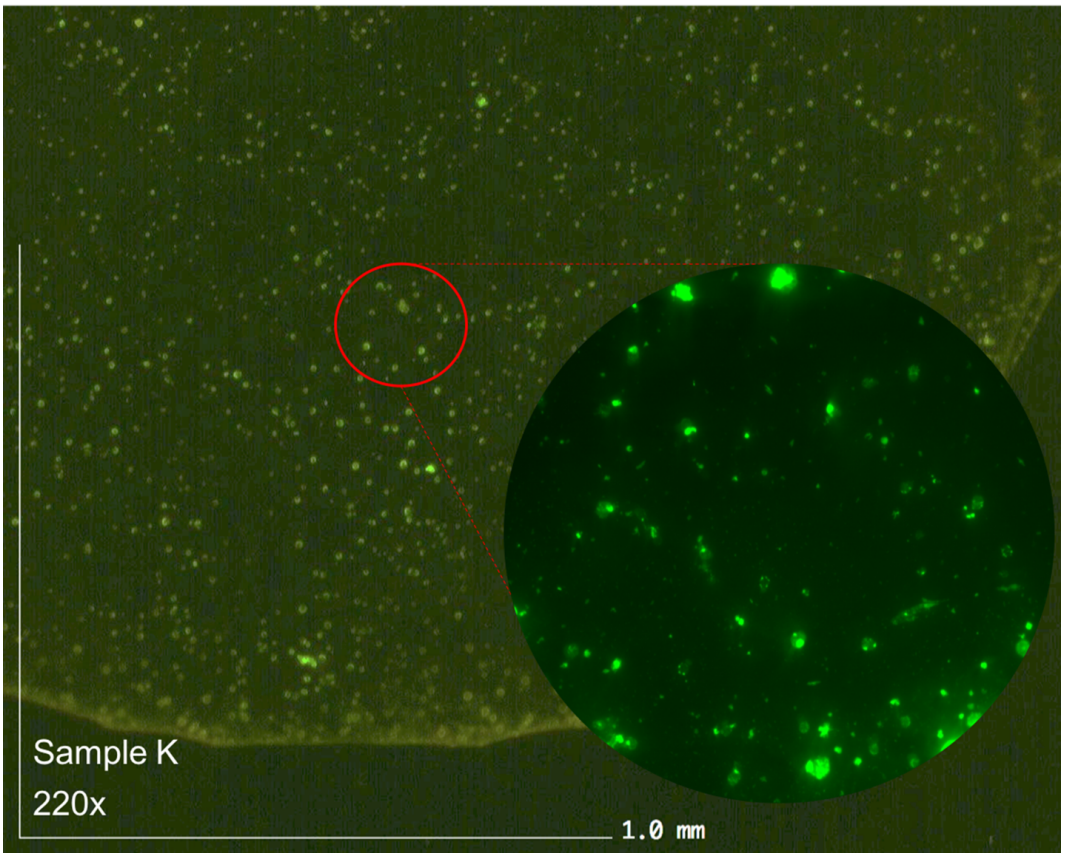


Figure 28: Magnification of matrix-free bone cells. The cells present in the supernatant from sample K. The sample was stained with 20x DD and observed at 220x magnification. The circle on the image indicates the area magnified and shown in the image. The scale bar is 1 mm.

3.3.5. Collection of matrix-free bone cells

A 60 µL aliquot of the supernatant containing the matrix-free bone cells was removed and stored in a new sterile 1.5 mL tube. All tubes were stored at -20 ºC prior to subsequent analysis.

A 2 µL aliquot of the supernatant was used for subsequent real-time PCR quantification.

3.3.6. DNA quantification

DNA quantification was performed using four replicates of the corresponding DNA extract and matrix-free bone samples for all 9 bone samples. A negative control was included. Quantifications were performed using a Rotor-Gene Q (QIAGEN) in combination with the Investigator® Quantiplex Pro RGQ kit (QIAGEN) as per the manufacturer's instructions. All data for human DNA, male DNA, degradation indices, presence of PCR inhibitors and mixture/degradation ratios were determined using the Q-Rex software and the QIAGEN Quantification Assay Data Handling Tool (QIAGEN). A degradation index value ≥ 10 is defined as 'possibly degraded' according to the Investigator® Quantiplex Pro RGQ kit (QIAGEN).

3.3.7. Short Tandem Repeat (STR) profiling

DNA (1 ng) of all 9 bone samples including matrix-free cell samples (supernatants) and DNA extracts was amplified with the AmpFLSTR™ Identifiler™ Plus PCR Amplification Kit (ThermoFisher Scientific, Waltham MA, USA) for 29 cycles according to manufacturer's instructions. Male 007 DNA 5 µL (1 ng) positive control and negative controls (nuclear-free water) were included in each PCR batch.

PCR products were separated using a 36 cm capillary through POP-4 polymer on a 3500xL Genetic Analyzer (ThermoFisher Scientific) using a 15 second injection. The internal lane DNA standard LIZ 600 was used to determine the length of PCR products. The data were analyzed using GeneMapper ID-X v1.4 (ThermoFisher Scientific) with an analytical threshold of 50 RFU (relative fluorescent units). Stutter peaks (n-4) lower than 15% of the parent peak were removed and two peaks at the same locus were considered heterozygous if their peak height ratio exceeded 70%. A locus was designated as homozygous if a single allele was present at greater than 150 RFU (based on an internal validation study). DNA profiles obtained from the matrix-free cells approach were compared with the DNA profiles obtained after DNA extraction.

3.3.8. DNA data analysis

The average peak height (APH) of resultant DNA profiles was calculated by the sum of the peak heights of all the autosomal alleles divided by the number of alleles recorded. Statistical significance was assessed by using R software (version 3.6.3) and two-sided paired t-test, $p \leq 0.05$ was accepted for a significance level.

3.4. Results

3.4.1. Matrix-free bone cell visualization and assessment

The presence of cells released from the mineral matrix can be seen in Fig. 27. The morphology of matrix-free cells was assessed by increasing the magnification to 220x to assess the presence of the nuclei (Fig. 28). The large number of nucleated cells were a viable target for subsequent DNA analysis.

3.4.2. Comparison of DNA extraction and matrix-free cells approach

The highest DNA yields using the commercial DNA Investigator kit were for specimens D (1180.9 ± 37.7 ng) and O (1023.2 ± 170.7 ng). The highest DNA yield for the matrix-free cells approach was for sample O (161.9 ± 27.3 ng). All DNA yields obtained via the DNA extraction process were higher than for the matrix-free cells approach (Table 6) ($p = 3.69 \times 10\text{-}5$). Analyses by real-time PCR indicated no inhibition (data not shown) and low degradation indices (DI < 10) for both methods. There were 9 samples recorded low DI for the traditional method, while 8 samples provided low DI for the matrix-free cell method. The only exception was sample A for the matrix-free samples where the average DI was 11.7 (Table 6).

Table 6: Comparison of DNA yield and quality for nine bone samples. The samples undergoing either traditional DNA extraction or the matrix-free cells approach.

Method	Average DNA yield (ng)	Degradation index (DI)	Average peak height (RFU)	% of STR loci completed
Traditional method	383.5 ± 31.5	1.5 ± 0.2	5534.2	100
Matrix-free cells method	34.0 ± 4.5	4.7 ± 1.3	5372.4	93.3

Data of DNA yield and degradation index are presented as mean ± 1 SD (n = 4 replicates).

3.4.3. Autosomal STR profiles

Data pertaining to APH and profile completeness are shown in Table 6. STR profiles generated from samples undergoing the traditional DNA extraction method were generated from 1 ng of DNA template. Complete single-source DNA profiles were recorded from all 9 samples (100% of STR loci successfully typed). Positive control (DNA Control 007) gave a complete DNA profile (data not shown). Elimination comparisons to the author and other reseachers were done to ensure they did not contribute the DNA profiles obtained. In addition, none of the samples matched one another.

For the matrix-free cells method, complete single-source DNA profiles were recorded from 7 samples (100% of STR loci successfully typed) using 1 ng of DNA template. Two of the matrix-free samples, samples A and K, generated 28 of the possible 30 alleles (93.3% of STR loci successfully typed), with much lower APH values. Samples A and J had the lowest yield of DNA and hence the maximum template that could be added to the PCR was only 0.1 ng. The average DI values for matrix-free cell samples were higher than for traditional DNA extracts for all 9 samples ($p = 0.02231$). The STR profiles of sample O obtained by the two methods are illustrated in Fig. 29.

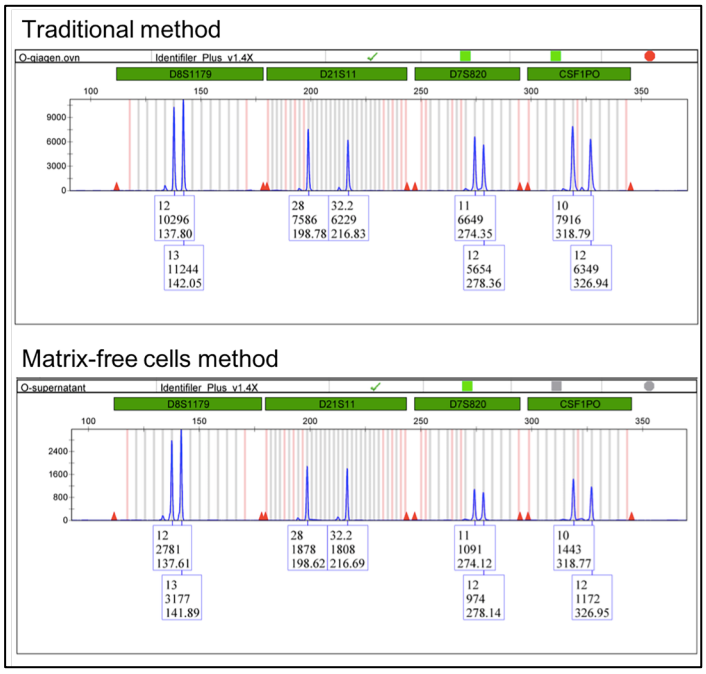


Figure 29: STR profiles from sample O (6-FAM™ (blue) dye lane only). Top panel: amplification of 1 ng DNA obtained via traditional DNA extraction. Bottom panel: direct amplification of 1 ng of matrix-free supernatant DNA.

3.5. Discussion

DNA recovery from bone powder is commonly used in forensic laboratories as template for DNA profiling [2, 12, 13, 53, 145]. With no means of knowing whether DNA is present, or the mass of DNA in the powder isolated from within a bone section, inevitably varying success of STR profiling will ensue. A partial or complete demineralization step using EDTA to remove calcium from bones is often unavoidable if bones are compromised [53, 58, 146]. Any residual EDTA can inhibit subsequent enzymatic analyses due to the formation of Mg^{2+} complexes. When examining fresh bones, a protocol such as the QIAamp DNA Investigator Kit (QIAGEN) omits a decalcification step, but an overnight incubation in lysis buffer is required. An approach of rapidly isolating matrix-free cells, as described in this study, provides a simplified and time-effective method for STR profiling from bone. By targeting matrix-free cells, decalcification and DNA extraction steps are omitted and addition of inhibitors of PCR, such as EDTA, avoided.

Currently, the standard process for DNA analysis of bone uses a DNA extraction, yet any such process results in loss of DNA [60, 147]. The semi-direct approach applied in this report offers an alternative procedure to minimize the loss of DNA in downstream analyses. However, limitations of any method that omits a quantification step, and removal of inhibitors, can lead to unknown quality and quantity of DNA as the template for STR typing; leading potentially to off-scale or pull-up peaks, or allelic drop-out in samples with low DNA concentration [81]. Here, matrix-free bone cells were initially diluted in deionized water, which resulted in the ability to first visualize the stained nuclei and quantify the available DNA material with real-time PCR.

Degradation indices (DI) of DNA obtained from the matrix-free cells method were higher than for the traditional extraction method. Our data show that two samples (A and K) gave the highest DI for DNA from the matrix-free cells and also had a low amount of template DNA; a combination of these factors is most likely the reason for obtaining incomplete STR profiles.

3.6. Conclusion

Diamond Dye staining was shown to confirm the release of nucleated bone cells from the matrix. The stained nuclei were observed when released into the supernatant during a stream-lined DNA isolation method as described. Amplification of these matrix-free bone cells provided a simple approach which resulted in informative DNA profiles without the need for demineralization and a lengthy DNA extraction process. It offers a rapid and reliable approach for STR profiling from bone specimens within 8 hours. A prime reason for any standard DNA extraction process is to remove inhibitors of PCR, such as calcium in the case of bone; yet the matrix-free cells approach described in this paper was able to successfully generate DNA profiles in the absence of these steps [2, 11, 129, 145, 146]. For highly compromised samples, the matrix-free cells method still provided an informative profile, rather than a complete profile with all loci typed. The residuals such as the pellets may be beneficial to the state of the traditional extraction method. Significantly, the matrix-free cells method does not require the use of mechanical devices to powder the bone nor liquid nitrogen to freeze the bone powders. This simple method of matrix-free cells has potential use for rapid human identification when working with bone samples but also for other areas where human or other animal bones are encountered.

3.7. Highlights

- Matrix-free bone cells are a potential template for autosomal DNA profiling without decalcification and extraction.
- A combination of morphological assessment and real-time PCR provides an efficient tool for STR-based identification from bones.
- PCR of matrix-free osteocytes allows a quick turn-around time for osteochondral bone samples.
- Osteochondral bone is a rich source for high quantities of nuclear DNA in bone-sample selection.

3.8. Conflict of interest

The authors declare no conflict of interest.

3.9. Acknowledgement

The authors are grateful for access and use of the instruments at the Flinders Microscopy (Light Microscopy), Flinders Medical Centre; and Centre for Orthopedic and Trauma Research (The University of Adelaide). This research was funded by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund.

SUPPLEMENTARY TABLE: Data of DNA yield and quality for nine bone samples. Conventional DNA extraction and the matrix-free cells approach are shown.

Sample ID	Age	Method	Average DNA yield (ng)	Degradation index (DI)	Average peak height (RFU)	Number of alleles (30)
A	59	Extraction	7.0 ± 0.8	1.8 ± 0.2	5,505	30
		Matrix-free cells	0.9 ± 0.2	11.7 ± 5.1	294	28
C	69	Extraction	72.3 ± 11.3	1.2 ± 0.0	3,334	30
		Matrix-free cells	5.7 ± 0.8	3.4 ± 0.6	1,276	30
D	-	Extraction	1180.9 ± 37.7	1.0 ± 0.1	415	30
		Matrix-free cells	37.0 ± 2.2	2.4 ± 0.2	2,608	30
E	81	Extraction	520.0 ± 26.6	1.0 ± 0.1	11,835	30
		Matrix-free cells	15.5 ± 3.9	2.3 ± 0.3	12,536	30
F	63	Extraction	402.1 ± 19.5	1.1 ± 0.1	1,382	30
		Matrix-free cells	73.6 ± 4.4	2.0 ± 0.1	11,149	30
J	61	Extraction	13.7 ± 2.3	1.6 ± 0.3	9,990	30
		Matrix-free cells	1.0 ± 0.2	6.5 + 2.1	884	30
K	76	Extraction	6.9 ± 1.3	3.4 ± 0.3	5,407	30
		Matrix-free cells	6.8 ± 0.6	8.2 ± 2.3	249	28
O	58	Extraction	1023.2 ± 170.7	0.9 ± 0.2	10,204	30
		Matrix-free cells	161.9 ± 27.3	1.5 ± 0.3	18,889	30
T	69	Extraction	225.5 ± 13.5	1.1 ± 0.1	1,736	30
		Matrix-free cells	3.2 ± 0.8	4.4 ± 0.6	467	30

DNA yield is based on real-time quantification data, using four replicates for each sample. Real-time data were used to record the degradation indices. A DI value ≥ 10 is defined as 'possibly degraded' according to the Investigator® Quantiplex Pro RGQ kit (QIAGEN). The average RFU data is based on 15 STR loci within the Identifiler Plus kit. The highest number of alleles was 30 with loci designated as homozygous counted as two alleles. Data of DNA yield and degradation index are presented as mean ± 1 SD (n = 4 replicates).

CHAPTER 4: CONCLUSION

4.1. Preface

The central aim of this MSc project was to provide a simple and effective means to isolate high quality DNA from bone. This aim was addressed by use of a DNA binding dye to stain and then visualise cellular material within bone sections. Directly related to the ability to stain cells within bone sections was the opportunity to generate STR DNA profiles from the cells and determine a relationship for future analyses between the number of cells present and the chance to generate a DNA profile. Isolating DNA using processes that are quicker than previous and current methods, that reduce the opportunity to introduce contaminating DNA, and conduct the process without specialist equipment were addressed in this MSc thesis.

4.2. Concluding remarks

Based on a wide range applifications of staining biological material with DD (such as hairs, sperms, latent touch DNA), it was demonstrated DD is a safe dye that not impact on the quantity and quality of DNA molecules in downstream DNA analysis process. This thesis details the first use of DD staining applied to detecting DNA within bone tissues.

The visualisation of the number and location of cells containing nuclei provided a key means to assessed the chance of obtaining STR profiles from bone formalin fixed and paraffin embedded sections. DD stained cells also confirmed that osteochondral specimens are a potential DNA template for the recovery of DNA from bone samples. This finding also opens a possibility of a novel triage approach. Compared to other dyes used in histology (such as hematoxylin, eosin, fuchsin), DD staining does not require any rinses or well trained techniques. Images of cells were visualised after 10 seconds after pipetting a small amount of DD (5 – 10 µL of 20x diluted dye) on the surface of the bone sections fixed on slide. The process of recording cells is aided greatly by use of a mini-fluorescent microscope that can be attached to a laptop or tablet. The software is free to download for visualising the cells. The staining detected nucleated cells in several types of bone cells such as chondrocytes, osteoblasts, osteoclasts and osteocytes. It can be concluded that DD is a promissing dye used in forensic DNA, but also in forensic pathology laboratories or histological studies.

In order to achieve the best recovery of DNA from bone specimens, some improvements have been performed in the pre-lysis process. For example, a total decalcification requires many

hours or days to dissolve completely bone powder, resulting in efficient release of DNA molecules. The recent procedures can be a time-consuming protocol from the begining steps to DNA profiling. The presence of the matrix-free cells was visualised in the supernatant allowing a speedy process of DNA profiling from bones. There is no requirements of powdering, specific related equipment and reagent (such as drilling tool, liquid nitrogen) as well as lengthy demineralisation; the net outcome is the recovery of DNA from bones can be proceeded within 8 hours. Additionally, the process described in this thesis avoids having to perform multiple changes of decalcified/lysis solution or tube changes via a series of washes in extraction process; the rapid process minimises introduction of contaminating DNA and prevention of DNA loss. This investigation offers a rapid, simple and reliable protocol for laboratories with limited funds. Labor-intensive manual procedures or outsource to specialised laboratories is minimised. Commercial extraction kits and large volume of lysis buffer are also not required due to the direct quantification and amplification of matrix-free cells. The isolation and release of matrix-free bone cells has been investigated in this study which opens up a novel approach for challenging skeletal samples such as degraded bones or ancient bones. As shown in this thesis, direct amplification of matrix-free cells increased the success of STR profiling from samples presenting low number of cells or degraded DNA.

The study of STR profiling from FFPE bone specimens (recorded in the first publication) involves some limitations. Fixation in formalin and embedding in paraffin is a lengthy process for bone blocks prior to DNA analysis. Formalin is recorded as a reagent which is responsible for DNA degradation because of the process of cross linkage. Bone samples needed to be cut into extremely thin slices known as sections (5 µm thickness) from paraffin bone blocks, resulting in slicing through the cells. In this process, chromatids may be also sliced with the result of part of a chromatid being in one section and part in another and lost locations, allowing a chance for success of amplification. Hence, the thickness of bone sections is an essential factor in yielding the best recovery of DNA and typing informative STR profiles. In this study, based on the dimensions of bone cells, the thickness was ideally chosen as 5 µm in order to enhance subsequent DNA process. If the bone is cut into thicker sections, this may inevitably contain higher amount of inorganic materials (calcium), thus requiring a longer decalcification instead of overnight process. In case the bone is sliced into sections where chromatids are cut through leading to poor access to successful STR amplification. Due to these limitations of FFPE bone specimens, a fluorescent microscope was required to observe and monitor the presence of cells containing nuclei as well as

the number of these nucleated cells, allowing greater confidence in the opportunity to obtain a DNA profile, or opt not to proceed further. Given the aforementioned, it is therefore not surprising that FFPE bone blocks have not been the focus of study for DNA profiling in forensic laboratories.

Second publication describes the release of matrix-free cells from fragmented bone specimens. The process outlined in the thesis resulted in an adequate amount of DNA for amplification resulting in informative STR profiles. While low degradation indices (DI < 10) were recorded from 7 out of 9 samples, there were two samples that showed higher DI, resulting in incomplete profiles typed. In this case, the remaining pellets containing fragmented bone samples will be administered to a traditional extraction to yield sufficient DNA for STR profiling.

4.3. Impact to forensic identification and further investigation

Archived bone materials are commonly used in pathology, but they are not a common template in DNA in forensic casework. Hence, less considerations have been given to the use of DNA profiling from this type of material. If there are any cases where archived bones are the only source of ante-mortem material for identification purposes, forensic analysts will be faced with some challenging factors, such as degradation and inhibition prior to typing STR profiles. To aid the workflow of STR profiling from formalin-fixed and paraffin embedded human bones, an inspection of cells containing DNA was carried out using DD prior to downstream DNA analysis. The number of nucleated cells or empty-cell lacunae were recorded in relation to the degradation status of samples and success rate of STR profiling. Publication I (chapter 2) reported on the application of DD to human bone samples, all types of bone cells were visualised rapidly within 10 seconds. DD staining can be applied to further studies of human or animal skeletal remains, such as histology, paleontology, or archaeology exploring prehistoric life forms.

Fragmented bone samples are more commonly encountered compared to preserved sections. A process is reported where bone cells can be release from the matrix and removed to a solution. Here the cells remain suspended in the supernatant. These are the initial steps in an alternative approach to successful STR profiling from archived bones (publication II – chapter 3). The presence of population of cells released from the mineral matrix of the bone (known as matrix-free cells in this study) was confirmed by DD staining, allowing a subsequent direct quantification and amplification to have been performed. A lengthy process of removal of calcium was not required, avoiding a waste of large volume of decalcification solution (approximately 3 – 5 mL of

0.5 M EDTA per day). It is crucial to notice that any undissolved bone material present in the supernatant will also disposed along with EDTA changes. This will result in a loss of cells, reducing the template for downstream DNA processes. By using the matrix-free cells suspended in the supernatant for direct STR profiling, any risk of cell loss or introduction of contaminatant is minimised. This approach offers a promising method for forensic analysts due to the simplification and reliability. Further, the matrix-free cells method provides the flexibility for forensic technicians to assess the possibility of complete STR profiling in any standard DNA laboratory, which does not have bone-related equipments and reagents. Ultimately, the technique broadens further applications for skeletal samples with wide ranges of degradation levels such as ancient bones and teeth in an identification of forensic biology, anthropology or paleopedology.

It is conjectured that based on the success of direct STR profiling from the matrix-free cells demonstrated in this thesis, a number of human bone remains found from mass disaster and wars could be examined in the same way. These remains may have been subjected to a wide range of degradation status due to environmental exposures. Biological materials containing DNA collected from the supernatant using the matrix-free cells method, the cell number quantified, and DNA amplified directly to generate nuclear STR profiles. These data could complement that from the mitchodrondrial genome (involving two hypervariable regions (HV1, HV2) of the D-loop of mitochondrial DNA). The morphology of bone cells containing nuclei can be visualised using DD staining in parallel of direct quantification and amplification from matrix-free cells template. The use of traditional DNA typing process will be performed to compare the results. A total decalcification will be carried out prior to DNA extraction procedure.

4.4. Final statement

This thesis has outlined a novel process of cell assessment from bone samples. The approach to locate and release matrix-free cells into a forensic science workflow speeds current methods. Cell morphology shows the presence of nuclei, aids the prediction of a success of STR profiling. An approach of matrix-free cells population boosts the recovery of DNA from bone specimens by preventing cell/DNA loss through demineralisation and DNA isolation process. This is a promising technique to yield the best DNA from challenging samples with degradation in further studies. Also, financial barriers are minimised due to no requirements of commercial isolation kit, reagents and specific equipments. The method will be studied in molecular biology laboratories (even with

limited funds) in order to generate a comprehensive database of nuclear STR profiles and mitochondrial profiles from skeletal specimens.

CHAPTER 5: APPENDICES

5.1. Images of Diamond dye stained cells

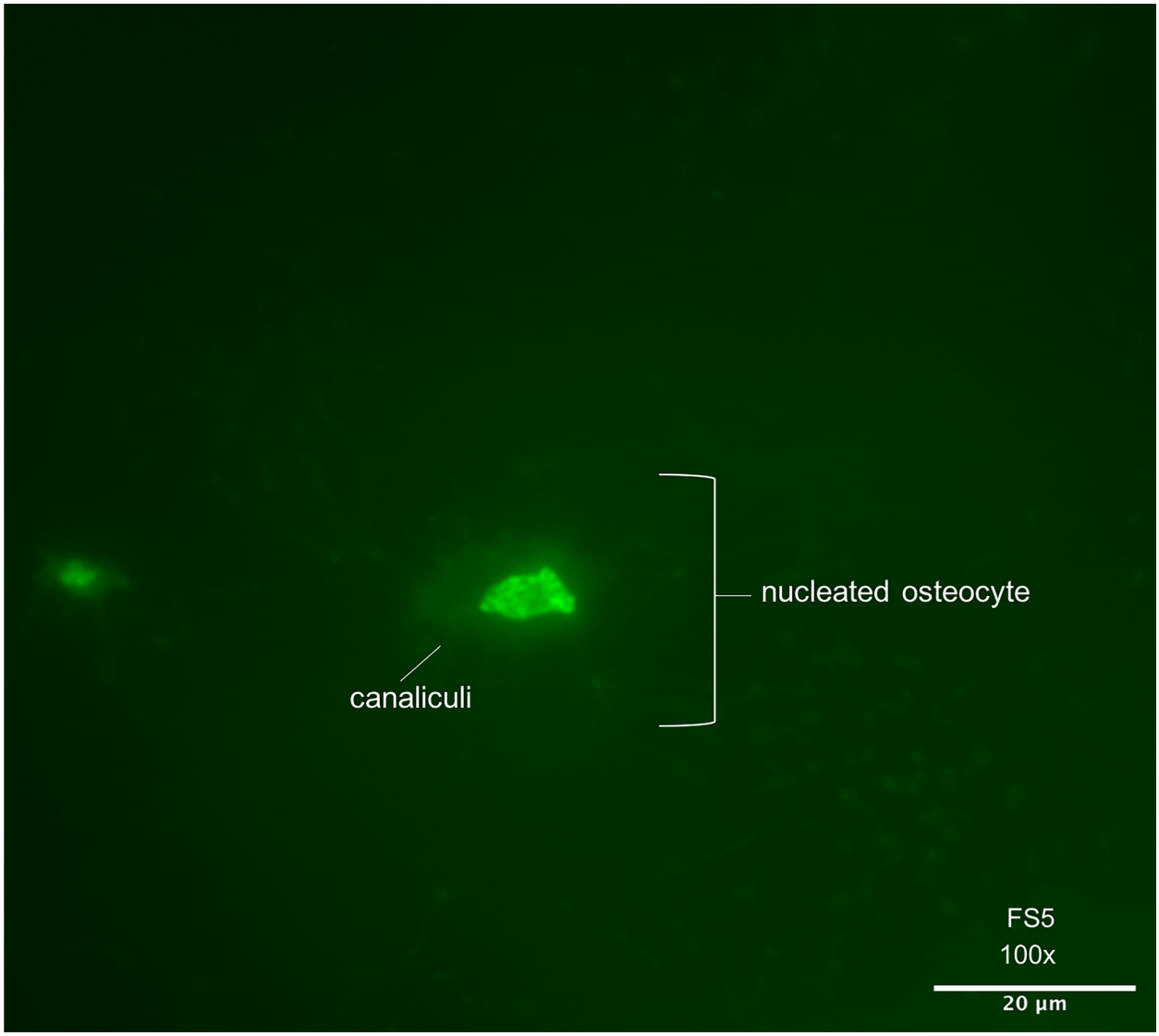
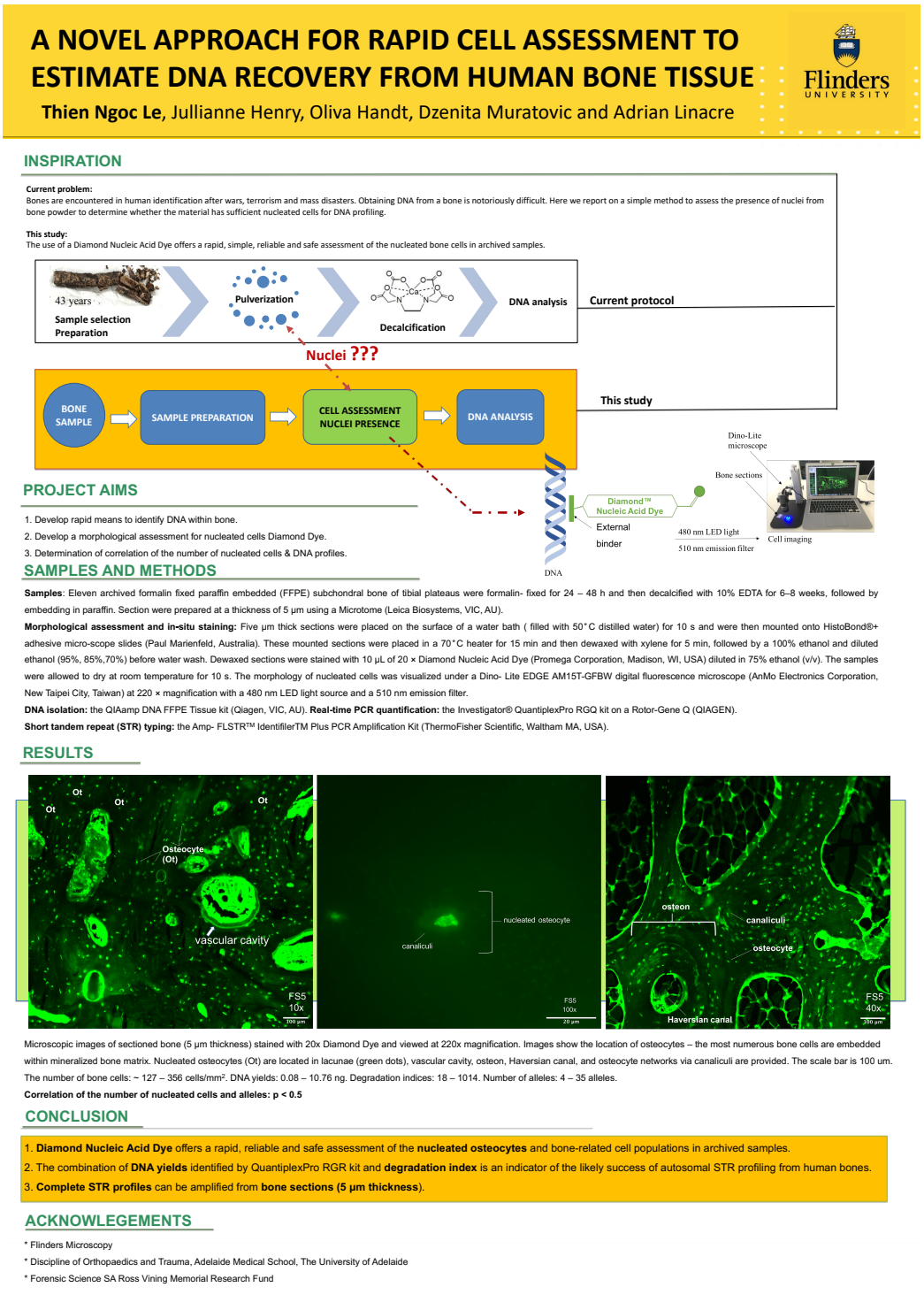


Figure 30: Morphology of an individual nucleated osteocyte. Showing a formalin-fixed and resin-embedded section of an osteochondral bone (sample FS5) after staining with 20x DD. An individual nucleated osteocyte with the lacuno-canalicular network is visualised. The image photographed at 100x magnification. The scale bar is 20 µm.

5.2. Poster presentation

The poster presented at 1st Annual College of Science and Engineering Higher Degree by Research (HDR) Symposium, Flinders University, Adelaide, Australia, November 2021.



CHAPTER 6: REFERENCES

[1] C. Murray, C. McAlister, K. Elliott, Identification and isolation of male cells using fluorescence in situ hybridisation and laser microdissection, for use in the investigation of sexual assault, Forensic Sci Int Genet 1(3) (2007) 247-252.
[2] M.J. Dukes, A.L. Williams, C.M. Massey, P.W. Wojtkiewicz, Technical note: Bone DNA extraction and purification using silica-coated paramagnetic beads, Am J Phys Anthropol 148(3) (2012) 473-482.
[3] M. Harrel, S. Hughes-Stamm, A Powder-free DNA Extraction Workflow for Skeletal Samples, J Forensic Sci 65(2) (2020) 601-609.
[4] K. Imaizumi, Forensic investigation of burnt human remains, Res. rep. forensic med. sci 5 (2015) 67-74.
[5] E.S.M. Iwamura, C.R.G.C.M. Oliveira, J.A. Soares-Vieira, S.A.B. Nascimento, D.R. Muñoz, A qualitative study of compact bone microstructure and nuclear short tandem repeat obtained from femur of human remains found on the ground and exhumed 3 years after death, Am J Forensic Med Pathol 26(1) (2005) 33-44.
[6] J. Jakubowska, J. Jakubowska, A. Maciejewska, A. Maciejewska, R. Pawłowski, R. Pawłowski, Comparison of three methods of DNA extraction from human bones with different degrees of degradation, Int J Legal Med 126(1) (2012) 173-178.
[7] I. Zupanic Pajnic, B. Gornjak Pogorelc, J. Balazic, T. Zupanc, B. Stefanic, Highly efficient nuclear DNA typing of the World War II skeletal remains using three new autosomal short tandem repeat amplification kits with the extended European Standard Set of loci, Croat Med J 53(1) (2012) 17-23.
[8] A. Wheeler, N. Czado, D. Gangitano, M. Turnbough, S. Hughes-Stamm, Comparison of DNA yield and STR success rates from different tissues in embalmed bodies, Int J Legal Med 131(1) (2017) 61-66.
[9] D.Y. Yang, B. Eng, J.S. Waye, J.C. Dudar, S.R. Saunders, Improved DNA extraction from ancient bones using silica-based spin columns, Am. J. Phys. Anthropol. 105(4) (1998) 539-543.
[10] N. Rohland, M. Hofreiter, Ancient DNA extraction from bones and teeth, Nature protocols 2(7) (2007) 1756.
[11] S. Desmyter, C. De Greef, A more efficient extraction method of human bone resulting in improved DNA profiling, Forensic Sci. Int. Genet. 1(1) (2008) 24-25.
[12] QiagenTeam, Isolation of Total DNA from Bones and Teeth, 2020.
[13] P. Corporation, Bone Extraction Protocol to be Used With the DNA IQ™ System, 2008.
[14] C. Bart, Normal Bone Anatomy and Physiology, Clin J Am Soc Nephrol 3(Supplement 3) (2008) S131-S139.
[15] S. Tadano, B. Giri, X-ray diffraction as a promising tool to characterize bone nanocomposites, Science and technology of advanced materials (2012).
[16] H.K. Datta, W.F. Ng, J.A. Walker, S.P. Tuck, S.S. Varanasi, The cell biology of bone metabolism, J. Clin. Pathol. 61(5) (2008) 577-587.
[17] J. Klein-Nulend, L. Bonewald, Chapter 8 - The Osteocyte, in: J.P. Bilezikian, L.G. Raisz, T.J. Martin (Eds.), Principles of Bone Biology (Third Edition), Academic Press, San Diego, 2008, pp. 153-174.
[18] T.N. Le, Dzenita Muratovic, O. Handt, J. Henry, A. Linacre, A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue, Forensic Science, Medicine and Pathology (2021).

[19] T. Nakano, Y. Tabata, Y. Umakoshi, Texture and Bone Reinforcement, in: K.H.J. Buschow, R.W. Cahn, M.C. Flemings, B. Ilschner, E.J. Kramer, S. Mahajan, P. Veyssière (Eds.), Encyclopedia of Materials: Science and Technology, Elsevier, Oxford, 2005, pp. 1-8.
[20] J.A. Buckwalter, M.J. Glimcher, R.R. Cooper, R. Recker, Bone biology. I: Structure, blood supply, cells, matrix, and mineralization, Instr. Course Lect. 45 (1996) 371-386.
[21] P.A. Downey, M.I. Siegel, Bone biology and the clinical implications for osteoporosis, Physical therapy 86(1) (2006) 77-91.
[22] B. Clarke, Normal bone anatomy and physiology, Clinical journal of the American Society of Nephrology 3(Supplement 3) (2008) S131-S139.
[23] V. Everts, J.M. Delaisse, W. Korper, D.C. Jansen, W. Tigchelaar-Gutter, P. Saftig, W. Beertsen, The bone lining cell: its role in cleaning Howship's lacunae and initiating bone formation, J Bone Miner Res 17(1) (2002) 77-90.
[24] A.P. Singh, Bone Anatomy and Physiology. <https://boneandspine.com/cortical-bone-and-cancellous-bone/#what-is-cortical-bone>, (accessed 27 May.2022).
[25] R. Florencio-Silva, G.R.d.S. Sasso, E. Sasso-Cerri, M.J. Simões, P.S. Cerri, Biology of Bone Tissue: Structure, Function, and Factors That Influence Bone Cells, Biomed Res Int 2015 (2015) 1-17.
[26] S.C. Manolagas, A.M. Parfitt, What old means to bone, Trends Endocrinol Metab 21(6) (2010) 369-374.
[27] J.D. Currey, The many adaptations of bone, J Biomech 36(10) (2003) 1487-1495.
[28] K. Matsuo, N. Irie, Osteoclast–osteoblast communication, Arch Biochem Biophys 473(2) (2008) 201-209.
[29] S.C. Miller, L. de Saint-Georges, B.M. Bowman, W.S. Jee, Bone lining cells: structure and function, Scanning Microsc 3(3) (1989) 953-60; discussion 960-1.
[30] Bone health and osteoporosis : a report of the surgeon general, Office of the Surgeon General US2004.
[31] J. Green, S. Schotland, D.J. Stauber, C.R. Kleeman, T.L. Clemens, Cell-matrix interaction in bone: type I collagen modulates signal transduction in osteoblast-like cells, Am. J. Physiol. Cell Physiol. 268(5) (1995) C1090-C1103.
[32] S.W. Verbruggen, L.M. McNamara, Chapter 6 - Bone mechanobiology in health and disease, in: S.W. Verbruggen (Ed.), Mechanobiology in Health and Disease, Academic Press2018, pp. 157-214.
[33] U. Kini, B.N. Nandeesh, Physiology of bone formation, remodeling, and metabolism, Radionuclide and hybrid bone imaging, Springer2012, pp. 29-57.
[34] R. Owen, G.C. Reilly, In vitro models of bone remodelling and associated disorders, Frontiers in bioengineering and biotechnology (2018) 134.
[35] Y.M. Coulson-Thomas, A.L. Norton, V.J. Coulson-Thomas, R. Florencio-Silva, N. Ali, S. Elmrghni, C.D. Gil, G.R.S. Sasso, R.A. Dixon, H.B. Nader, DNA and bone structure preservation in medieval human skeletons, Forensic science international 251 (2015) 186-194.
[36] S. Charlton, T. Booth, I. Barnes, The problem with petrous? A consideration of the potential biases in the utilization of pars petrosa for ancient DNA analysis, World archaeology 51(4) (2019) 574-585.
[37] D. Gaudio, D.M. Fernandes, R. Schmidt, O. Cheronet, D. Mazzarelli, M. Mattia, T. O'Keeffe, R.N.M. Feeney, C. Cattaneo, R. Pinhasi, Genome-wide DNA from degraded petrous bones and the

assessment of sex and probable geographic origins of forensic cases, Scientific reports 9(1) (2019) 1-11.
[38] Z.M. Budimlija, M.K. Prinz, A. Zelson-Mundorff, J. Wiersema, E. Bartelink, G. MacKinnon, B.L. Nazzaruolo, S.M. Estacio, M.J. Hennessey, R.C. Shaler, World Trade Center human identification project: experiences with individual body identification cases, Croat Med J 44(3) (2003) 259-263.
[39] G.C. Calacal, B.G. Gallardo, D.L.T. Apaga, M.C.A. De Ungria, Improved autosomal STR typing of degraded femur samples extracted using a custom demineralization buffer and DNA IQ™, Forensic Science International: Synergy 3 (2021) 100131.
[40] S. Desmyter, C. De Greef, A more efficient extraction method of human bone resulting in improved DNA profiling, Forensic science international. Genetics supplement series 1(1) (2008) 24-25.
[41] A. Mundorff, J.M. Davoren, Examination of DNA yield rates for different skeletal elements at increasing post mortem intervals, Forensic Science International: Genetics 8(1) (2014) 55-63.
[42] J.A. Ramos-Vara, Technical aspects of immunohistochemistry, Vet Pathol 42(4) (2005) 405-426.
[43] R.A. Skinner, Decalcification of Bone Tissue, in: Y.H. An, K.L. Martin (Eds.), Handbook of Histology Methods for Bone and Cartilage, Humana Press, Totowa, NJ, 2003, pp. 167-184.
[44] D.C. Sheehan, Theory and practice of histotechnology, Saint Louis, C. V. Mosby Co., Saint Louis, 1973.
[45] S. Taqi, S. Sami, L. Sami, S. Zaki, A review of artifacts in histopathology, J Oral Maxillofac Pathol 22(2) (2018) 279-279.
[46] D.H. Chow, L. Zheng, L. Tian, K.-S. Ho, L. Qin, X. Guo, Application of ultrasound accelerates the decalcification process of bone matrix without affecting histological and immunohistochemical analysis, J Orthop Translat 17 (2019) 112-120.
[47] G.O. Rolls, Difficult blocks and reprocessing, Wetzlar: Leica Microsystems (2011).
[48] H.M. Frost, In vivo osteocyte death, JBJS 42(1) (1960) 138-143.
[49] O.T. Avery, C.M. MacLeod, M. McCarty, Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III, J. Exp. Med 79(2) (1944) 137-158.
[50] J.D. Watson, F.H.C. Crick, Molecular structure of nucleic acids: a structure for deoxyribose nucleic acid, Nature 171(4356) (1953) 737-738.
[51] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular cloning: a laboratory manual, Cold spring harbor laboratory press1989.
[52] S. Köchl, H. Niederstätter, W. Parson, DNA extraction and quantitation of forensic samples using the phenol-chloroform method and real-time PCR, Forensic DNA typing protocols, Springer2005, pp. 13-29.
[53] O.M. Loreille, T.M. Diegoli, J.A. Irwin, M.D. Coble, T.J. Parsons, High efficiency DNA extraction from bone by total demineralization, Forensic Sci. Int. Genet. 1(2) (2007) 191-195.
[54] P.S. Walsh, D.A. Metzger, R. Higuchi, Chelex 100 as a medium for simple extraction of DNA for PCR-based typing from forensic material, Biotechniques 10(4) (1991) 506-13.
[55] H.E. McKiernan, P.B. Danielson, Molecular Diagnostic Applications in Forensic Science, 2017, pp. 371-394.

[56] D.Y. Yang, B. Eng, J.C. Dudar, S.R. Saunders, J.S. Waye, Removal of PCR inhibitors using silica-based spin columns: application to ancient bones, Can Soc Forensic Sci 30(1) (1997) 1-5.
[57] J. Rothe, M. Nagy, Comparison of two silica-based extraction methods for DNA isolation from bones, Leg. Med. 22 (2016) 36-41.
[58] J. Davoren, D. Vanek, R. Konjhodzić, J. Crews, E. Huffine, T.J. Parsons, Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves, Croatian medical journal 48(4) (2007) 478.
[59] E. Ozdemir-Kaynak, O. Yesil-Celiktas, Microwave-assisted digestion combined with silica-based spin column for DNA isolation from human bones, Anal. Biochem. 486 (2015) 44-50.
[60] A. Barbaro, N. Staiti, P. Cormaci, L. Saravo, DNA profiling by different extraction methods, Int. congr. ser. 1261(C) (2004) 562-564.
[61] N. Rohland, M. Hofreiter, Comparison and optimization of ancient DNA extraction, Biotechniques 42(3) (2007) 343-352.
[62] P.L. Marshall, M. Stoljarova, S.E. Schmedes, J.L. King, B. Budowle, A high volume extraction and purification method for recovering DNA from human bone, Forensic Sci. Int. Genet. 12 (2014) 155-160.
[63] B.B. Panda, A.S. Meher, R.K. Hazra, Comparison between different methods of DNA isolation from dried blood spots for determination of malaria to determine specificity and cost effectiveness, J Parasit Dis. 43(3) (2019) 337-342.
[64] A. Linacre, J.E.L. Templeton, Forensic DNA profiling: state of the art, Res. rep. forensic med. sci. 4 (2014) 25-36.
[65] F. Di Pasquale, S. Cornelius, M. König, M. Scherer, A. Prochnow, R. Peist, Analysis and interpretation of difficult samples—The interaction of a DNA quantification kit with the right STR assay may facilitate processing of critical trace material, Forensic Sci. Int. Genet. 5 (2015) e407-e408.
[66] T.F. Scientific, AmpFlSTR® Identifiler® Plus PCR Amplification Kit User's Guide, Waltham, MA, USA: Thermo Fisher Scientific (2012).
[67] T.F. Scientific, Quantifiler(R) HP and Trio DNA Quantification Kits User Guide 2017.
[68] I. Technologies, InnoQuant(R) HY User Guide v1.5 Dec 2016.
[69] P. Corporation, PowerQuant System Technical Manual March 2015.
[70] QIAGEN, Investigator® Quantiplex® Pro RGQ Kit Handbook, Feb 2018.
[71] QIAGEN, Evaluation of the Investigator Quantiplex Pro RGQ Kit for DNA quantification and quality assessment of forensic casework samples, 2018.
[72] A.S. Holmes, R. Houston, K. Elwick, D. Gangitano, S. Hughes-Stamm, Evaluation of four commercial quantitative real-time PCR kits with inhibited and degraded samples, Int. J. Legal Med. 132(3) (2018) 691-701.
[73] A. Barbaro, S. Samar, G. Falcone, A. La Marca, Highly efficient and automated extraction of DNA from human remains using a modified EZ1 protocol, Forensic Sci. Res. 6(1) (2021) 59-66.
[74] T.F. Scientific, GlobalFiler™ and GlobalFiler™ IQC PCR Amplification Kits: User Guide., Waltham, MA, USA: Thermo Fisher Scientific (2019).
[75] L.I. Zon, D.M. Dorfman, S.H. Orkin, The polymerase chain reaction colony miniprep, Biotechniques 7(7) (1989) 696-698.
[76] D.Y. Wang, C.-W. Chang, N.J. Oldroyd, L.K. Hennessy, Direct amplification of STRs from blood or buccal cell samples, Forensic Sci. Int. Genet. 2(1) (2009) 113-114.

[77] D. Wieczorek, B.E. Krenke, Direct amplification from buccal and blood samples preserved on cards using the PowerPlex® 16 HS System, Profiles in DNA, 2009.
[78] R. Roy, STR typing from bloodstained fabric without DNA extraction.
[79] S. Verheij, J. Harteveld, T. Sijen, A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples, Forensic Sci. Int. Genet. 6(2) (2012) 167-175.
[80] A. Linacre, V. Pekarek, Y.C. Swaran, S.S. Tobe, Generation of DNA profiles from fabrics without DNA extraction, Forensic Sci Int: Genetics 4(2) (2009) 137-141.
[81] B. Martin, A. Linacre, Direct PCR: A review of use and limitations, Sci. Justice 60(4) (2020) 303-310.
[82] J. Hedman, A. Nordgaard, C. Dufva, B. Rasmusson, R. Ansell, P. Rådström, Synergy between DNA polymerases increases polymerase chain reaction inhibitor tolerance in forensic DNA analysis, Anal. Biochem. 405(2) (2010) 192-200.
[83] N. Zahra, S. Hadi, J.A. Smith, A. Iyengar, W. Goodwin, Development of internal amplification controls for DNA profiling with the AmpFℓSTR® SGM Plus® kit, Electrophoresis 32(11) (2011) 1371-1378.
[84] R. Weispfenning, K. Oostdik, M. Ensenberger, B. Krenke, C. Sprecher, D. Storts, Doing more with less: Implementing direct amplification with the PowerPlex® 18D System, Forensic Sci. Int. Genet. 3(1) (2011) 409-410.
[85] C.H. Phua, T. Kitpipit, J. Pradutkanchana, P. Duangsuwan, W. Samai, P. Thanakiatkrai, Direct STR typing from human bones, Forensic Sci Int 330 (2022) 111099.
[86] J.M. Butler, Forensic DNA typing biology, technology, and genetics of STR markers, 2nd ed., London : Elsevier Academic Press, London, 2005.
[87] J.L. Weber, Human DNA polymorphisms and methods of analysis, Curr Opin Biotechnol 1(2) (1990) 166-171.
[88] P.M. Schneider, K. Bender, W.R. Mayr, W. Parson, B. Hoste, R. Decorte, J. Cordonnier, D. Vanek, N. Morling, M. Karjalainen, C. Marie-Paule Carlotti, M. Sabatier, C. Hohoff, H. Schmitter, W. Pflug, R. Wenzel, D. Patzelt, R. Lessig, P. Dobrowolski, G. O'Donnell, L. Garafano, M. Dobosz, P. de Knijff, B. Mevag, R. Pawlowski, L. Gusmão, M. Conceicao Vide, A. Alonso Alonso, O. García Fernández, P. Sanz Nicolás, A. Kihlgreen, W. Bär, V. Meier, A. Teyssier, R. Coquoz, C. Brandt, U. Germann, P. Gill, J. Hallett, M. Greenhalgh, STR analysis of artificially degraded DNA—results of a collaborative European exercise, Forensic Sci Int 139(2) (2004) 123-134.
[89] A.M. Haines, S.S. Tobe, H. Kobus, A. Linacre, Duration of in situ fluorescent signals within hairs follicles, Forensic Sci. Int. Genet. 5 (2015) e175-e176.
[90] A.M. Haines, S.S. Tobe, H.J. Kobus, A. Linacre, Effect of nucleic acid binding dyes on DNA extraction, amplification, and STR typing, Electrophoresis 36(20) (2015) 2561-2568.
[91] A.M. Haines, S.S. Tobe, H.J. Kobus, A. Linacre, Properties of nucleic acid staining dyes used in gel electrophoresis, Electrophoresis 36(6) (2015) 941-944.
[92] A.M. Haines, S.S. Tobe, A. Linacre, Optimization of Diamond Nucleic Acid Dye for quantitative PCR, Biotechniques 61(4) (2016) 183-189.
[93] P. Kanokwongnuwut, K.P. Kirkbride, A. Linacre, Visualising latent DNA on tapes, Forensic Sci Int Genet Suppl Ser. 7(1) (2019) 237-239.
[94] P. Kanokwongnuwut, P. Kirkbride, A. Linacre, Visualising latent DNA on swabs, Forensic Sci Int 291 (2018) 115-123.

[95] S.A. Kamel-ElSayed, L.M. Tiede-Lewis, Y. Lu, P.A. Veno, S.L. Dallas, Novel approaches for two and three dimensional multiplexed imaging of osteocytes, Bone 76 (2015) 129-140.
[96] S. Eisenberger, G. Hoppe, W. Pyerin, K. Ackermann, High-quality RNA preparation for transcript profiling of osteocytes from native human bone microdissections, Anal Biochem 335(2) (2004) 260-266.
[97] T. Ehrig, S.A. Abdulkadir, S.M. Dintzis, J. Milbrandt, M.A. Watson, Quantitative Amplification of Genomic DNA from Histological Tissue Sections after Staining with Nuclear Dyes and Laser Capture Microdissection, J Mol Diagn 3(1) (2001) 22-25.
[98] S. Seidl, R. Burgemeister, R. Hausmann, P. Betz, T. Lederer, Contact-free isolation of sperm and epithelial cells by laser microdissection and pressure catapulting, Forensic Sci Med Pathol 1(2) (2005) 153-157.
[99] T. Murase, H. Inagaki, T. Eimoto, Influence of Histochemical and Immunohistochemical Stains on Polymerase Chain Reaction, Mod Pathol 13(2) (2000) 147-151.
[100] M.P. Burton, B.G. Schneider, R. Brown, N. Escamilla-Ponce, M.L. Gulley, Comparison of Histologic Stains for Use in PCR Analysis of Microdissected, Paraffin-Embedded Tissues, Biotechniques 24(1) (1998) 86-92.
[101] T.F. Scientific, The Molecular Probes Handbook—A Guide to Fluorescent Probes and Labeling Technologies. , Thermo Fisher Scientific, Thermo Fisher Scientific website, pp. https://www.thermofisher.com/au/en/home/references/molecular-probes-the-handbook/nucleic-acid-detection-and-genomics-technology/nucleic-acid-stains.html.
[102] B. Martin, R. Blackie, D. Taylor, A. Linacre, DNA profiles generated from a range of touched sample types, Forensic Sci Int Genet 36 (2018) 13-19.
[103] T.N. Le, D. Muratovic, O. Handt, J. Henry, A. Linacre, DNA profiling from human bone cells in the absence of decalcification and DNA extraction, J. Forensic Sci. 67(4) (2022) 1690-1696.
[104] A.C. Nechifor-Boilă, A. Loghin, V. Vacariu, V.B. Halaţiu, A. Borda, The storage period of the formalin-fixed paraffin-embedded tumor blocks does not influence the concentration and purity of the isolated DNA in a series of 83 renal and thyroid carcinomas, Rom J Morphol Embryol 56(2 Suppl) (2015) 759-763.
[105] D. Muratovic, D.M. Findlay, F.M. Cicuttini, A.E. Wluka, Y.R. Lee, S. Edwards, J.S. Kuliwaba, Bone marrow lesions in knee osteoarthritis: regional differences in tibial subchondral bone microstructure and their association with cartilage degeneration, Osteoarthritis Cartilage 27(11) (2019) 1653-1662.
[106] R. Baak-Pablo, V. Dezentje, H.-J. Guchelaar, T. van der Straaten, Genotyping of DNA Samples Isolated from Formalin-Fixed Paraffin-Embedded Tissues Using Preamplification, J Mol Diagn 12(6) (2010) 746-749.
[107] A. Guyard, A. Boyez, A. Pujals, C. Robe, J. Tran Van Nhieu, Y. Allory, J. Moroch, O. Georges, J.-C. Fournet, E.-S. Zafrani, K. Leroy, DNA degrades during storage in formalin-fixed and paraffin-embedded tissue blocks, Virchows Arch 471(4) (2017) 491-500.
[108] R.W. Dapson, Macromolecular changes caused by formalin fixation and antigen retrieval, Biotech Histochem 82(3) (2007) 133-140.
[109] R.W. Dapson, Glyoxal fixation: how it works and why it only occasionally needs antigen retrieval, Biotech Histochem 82(3) (2007) 161-166.
[110] C.H. Fox, F.B. Johnson, J. Whiting, P.P. Roller, Formaldehyde fixation, J Histochemistry & Cytochemistry 33(8) (1985) 845-853.

[111] R. Thavarajah, V.K. Mudimbaimannar, J. Elizabeth, U.K. Rao, K. Ranganathan, Chemical and physical basics of routine formaldehyde fixation, J Oral Maxi Patho: JOMFP 16(3) (2012) 400-405.
[112] J.A. Kiernan, Formaldehyde, formalin, paraformaldehyde and glutaraldehyde: what they are and what they do, Microscopy today 8(1) (2000) 8-13.
[113] I. Eltoum, J. Fredenburgh, R.B. Myers, W.E. Grizzle, Introduction to the theory and practice of fixation of tissues, J Histo 24(3) (2001) 173-190.
[114] M. Kawanishi, T. Matsuda, T. Yagi, Genotoxicity of formaldehyde: molecular basis of DNA damage and mutation, Front Environment Sci 2 (2014) 1-8.
[115] S. Bonin, F. Petrera, B. Niccolini, G. Stanta, PCR analysis in archival postmortem tissues, Mol Pathol 56(3) (2003) 184-186.
[116] A. Farrugia, C. Keyser, B. Ludes, Efficiency evaluation of a DNA extraction and purification protocol on archival formalin-fixed and paraffin-embedded tissue, Forensic Sci Int 194(1) (2009) 25-28.
[117] J.B.A. Okello, J. Zurek, A.M. Devault, M. Kuch, A.L. Okwi, N.K. Sewankambo, G.S. Bimenya, D. Poinar, H.N. Poinar, Comparison of methods in the recovery of nucleic acids from archival formalin-fixed paraffin-embedded autopsy tissues, Anal Biochem 400(1) (2010) 110-117.
[118] J. Lin, S.H. Kennedy, T. Svarovsky, J. Rogers, J.W. Kemnitz, A. Xu, K.T. Zondervan, High-quality genomic DNA extraction from formalin-fixed and paraffin-embedded samples deparaffinized using mineral oil, Anal Biochem 395(2) (2009) 265-267.
[119] N. Ludyga, B. Grünwald, O. Azimzadeh, S. Englert, H. Höfler, S. Tapio, M. Aubele, Nucleic acids from long-term preserved FFPE tissues are suitable for downstream analyses, Virchows Arch 460(2) (2012) 131-140.
[120] A. Trindade-Filho, C. Mendes, S. Ferreira, S. Oliveira, A. Vasconcelos, F. Maia, H. Pak, K. Paula, DNA obtained from decomposed corpses cartilage: A comparison with skeleton muscle source, Forensic Sci Int Genetics Suppl Series 1(1) (2008) 459-461.
[121] K.E. Latham, J.J. Miller, DNA recovery and analysis from skeletal material in modern forensic contexts, Forensic Sci Res 4(1) (2018) 51-59.
[122] S.M. Edson, M. Roberts, Determination of materials present in skeletonized human remains and the associated DNA: Development of a GC/MS protocol, Forensic Sci Int: Synergy 1 (2019) 170-184.
[123] P. Kanokwongnuwut, B. Martin, D. Taylor, K.P. Kirkbride, A. Linacre, How many cells are required for successful DNA profiling?, Forensic Sci Int: Genetics 51 (2021) 1-10.
[124] D. Muratovic, D.M. Findlay, F.M. Cicuttini, A.E. Wluka, Y.R. Lee, J.S. Kuliwaba, Bone matrix microdamage and vascular changes characterize bone marrow lesions in the subchondral bone of knee osteoarthritis, Bone 108 (2018) 193-201.
[125] R.C. Team, R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/, 2013.
[126] B. Busse, D. Djonic, P. Milovanovic, M. Hahn, K. Püschel, R.O. Ritchie, M. Djuric, M. Amling, Decrease in the osteocyte lacunar density accompanied by hypermineralized lacunar occlusion reveals failure and delay of remodeling in aged human bone, Aging Cell 9(6) (2010) 1065-1075.
[127] G.R. Cindy Sampias, H&E Staining Overview: A Guide to Best Practices, Leica Biosystems.
[128] J.P. Henry, B. Bordoni, Histology, Osteoblasts, StatPearls, StatPearls Publishing

[129] J. Davoren, D. Vanek, R. Konjhodzić, J. Crews, E. Huffine, T.J. Parsons, Highly effective DNA extraction method for nuclear short tandem repeat testing of skeletal remains from mass graves, Croat Med J 48(4) (2007) 478-485.
[130] M. Vandewoestyne, F. Van Nieuwerburgh, D. Van Hoofstat, D. Deforce, Evaluation of three DNA extraction protocols for forensic STR typing after laser capture microdissection, Forensic Sci Int Genet 6(2) (2011) 258-262.
[131] M. Meredith, J.-A. Bright, S. Cockerton, S. Vintiner, Development of a one-tube extraction and amplification method for DNA analysis of sperm and epithelial cells recovered from forensic samples by laser microdissection, Forensic Sci Int Genet 6(1) (2011) 91-96.
[132] J. Morrison, S. McColl, J. Louhelainen, K. Sheppard, A. May, L. Girdland-Flink, G. Watts, N. Dawnay, Assessing the performance of quantity and quality metrics using the QIAGEN Investigator® Quantiplex® pro RGQ kit, Sci. Justice 60(4) (2020) 388-397.
[133] J.S. Buckleton, J.A. Bright, S. Gittelson, T.R. Moretti, A.J. Onorato, F.R. Bieber, B. Budowle, D.A. Taylor, The Probabilistic Genotyping Software STRmix: Utility and Evidence for its Validity, J Forensic Sci 64(2) (2019) 393-405.
[134] H.M. Frost, In vivo osteocyte death, The Journal of Bone & Joint Surgery 42(1) (1960) 138-143.
[135] S. Iyavoo, S. Hadi, W. Goodwin, Evaluation of five preservation methods for recovery of DNA from bone, Forensic Science International: Genetics Supplement Series 7(1) (2019) 200-202.
[136] J. Rothe, M. Nagy, Comparison of two silica-based extraction methods for DNA isolation from bones, Legal Medicine 22 (2016) 36-41.
[137] O.M. Loreille, T.M. Diegoli, J.A. Irwin, M.D. Coble, T.J. Parsons, High efficiency DNA extraction from bone by total demineralization, Forensic Science International: Genetics 1(2) (2007) 191-195.
[138] F.E. Duijs, T. Sijen, A rapid and efficient method for DNA extraction from bone powder, Forensic Science International: Reports 2 (2020) 100099.
[139] H. Khosravinia, K.P. Ramesha, Influence of EDTA and magnesium on DNA extraction from blood samples and specificity of polymerase chain reaction, African Journal of Biotechnology 6(3) (2007).
[140] N. Rohland, M. Hofreiter, Comparison and optimization of ancient DNA extraction, Biotechniques 42(3) (2007) 343-52.
[141] P.L. Marshall, M. Stoljarova, S.E. Schmedes, J.L. King, B. Budowle, A high volume extraction and purification method for recovering DNA from human bone, Forensic Sci Int Genet 12 (2014) 155-60.
[142] R.A.H. van Oorschot, D.G. Phelan, S. Furlong, G.M. Scarfo, N.L. Holding, M.J. Cummins, Are you collecting all the available DNA from touched objects?, International Congress series 1239(C) (2003) 803-807.
[143] A. Barbaro, N. Staiti, P. Cormaci, L. Saravo, DNA profiling by different extraction methods, International Congress series 1261(C) (2004) 562-564.
[144] T.N. Le, Dzenita Muratovic, O. Handt, J. Henry, A. Linacre, A novel approach for rapid cell assessment to estimate DNA recovery from human bone tissue, Forensic Sci Med Pathol 17(4) (2021) 649-659.
[145] F.E. Duijs, T. Sijen, A rapid and efficient method for DNA extraction from bone powder, Forensic Sci. Int. Rep. 2 (2020) 100099.

[146] G.C. Calacal, B.G. Gallardo, D.L.T. Apaga, M.C.A. De Ungria, Improved autosomal STR typing of degraded femur samples extracted using a custom demineralization buffer and DNA IQ™, Forensic Sci. Int.: Synergy 3 (2021) 100131.
[147] R.A.H. van Oorschot, D.G. Phelan, S. Furlong, G.M. Scarfo, N.L. Holding, M.J. Cummins, Are you collecting all the available DNA from touched objects?, Int. congr. ser. 1239(C) (2003) 803-807.