A Direct Amplification for STR Typing from Human Bones

Cheng Ho Phua

A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Forensic Science
Prince of Songkla University
2020

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

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ABSTRACT

Bones are the most prevalent evidence in incidences of mass disaster or long-occurred forensic cases. Individualisation and identification by conventional STR analysis is routine yet suffers from being costly and time-consuming mainly due to the DNA extraction step which also introduces risk of contamination and yield loss (up to 76%). Bone samples may need to be decalcified overnight or for a couple of days as a pre-treatment step. Past studies also found that different skeletal elements have different DNA yield and consequently different STR typing success rates. To streamline STR typing from bones and choose the best bone element for direct STR typing, this study aimed to develop a direct STR typing protocol from human bones. An optimized direct PCR protocol for STR typing from human bones was successfully developed. One hundred mg bone powder in 300 µL PBS buffer was determined to be the optimal condition. The mixture was heated at 98°C for three minutes, with the supernatant ready for subsequent DNA amplification. IDplex Plus was found to perform better than Identifiler Plus for direct STR typing (median allele recovered of 31 and median peak height of 980 RFU at optimal conditions). Fifteen of each bone elements (1st distal phalange of the hand, capitate, patella, metacarpal 4, talus and tibia) (N=105) were then subjected to direct STR typing with 92.4% of the samples resulting in high partial to full profiles. Median peak height from the profiles generated from cancellous bones were significantly better than from compact bones (p=0.033) and was also significantly different across the different bone elements (p<0.001). Bone samples of two days PMI generated significantly higher median number of allele (32±2) as compared to (28±7) for five days PMI (p<0.001). However, low success rates were obtained when applying the developed protocol on casework samples. In conclusion, the developed protocol is robust as performance was similar for all the seven bone elements investigated, as well as being rapid and easy to be applied. Further optimisation may increase success rates for casework bones and allows implementation in casework samples.

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List of Abbreviations

°C	degree Celsius
µL	microlitre
AMELY	amelogenin, Y isoform
ANOVA	analysis of variance
cm	centimetre
CODIS	Combined DNA Index System
DNA	deoxyribonucleic acid
et al.	et alia
etc.	et cetera
g	gram
ISO	International Organization for Standardization
kV	kilovolt
mg	milligram
min	minute
mL	millilitre
ng	nanogram
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
RFU	relative frequency unit
rpm	revolutions per minute
s	second

CHAPTER 1
Introduction

1.1 Background and rationale

Bones are the most enduring biological tissue, as they are able to last longer in various conditions compared to other tissues such as skin and muscle. Well-preserved bones tend to survive up to thousands of years; but it also has to take into account many factors. Putting aside natural death, bones are usually found as unidentified remains from numerous instances: plague (Deepak, 2005; Feldman *et al.*, 2016), terrorism (Holland, Cave, Holland, & Bille, 2003; Olaisen, Stenersen, & Mevåg, 1997; Sudoyo *et al.*, 2008), war (Corach *et al.*, 1997; Marjanović *et al.*, 2009; Primorac *et al.*, 1996), mass disaster (Goodwin & Simmons, 2012), ancient human and animal skeletal remains (Hagelberg *et al.*, 1994; Handt *et al.*, 1994; Höss & Pääbo, 1993), and even illegal immigrants (Cattaneo *et al.*, 2010; Hinkes, 2008). Past local events in Thailand, in which remains of victims ranging from fresh and intact bodies to only body parts and bone remnants were discovered were the 2004 Boxing day Tsunami (Holmes, 2017; Kieser, Laing, & Herbison, 2006; Sribanditmongkol *et al.*, 2007), and the exhumed Rohingya human-trafficking victims in a shallow grave in Songkhla province in 2015 (Saedon *et al.*, 2017). They can be the most prevalent evidence found in cases that had occurred for quite some time, or in extreme circumstances such as fire incidents and buried bodies, as they are made of a very tough structure and composition (Marks & Odgren, 2002).

Standard protocol for identification when encountering bones is through morphological observation to estimate the victim's age, sex, and stature (Budowle & van Daal, 2008; Ciaffi, Gibelli, & Cattaneo, 2011; Hillewig *et al.*, 2011). However, incidences like this may result in comingling of remains from many individuals. Individualisation and identification are difficult when the bones are scattered or intermixed. Bones are also possible to be too degraded or deformed for morphology. This is where DNA-based methods come into play. DNA provides a wide range of information for phylogenetic and diversity studies, population and evolutionary studies,

and also as genetic fingerprinting (Andréasson, Nilsson, Budowle, Frisk, & Allen, 2006; Børsting & Morling, 2015; Budowle, Bieber, & Eisenberg, 2005; Budowle, Planz, Campbell, & Eisenberg, 2004; Mitchelson, 2003; Rankin, Narveson, Birkby, & Lai, 1996). Although there are many methods for typing of human DNA for individualisation such next-generation sequencing (NGS) (Børsting & Morling, 2015) and single-nucleotide polymorphism (SNP) array (Cornelis, Gansemans, Deleye, Deforce, & Van Nieuwerburgh, 2017), the most widely used method is still through short tandem repeat (STR) analysis and capillary electrophoresis (CE). It is also noteworthy that different elements of the bone and different post-mortem intervals (Mundorff & Davoren, 2014) have been shown to contain different amounts of DNA (Miloš *et al*., 2007).

The general overview for STR typing involves decontamination and cleaning of the sample, DNA extraction, DNA amplification, STR typing by capillary electrophoresis, and data interpretation. The extraction process for obtaining DNA from samples is crucial for recovering them from within the sample, especially when encountering trace and degraded bone samples. The standard practice to extract DNA from bones is by organic extraction method. However, the chemicals used are highly toxic, and the process is labour-intensive and time-consuming (Marshall *et al*., 2014) though the DNA obtained is of high purity. Various other protocols for extracting DNA from bones include total demineralisation (Amory *et al.*, 2012; Dukes *et al.*, 2012; Hasap *et al*., 2020; Jakubowska *et al*., 2012; Loreille *et al*., 2007), silica membrane (Dukes *et al*., 2012; Höss & Pääbo, 1993; Marshall *et al*., 2014; Rothe & Nagy, 2016; Yang *et al*., 1998), silica-based column (Ambers *et al*., 2014; Hasap *et al*., 2020; Marshall *et al*., 2014), crystal aggregation (Jakubowska *et al*., 2012), and proprietary PrepFiler® BTA (Barbaro, Cormaci, & Falcone, 2011; Ding, Zhang, & Gao, 2017; Hasap *et al*., 2020; Liu *et al*., 2018). While purity is superior using organic extraction, Hi-Flow silica and total demineralisation method fair better in terms of quantity of DNA (Hasap *et al*., 2020; Jakubowska *et al*., 2012).

Alternately, a direct method of PCR which omits the requirement for DNA extraction has been gaining popularity in many fields of research and applications ranging from microbiology, clinical chemistry, food industry, and even in human and wildlife forensic DNA analysis (Ambers *et al*., 2018; Dangsriwan *et al*., 2017; Geiger *et al*., 2019; Kang, 2019; Kitpipit, Sittichan, & Thanakiatkrai, 2014b; Kitpipit, Thanakiatkrai, & Chotigeat, 2013; Templeton *et al*., 2013; Templeton *et al*., 2015; Thanakiatkrai *et al*., 2019). Direct PCR is better than conventional PCR that requires DNA extraction in the sense that only a small amount of sample is required to achieve similar results with that using extraction. Trace and latent DNA could be used for STR typing while still generating substantial peak heights and numbers of alleles (Ambers *et al*., 2018; Dangsriwan *et al*., 2017; Tonkrongjun *et al*., 2017) whereby if extraction was done, DNA loss is unavoidable (up to 76%) (Ottens, Templeton, Paradiso, Taylor, & Abarno, 2013; Templeton *et al*., 2013; van Oorschot *et al*., 2003; van Oorschot, Ballantyne, & Mitchell, 2010). Depending on the type of sample, a simple additional step termed 'modified dilution protocol' may improve results of direct PCR, as reported by Kitpipit *et al*. (2014) in successful DNA amplifications from wildlife bones. This protocol is advantageous over conventional method in terms of costs involved (extraction kits and chemicals) and overall processing time. However, there is a lack of study on the application of direct PCR on human bone samples, especially for STR analysis (Gausterer, Fichtinger, & Stein, 2007; Kitpipit *et al*., 2014a; Verheij, Harteveld, & Sijen, 2012). It is expected that additional steps or processes may be required before utilising the solution in downstream process. This is because calcium is the main component of bone and is a PCR inhibitor, and DNA is found to be within and bound to the carbonated hydroxyapatite crystal (Salamon, Tuross, Arensburg, & Weiner, 2005) and the collagen in bone (Campos *et al*., 2012; Kitamura, Iwamoto, Sakairi, Tokura, & Nishi, 1997). Both calcium and collagen may interfere with STR amplification (Opel, Chung, & McCord, 2010).

Additionally, the success of an STR amplification depends on many factors other than DNA concentration and PCR inhibitor present in the DNA. Commercial STR kits has undergone development and validation processes with optimisation for performance efficiency (Green, Lagacé, Oldroyd, Hennessy, & Mulero, 2013; Promega

Corporation, 2017). Each component of an STR kit plays important function and has been carefully chosen and optimised to give the best performance: DNA polymerase (Abu Al-Soud & Râdström, 1998; Purzycka, Olewiecki, Soltyszewski, Pepinski, & Janica, 2006), primer sets and target amplicon size (Mulero *et al.*, 2008), reaction buffer and additives (Ahmad & Ghasemi, 2007), and reaction conditions optimised relative to the above components and reaction purpose. This is complicated further if a modified protocol, such as direct STR typing, is used. Hence, the choice for using one STR kit over another may require considerations on the aspects of expected result, sample type and availability, time constraint, operational cost, and instrument availability.

Overall, DNA analysis from skeletal or bone remains can be streamlined. This study aim to 1) perform and optimise various pre-treatment conditions on bone samples without performing extraction (direct PCR) for optimal STR analysis, 2) compare and determine the performance of different STR kits for typing from bone samples without extraction process, 3) study the performance of direct STR analysis from different bone elements, and 4) apply the optimised conditions obtained from the previous experiments to test feasibility for typing from bones of different conditions normally found in forensic cases by using real casework samples.

1.2 Literature review

1.2.1 Brief history of forensic DNA analysis

Sir Alec Jeffrey was responsible behind the driving force of utilisation of DNA for forensic analysis whereby in 1985, he started with a series of publications and developed technique for human identification. He reported his accidental discovery of the unique DNA patterns in each individual giving each person a distinctive DNA 'fingerprint' due to the wide-ranged variability in his population of study (Gill, Jeffreys, & Werrett, 1985; Jeffreys, Wilson, & Thein, 1985a, 1985b). This was then termed as minisatellites or variable number tandem repeat (VNTR). Since then, it had gained traction as one of the most used evidence in the upholding of law. DNA analysis for the purpose of identification is robust and convenient for the following reasons: the

interpersonal variation or the polymorphic loci within the DNA give uniqueness and a high degree of confidence for distinguished profiling and individualisation (Berglund, Kiialainen, & Syvänen, 2011; Budowle & van Daal, 2008); most biological materials contain DNA; and DNA are very stable with half-life of 521 years (Allentoft *et al.*, 2012). The earliest technique of profiling using DNA was the restriction fragment length polymorphism (RFLP) which utilises the unique signature of fragment sizes patterns when cut using restriction enzymes (A. J. Jeffreys, Wilson and Thein, 1985; A. J. Jeffreys, Wilson and Thein, 1985). In 1991, Hochmeister *et al.* (1991) employed RFLP on VNTR on nuclear DNA that was extracted from the femoral bone of a corpse that has been submerged for 18-months and also an 11-years-old mummified corpse for identification (Hochmeister *et al.*, 1991; Iwamura, Soares-Vieira, & Muñoz, 2004). DNA fingerprinting was once laborious – requiring over eight weeks for processing each sample; the process also required an impractical amount of 50 ng of DNA with at least 10,000 intact base pair (bp) fragments (Butler, 2005; Giusti & Budowle, 1995).

Since then, various techniques had been developed and implemented for DNA analysis. PCR-based technique is robust and multipurpose with the required sensitivity and specificity needed especially in molecular work (Ziętkiewicz *et al.*, 2012). PCR-based methods techniques combine the capability of multitarget amplification with the sensitivity to analyse as minute as picograms of DNA (Budowle & van Daal, 2008). To increase the discriminating power of DNA fingerprinting, a subclass of the variable number tandem repeat called short tandem repeat (STR) or sometimes known as microsatellites is employed. The region containing the STR can be amplified with PCR which the fragments are then detected using capillary electrophoresis. STR are regions in the DNA containing 2-10 nucleotide repeats consecutively. STR analysis targets specific regions on the chromosomes called loci, with the segments on both chromosomes are called the alleles. In STR profiling, the allele number are called based on how many tandem repeats there are in each locus (Butler, 2005). The UK was the pioneer in establishing their country's DNA database based on STR profile. The database currently has over 6.2 million individual profiles, and it had aided in matching 65.5% of the crime scene profile with those in the Database, hence showing its enormous help in crime solving (National Police Chiefs' Council, 2019). In 1989, the

Federal Bureau of Investigation (FB1) established their own national DNA database called CODIS with purpose of combining DNA index system of DNA profiles from offenders, missing persons, and unidentified human remains under one system for matching and comparison of DNA profiles from evidence samples to the ones in database to find for a match (Chimera & Dyer, 1992). With the convenience it brought, versatility in application and ease of technology in establishing and maintaining a database, as well as the use of statistics in reporting, STR typing is a valuable method for human individualisation in forensic laboratories worldwide.

1.2.2 Short tandem repeats (STR)

STR is advantageous compared to their predecessor techniques whereby fragment sizes amplified are only as big as 350 bp, which is considered very much shorter than those that had been required. As the fragment size is small, highly degraded samples are possible to be analysed. To have enough discriminating power, ten or up 20 STR loci according to are necessary (Butler, Coble, & Vallone, 2007; Decorte, 2010; Ziętkiewicz *et al*., 2012). For convenience and due to the advancement of technologies, multiplex autosomal STR loci are able to be amplified as fast as around one hour and 15 minutes (QIAGEN, 2015). Of course, there are many other commercial STR kits available with different performances and functions such as AmpFlSTR® Identifiler® PCR amplification kit (Applied Biosystems, USA), PowerPlex® 16 system (Promega, USA), AmpFlSTR® NGM™ PCR amplification kit (Life Technologies, USA), GlobalFiler® PCR Amplification kit (Thermo Fisher Scientific, USA), Investigator® 24plex QS kit (QIAGEN, Hilden, Germany) which amplifies core STR loci recommended by CODIS and additional loci including CSF1PO, D1S1656, D2S441, D2S1338, D3S1358, D5S818, D7S820, D8S1179, D10S1248, D12S391, D13S317, D16S539, D18S51, D19S433, D21S11, D22s1045, FGA, Penta D, Penta E, TH01, TPOX, and vWA (Butler, 2005; Butler *et al*., 2007; Collins *et al*., 2004; Krenke *et al*., 2002; Tan *et al*., 2017).

Various studies on comparison have been made for commercial STR kits as they had been individually developed and are proprietary. Hence, their performance and concordance of genotyping could vary. Commercial STR kits have undergone rigorous

optimisation and validation to make them robust and sensitive in addressing the increasing acceptance and usage range of STR profiling for human identification and individualisation (Butler, 2005; Cavalcanti *et al*., 2015, Wang *et al*., 2012). Concordance study for commercial STR kits had been done for specific population (e.g. demographic, ethnic) such as the study by Westen *et al*. (2014) which had compared concordance of six different autosomal STR kits (Promega's PowerPlex® 16, ESX-16 and ESI-17 Systems, Qiagen's Investigator® ESSplex Kit and Applied Biosystems' AmpFlSTR® Identifiler® and NGM PCR Amplification Kits) with a sample male Dutch population group. Nineteen autosomal allelic discordance were found among the six STR kits based on their genotyping results (from 2085 samples). Discordance were also found from 24 samples for the amelogenin locus (null allele was obtained for X) when NGM kit was used.

There had also been performance study on the direct amplification of three different STR kits from blood on paper discs by Caputo *et al*. (2017). They reported full concordance from 770 blood samples when compared to profiles reported by labs that submitted the blood samples, though the three STR kits performed with different efficiency when amplified directly. AmpFLSTR® Identifiler® Plus PCR amplification kit and QIAGEN® Investigator® IDplex Plus kit performance were also compared using control DNA and extracted casework samples (Mattayat *et al*., 2016) which they found that both kits performed statistically similar.

Internal validation of three latest generation STR kits (Thermo Fisher Scientific's GlobalFiler® Express, Promega's PowerPlex® Fusion 6C, and Qiagen's Investigator® 24plex GO!) was also done and reported as part of ISO implementation (Nongmanee *et al*., 2019). First-pass success rate when direct STR typing was done from 100 buccal swab samples differed among the three kits with Globalfiler® Express performed best (85%), followed by Fusion 6C (79%) and 24plex Go! (69%), in which they attributed the difference to the kits' reagents' chemistry and/or the individual kit's pre-treatment steps.

1.2.3 DNA extraction

A procedure for DNA isolation or DNA extraction is normally done to separate them from other cellular materials (proteins, membranes, etc.). It may be said that this step is most crucial and most labour-intensive before downstream processes e.g. PCR, hybridisation and electrophoresis (Cseke & Herdy, 2012; Elkins, 2013). Extraction of DNA can be briefly separated into three steps: lysis of cells, DNA separation from other cell components, and isolation of DNA. The lysis of cell involves disruption of the cell membrane and may be done using many different methods, but the easiest and faster detergent-based method is more commonly adapted (Walsh, Metzger, & Higuchi, 1991). Chelex, SDS, CHAPS, Triton-x, etc. are examples of surfactant used for disorganising the membrane's lipid bilayer. When the cells are broken apart, organelles and cell components are exposed to the environment. It is usually beneficial to have EDTA or any chelating agent which functions to chelate Mg^{2+} ions, a cofactor for DNase (DNA degrading enzyme deoxyribonuclease) to prevent them from digesting DNA (Goodwin, Linacre, & Hadi, 2011). Chelex-100 is also said to serve similar function through DNase inhibition (Walsh *et al.*, 1991). To break down as much cellular debris as possible for easing removal in the following step, a protease (normally the broad-spectrum serine protease) is used for denaturing proteins associated to DNA and other cellular proteins. Chemicals such as urea salts and guanidine hydrochloride are also used for protein aggregation and precipitation (Bennion & Daggett, 2003). Other bonds that may be present can also be broken with different chemicals: disulphide bonds in hair and bones can be reduced by dithiothreitol (DTT).

Finally, the DNA can be separated by many methods depending on convenience and applications, either by multistep organic solvent separation technique, silica beads one-tube extraction technique, and the widely used solid phase extraction method (silica-filter-based) (Hoff-Olsen *et al.*, 1999) where many commercial kits have been developed. Methods and techniques to be employed for one's work should be chosen with proper consideration of sample type, time consumption and budget, DNA purity, hazard, and application, among other factors (Elkins, 2013; Marsal, Boronat, Canals, Zamora, & Fort, 2013).

1.2.4 Inhibitors of PCR

No extraction method guarantees 100% efficiency and purity and that even high purity does not necessarily mean downstream application would be definite success (Abdel-Latif & Osman, 2017). PCR is a reaction involving enzymes and hence requires cofactors, hence at the same time susceptible to inhibitors. All substances affecting negatively on PCR are termed PCR inhibitors and may come from many sources (e.g. sample processing and DNA extraction). Impacts of these inhibitors on PCR are decreased sensitivity and reaction rate, and may lead to false negative results if total inhibition occurs (Schrader, Schielke, Ellerbroek, & Johne, 2012). Inhibitory effect of these substances is exerted either by direct interaction with DNA or that they interfere with the polymerase enzyme used (Bessetti, 2007). The positive two-charged magnesium ion is a crucial cofactor of DNA polymerase, similarly-charged ions (e.g. Ca^{2+}, Fe^{2+}) may be a competitive inhibitor, and substance that interfere binding can be a non-competitive inhibitor. Common forensically-significant samples that contain inhibitors are blood, fabric, soil and tissues, and they may also come from sample processing which tend to be organic compounds (e.g. salts and ionic detergents, ethanol and isopropanol, etc.) (Bessetti, 2007; Cseke & Herdy, 2012) as well as protein carry-overs (e.g. collagen, immunoglobins and proteinases) (Rådström, Knutsson, Wolffs, Lövenklev, & Löfström, 2004; Rossen, Nørskov, Holmstrøm, & Rasmussen, 1992).

Overcoming inhibitors' carry-over is the surest way for avoiding inefficient PCR or even failure. Sample collection method could be improved or adapted according to substrate. DNA purification would be another way to ensure that. Many commercial kits are widely available for convenience in different applications. Other than that, in cases where inhibitors could not be removed during purification or extraction, the DNA polymerase chosen for a PCR reaction could be of major impact (Katcher & Schwartz, 1994; Wiedbrauk, Werner, & Drevon, 1995). Most polymerases these days have been engineered for improved performance in terms of reaction rate, fidelity, proof-reading and base-addition error rates, and also inhibition tolerance (de Vega, Lázaro, Mencía, Blanco, & Salas, 2010; Elshawadfy *et al.*, 2014; Kranaster & Marx, 2010; Nilsson, Grånemo, Buś, Havsjö, & Allen, 2016; Ppyun *et al.*, 2013) for various genomic

applications. It is worth noting though, that most commercial STR kits uses AmpliTaq Gold® DNA polymerase, which has been studied to be among those highly sensitive to inhibitors (Abu Al-Soud & Râdström, 1998). Therefore, using increased amount of polymerase or the addition of additives may help. Bovine serum albumin (BSA) has found its way into more and more reaction mix for polymerase to provide and aid resistance against inhibitors (Comey *et al*., 1994).

In cases of DNA extracted from skeletal remains, possible remnants that could act as PCR inhibitors are humic material (Abbaszadegan, Huber, Gerba, & Pepper, 1993; Ijzerman, Dahling, & Fout, 1997) from the ground and soil, calcium ions which is the main mineral of bones (Buckleton *et al*., 2016; Corach *et al*., 1997; Powell, Gooding, Garrett, Lund, & Mckee, 1994), and collagen constituting the bone's matrix. Humic acid forms bonds with template DNA hence inhibiting reaction with polymerase even at low concentrations (Sutlović, Definis Gojanović, Andelinović, Gugić, & Primorac, 2005; Sutlovic, Gamulin, Definis-Gojanovic, Gugic, & Andjelinovic, 2008). The presence of detergents and calcium ions affect polymerase whereby it causes degradation (Powell *et al*., 1994; Rossen *et al*., 1992) and competitive inhibitory effect respectively (Abu Al-Soud & Râdström, 1998; Opel, Chung, & McCord, 2010). The collagen crosslinks with template DNA and hence changes its chemical properties (John, 1992; Opel, Chung, & McCord, 2010). EDTA, which is a chelating agent normally used for decalcifying calcium from bones, may also pose a negative effect in which it chelates the magnesium ions leading to loss of polymerase cofactor (Rossen *et al*., 1992).

1.2.5 Direct PCR

Studies had been done to investigate the extent and efficiency of DNA extraction and found that up to 76% of them is lost from the process (Ottens *et al*., 2013; Templeton *et al*., 2013; van Oorschot *et al*., 2003, 2010). Hence, it is hypothesised that a protocol that omits DNA extraction would allow all DNA available in a sample to be subjected to PCR amplification. Such technique is termed direct PCR and has been gaining favour for use in forensic applications (Ambers *et al*., 2018; Bickley *et al*., 1996; Dangsriwan *et al*., 2017; Kitpipit *et al*., 2014a, 2014b; Ottens *et al*., 2013; Swaran

& Welch, 2012; Templeton *et al*., 2015; Thanakiatkrai *et al*., 2019; Tonkrongjun *et al*., 2017; Verheij *et al*., 2012).

Commercial STR kits have also been optimised and developed for direct amplification from various samples (e.g. PowerPlex® STR kits by Promega, AmpFLSTR® Identifiler Direct PCR Amplification Kit and GlobalFiler Kits by Thermo Fisher Scientific, Investigator GO! Kits by QIAGEN). Buffers were optimised for increased inhibitor tolerance that may be present when samples are amplified directly along with higher sensitivity. Some STR kits also incorporated pre-treatment steps as part of sample "clean-up" or for pre-digestion for higher amplification success rate. Performance study of direct STR amplification for various kits had also been done with promising results. Gouveia *et al*. (2015) assessed and was able to amplify DNA directly from blood and buccal reference DNA samples obtaining full profile. Nongmanee *et al*. (2019) also performed internal validation of the performance of three latest generation direct STR kits (Thermo Fisher Scientific's GlobalFiler® Express, Promega's PowerPlex® Fusion 6C, and Qiagen's Investigator® 24plex GO!) for direct STR analysis as part of their ISO implementation which they found that the three kits performed differently which in turned reflects their robustness when processing the buccal swab samples. Caputo *et al*. (2017) also optimised pre-treatments on blood spots and studied efficiency of three different PowerPlex® STR kits for direct STR typing from those blood spots (treated and untreated). Concordance were observed from the profiles when analysed using the three different kits. Although they found that blood spots treated with Punch Solution™ reagent (Promega) prior to being subjected to amplification provided the highest percentage of high-quality profiles when amplified with PowerPlex® 21, the performance of the three kits varied with the different treatments applied to the blood spots. Different findings were obtained from the studies mentioned that had done various performance studies for different commercial STR kits. Thus, this demonstrated that there is a need for optimisation and validation (of treatments and/or PCR conditions) when direct PCR is employed and amplified using different STR kits.

Currently two protocols are available for direct PCR where they differ only slightly: direct protocol and dilution protocol. For direct protocol, sample is added into the reaction mix directly while for dilution protocol, a simple additional preparation step is required. Sample is added into normally buffer (but water can also be used) and heated to aid in cell lysis and thus releases DNA into the solution which could then be added to subsequent steps as per required. This solution is termed pre-PCR solution and is usually heated for three minutes at 98°C. This temperature also helps denature DNase that might damage DNA to be obtained. This pre-PCR solution or crude extract also acts as a dilution step - to dilute DNA to suitable concentration range for PCR, and also to dilute inhibitors that is expected to be present hence reducing their interference and effect (Kitpipit *et al*., 2014a). Kitpipit *et al*. also reported a higher success rate of amplification when using dilution protocol when compared to direct protocol in cases of trace and degraded samples: possibly due to the reasons explained previously. They too obtained successful amplification from samples of museum-sourced aged bones and antlers.

Quality of the STR profiles obtained from some studies were also better when done using direct PCR method than that with conventional PCR method (Swaran & Welch, 2012; Templeton *et al*., 2015), even with touch DNA samples that contains low-template DNA (Altshuler & Roy, 2015; Templeton *et al*., 2013). There was also a study on the feasibility of direct PCR on human bones by Gausterer, Fichtinger, and Stein (2010) with the approach of amplifying the 443 bp hypervariable region 1 (HV-1). They also varied the polymerase used, amplifying DNA from the direct addition of bone powder into the reaction mix and supernatant from their dilution protocol (containing TE buffer supplemented with DNARelease® Additive (Finnzymes, Finland)). The supernatant was also subjected to STR analysis with PowerPlex® S5 Kit (Promega). PCR product for the mtDNA HV-I region was successfully obtained when DNA from their developed dilution protocol was amplified, and also when nested PCR was done. Nine out of 10 alleles were also obtained when the same sample was amplified using PowerPlex® S5 Kit (Promega). This study thus demonstrated that direct STR analysis from human bones was indeed possible, though optimisation on treatment and

conditions were required, and more data are needed before it can be implemented with casework samples.

In summary, direct PCR has found its place and application in wide range of sample types. However, due to the complexity and low DNA-to-inhibitor ratio in bones, ivory, and horns, there had been few studies on using direct PCR with these samples (Gausterer *et al*., 2007; Kitpipit *et al*., 2014a; Verheij *et al*., 2012). This shows a gap in knowledge in the possibility of applying direct method of PCR using human bones. This would benefit the community in terms of sample amount required, time and chemical consumption, yield, and quality of STR typing from the reduced contamination that may be introduced in extraction steps.

1.2.6 Bones and skeletal remains

1.2.6.1 General information from bones

STR typing can be done from almost all types of biological material such as hair roots, blood, tissue, semen, saliva and even touch DNA from latent prints. However, it is unavoidable that those materials be damaged following effects of environmental conditions. Heat, moisture, bacteria and radiation cause degradation of genetic material in the cells (Alaeddini, Walsh, & Abbas, 2010; Iwamura *et al*., 2004; Pääbo, 1989) especially high molecular weight DNA. For this reason, in extreme cases where soft tissues are already decaying or decayed, the genetic material contained within could already be rendered unusable or has been contaminated by foreign DNA or even environmental inhibitors. Skeletal remains, in cases where they can be found, are useful as they can be preserved longer (Lundy, 1998).

Skeletal remains may be found as surface-scattered preyed by animals, buried underground in soil or sand, and submerged in water, among other possible conditions. Forensic anthropologists are the experts concerning human skeletons; hence it is more preferable that skeletal remains recovery are made by them to preserve them in their best conditions for analysis (Brooks, 1989; Hunter, Roberts, & Martin, 1997). After being cleaned in a medical facility, they are given a general physical description on the gender, age of death, race, and height. It might also be possible to tell any antemortem

or perimortem trauma, and cause of death if there are any (Lundy, 1998). Other than bones, identification through dental examination, or forensic odontology, is also a viable alternative or supplement (Senn & Weems, 2013).

1.2.6.2 DNA analysis from bones

Contrary to skin, muscle and organ tissues, bones decay at a much slower rate, lasting from a decade to hundreds of years depending on extrinsic and intrinsic factors. In order to obtain the preserved DNA from bone samples, DNA extraction is important for maximum recovery. Most extraction protocols are meant for use with fresh samples and tissue which contains intact cells and DNA (Rohland & Hofreiter, 2007a) but for bones they can be damaged in various ways, especially aged and ancient bones (Campos *et al*., 2012; Hagelberg *et al*., 1994; Liu *et al*., 2018; Pääbo, 1989; Rohland & Hofreiter, 2007a, 2007b). Also, standard protocol of extraction of DNA from bone samples tend to employ repeated incubation and washing of the samples with EDTA hence will also lead to loss in the amount of DNA remaining. As such, due to the delicate conditions of the DNA within those bones, harsh treatments and chemicals should be avoided when choosing or designing a method for extracting DNA from bones (Pääbo, 1989). Also, as ancient bones have been around and exposed to environmental conditions, PCR inhibitors too accumulate over time other than the calcium that is already present as the main constitution of bones. These inhibitors would affect downstream processes especially PCR if they were not dealt with (Rohland & Hofreiter, 2007b).

Phenol-chloroform method is the preferable choice when extracting DNA from bones due to its high purity and yield (Jakubowska *et al*., 2012); but phenol is highly toxic, requires many manual steps, and is time-consuming (Marshall *et al*., 2014). Research and publications in recent years have come up with different methods to extract DNA from bones (e.g. total demineralisation (Amory *et al*., 2012; Dukes *et al*., 2012; Jakubowska *et al*., 2012; Loreille *et al*., 2007), silica membrane (Dukes *et al*., 2012; Höss & Pääbo, 1993; Marshall *et al*., 2014; Rothe & Nagy, 2016; Yang *et al*., 1998), Hi-Flow silica-based column (Ambers *et al*., 2014; Marshall *et al*., 2014), crystal aggregation (Jakubowska *et al*., 2012), proprietary PrepFiler® BTA (Barbaro *et al*.,

2011; Ding *et al*., 2017; Liu *et al*., 2018)) and the developed bone extraction protocol by Hasap *et al*. (2020).

Hi-Flow silica extraction method employs large-volume buffer digestion with silica-based purification (Marshall *et al*., 2014). The method is convenience due to the use of a single tube large-volume silica column, which reduces handling steps and sample transfer (hence reducing risk of cross contamination). However, overnight incubation for digestion is required. Furthermore, the purchase of silica columns would be required, and 50 mL conical tubes centrifuge may not be readily available in general labs or may not be practical to be brought to field. As for the total demineralisation method, large volumes of extraction buffer are involved (9 to 18 mL depending on the amount of bone powder used) with overnight incubation (Loreille *et al*., 2007) and it requires centrifugal filter units.

While purity is superior for extraction using organic extraction, Hi-Flow silica and total demineralisation method fairs better in terms of quantity of DNA (Jakubowska *et al*., 2012). A recent study by Hasap *et al*. (2020) which compared extraction performance of Hi-Flow silica extraction method, total demineralisation method (TD), PrepFiler BTA method, and their developed extraction method found that their developed method and PrepFiler BTA performed better in removing inhibitors based on their internal positive control (IPC) results during DNA quantification. TD faired significantly better than PrepFiler BTA and Hi-Flow in terms of the median DNA concentration extracted from fresh bone samples, although the developed method by Hasap *et al*. performed best (Developed method: 135.85 ng/µL, TD: 134.53 ng/µL, PrepFiler BTA: 44.06 ng/µL, Hi-Flow: 0.19 ng/µL).

The DNA amount that could be recovered from bones is also dependent on the bone elements. Milos *et al*. stated that different skeletal elements gave rise to differing STR typing success rates. A success percentage of 86.9 was obtained when DNA was extracted from the femur and 46.2% success rate when typing from the long bone of the upper arm (Miloš *et al*., 2007). In terms of amount of DNA yield per gram of bone sample, the 1st distal phalange gave 448 ng/g but the tibia and femur only yielded 47 ng/g and 45 ng/g, with findings were of similar trend in the study by Hasap *et* al. (2020)

(1st distal phalange - 20.3 µg/g, 8.1 - µg/g, femur – 5.9 µg/g). Despite that, femur and tibia have been recommended and are more commonly used for DNA typing (Mundorff & Davoren, 2014). The reason is femur and tibia are weight-bearing long bones which are dense as compared to cancellous bones. Therefore, these bones are more efficient in protecting DNA against degradation due to more protective effect from hydroxyapatite molecules in the higher density compact bones (Alaeddini *et al.*, 2010; Campos *et al.*, 2012; Miloš *et al.*, 2007; Mundorff *et al.*, 2009; Pokines & Symes, 2013; Turingan *et al.*, 2019).

It could not be emphasised enough the important role of bones for forensic cases and investigations especially when it involves unidentified or mutilated remains. One of the major tragedies in humankind history that happened on the 11th of September 2001 resulted in the largest mass fatality in United States. Thirteen thousand skeletal fragments were analysed but only 8000 of them (65%) provided interpretable STR profile (Holland *et al.*, 2003). When STR typing failed, mitochondrial DNA (mtDNA) analysis was done instead to at least determine maternal lineage relatives (Budowle *et al.*, 2005). Another local disaster that happened was the 2004 Southeast Asian tsunami that took away lives of nearly 5,400 individuals. DNA typing also played a major role in giving the victims' families closure. Seawater and large impact force that was involved made victim recollection and identification of bodies difficult with the state of decay and scattering of the body parts and belongings. In this extreme case, organic DNA extraction was employed in tandem with ultrafiltration which resulted in 834 out of 1062 fragments yielding STR profiles (Deng *et al.*, 2005). mtDNA analysis was also done similar to the 9/11 incident (Kieser *et al.*, 2006). Although analysis from mtDNA provided a considerably high success rate of typing from those degraded samples, they still did not ensure the required high levels of certainty for positive identification of remains where only highly degraded or limited samples were available; Additionally mtDNA analysis could not distinguish siblings and maternal relatives (Budowle *et al.*, 2005).

In events involving mass fatality, human identification and individualisation by STR analysis of the bones is still the more commonly-employed method. The DNA

extraction step, although allows DNA recovery and co-contaminants removal, is tedious and time-consuming. Hence, a direct method of PCR that has been gaining acceptance in many fields of research and applications may be a solution, but this has not been explored systematically.

1.3 Objectives

1.3.1 To assess the optimal conditions and treatments on bone samples for direct amplification and STR analysis.

1.3.2 To compare and determine the performance of different STR kits for direct STR typing of bone samples.

1.3.3 To study the performance of direct STR analysis from different bone elements.

1.3.4 To apply and test feasibility of typing from bones of different conditions found in forensic cases.

CHAPTER 2

Methodology

This research comprised four experiments: 1) performed and optimised minimal treatment on bone samples without performing extraction with the end goal of obtaining optimal STR analysis, 2) compared and determined the performance of two STR kits for direct STR typing from bone samples, 3) studied the performance of direct STR analysis from seven different bone elements, and 4) applied the optimised conditions obtained from the previous experiments to test feasibility for STR typing from bones of different conditions normally found in forensic cases by using real casework samples (Figure 1). Experiments 1 to 3 were done to examine feasibility and efficacy of the developed protocol to be used for STR typing from fresh bone samples, including the performance of each bone elements, and performance of two different STR kits. In Experiment 4, the developed protocol was used on real casework samples to assess application and possibilities of contribution to improvements of the current established protocols, especially for urgent cases and disasters.

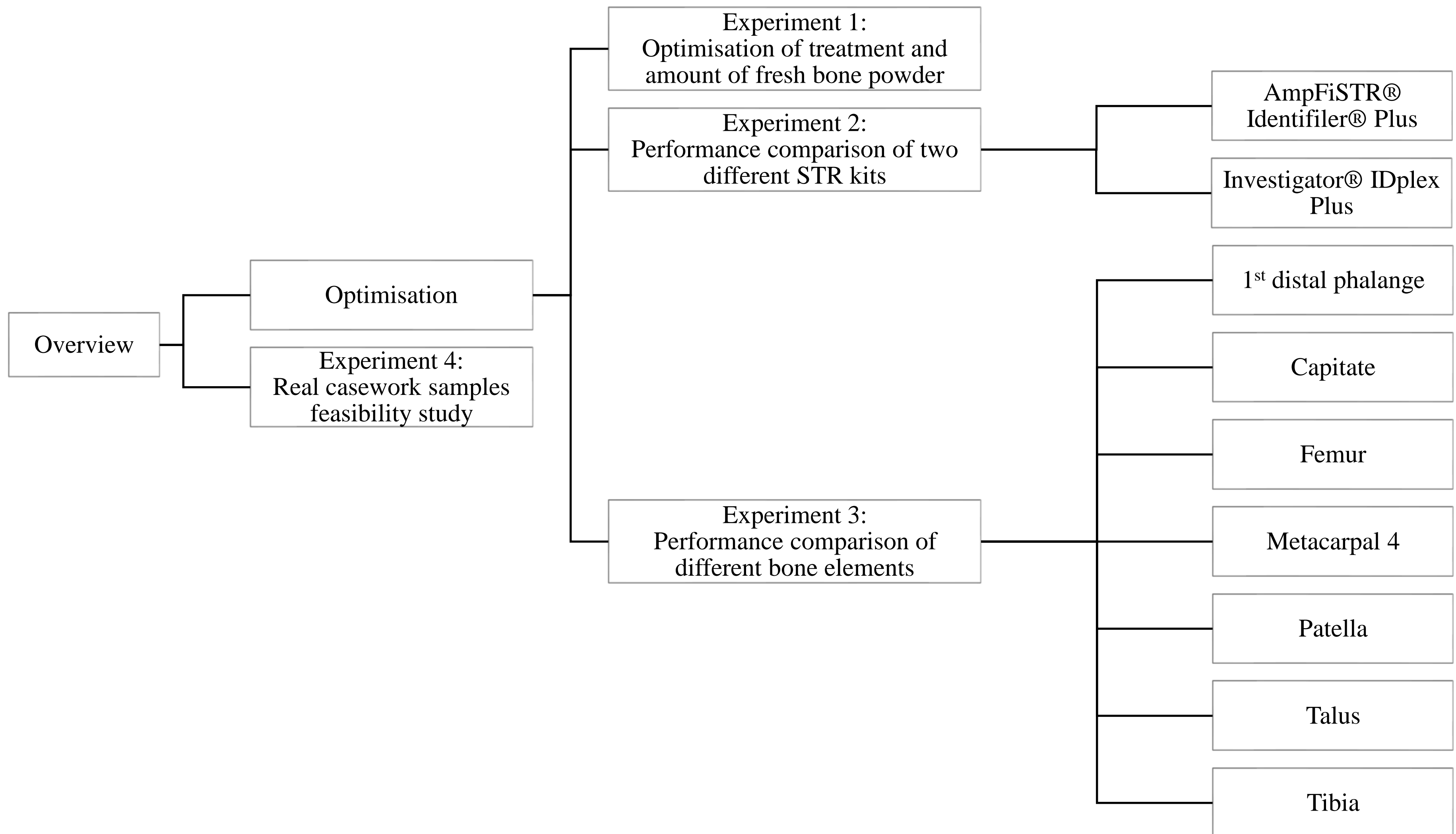


Figure 1 Flow chart showing overview of the experiments conducted in the present study with the optimisation part comprised of Experiments 1 to 3 and Experiment 4 being the real casework samples feasibility study.

2.1 Sample type and collection®

Bone samples studied consisted of two types: fresh bone samples from recently-deceased cadavers, and real casework samples exposed to different conditions. For fresh bone samples, they were collected from medical cadavers from the Department of Anatomy, Faculty of Science, Prince of Songkla University. The samples were acquired and processed according to the approval of the Prince of Songkla University Ethical Committee with ethical approval number REC.63-143-19-5 (Appendix A). The fresh bones from medical cadavers with post-mortem interval (PMI) of two days (five individuals) and five days (10 individuals), in which they had been exposed to air-conditioned room temperature (~25°C) during dissection for medical training. Bone collection was manually done using oscillating saw and scalpel by a trained technician. A total of 105 bone fragments were analysed. Fifteen of each bone element (femur, 1st distal phalange of the hand, capitate, patella, metacarpal 4, talus, and tibia) were used (Figure 2). As for casework samples, 19 casework samples were obtained from Forensic Medicine and Toxicology Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University (Table 1 and Table 2).

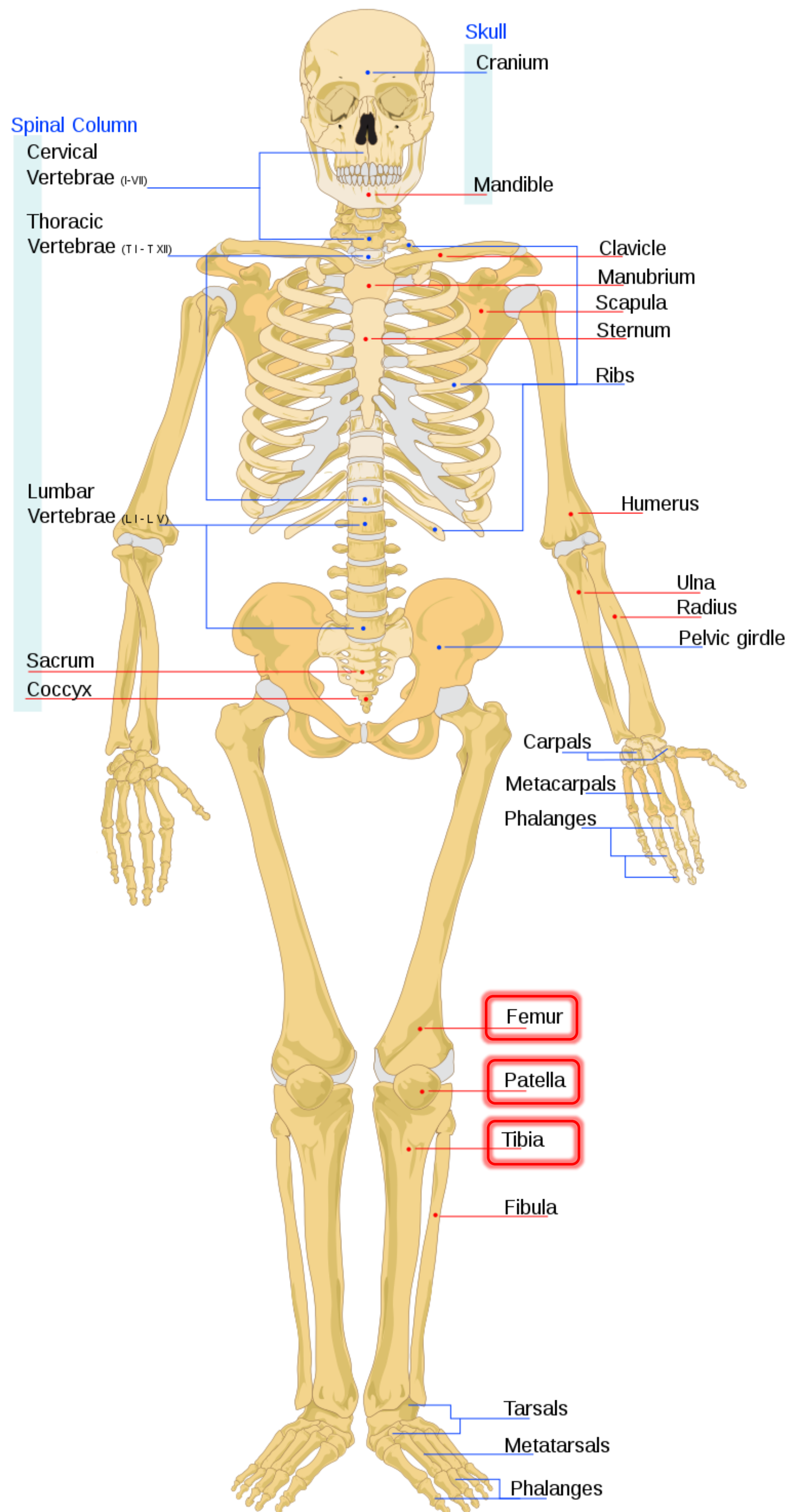
Table 1 The amount, bone elements and types of bones that were collected and used in this study.

Number of samples	Bone element	Type of bone	Source
15 each	Femur 1st distal phalange of the hand Capitate Patella Metacarpal 4 Talus Tibia	Fresh	Department of Anatomy, Faculty of Science, Prince of Songkla University
19	Femur, humerus, sternum, tibia	Real casework samples	Forensic Medicine and Toxicology unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University

Table 2 The characteristics of casework samples used in this study (n=19). Each individual sample had been assigned a unique sample ID by the Forensic Medicine and Toxicology Unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University.

Sample ID	PMI	Bone element	Gender	Condition
E60045	< 1 year	Femur	Male	Submerged in water
E60033	< 1 year	Humerus	Male	Exposed to sunlight
E60046	< 1 year	Humerus	Male	Exposed to sunlight
E60081	< 1 year	Humerus	Male	Submerged in water
E60082	< 1 year	Humerus	Male	Unknown
E60025	< 1 year	Sternum	Female	Exposed to sunlight
E58014	3 years	Femur	Male	Submerged in water
E58015	3 years	Femur	Male	Submerged in water
E58011	3 years	Humerus	Male	Burnt
E57041	4 years	Femur	Male	Exposed to sunlight
E57089	4 years	Femur	Male	Exposed to sunlight
E57095	4 years	Tibia	Male	Submerged in water
E57092	4 years	Humerus	Male	Exposed to sunlight
E57071	4 years	Sternum	Female	Exposed to sunlight
E57036	4 years	Tibia	Male	Submerged in water
E57009	4 years	Femur	Male	Unknown
E56112	5 years	Femur	Male	Exposed to sunlight
E56004	5 years	Unknown	Male	Unknown
E54091	7 years	Humerus	Male	Burnt

(a)



(b)

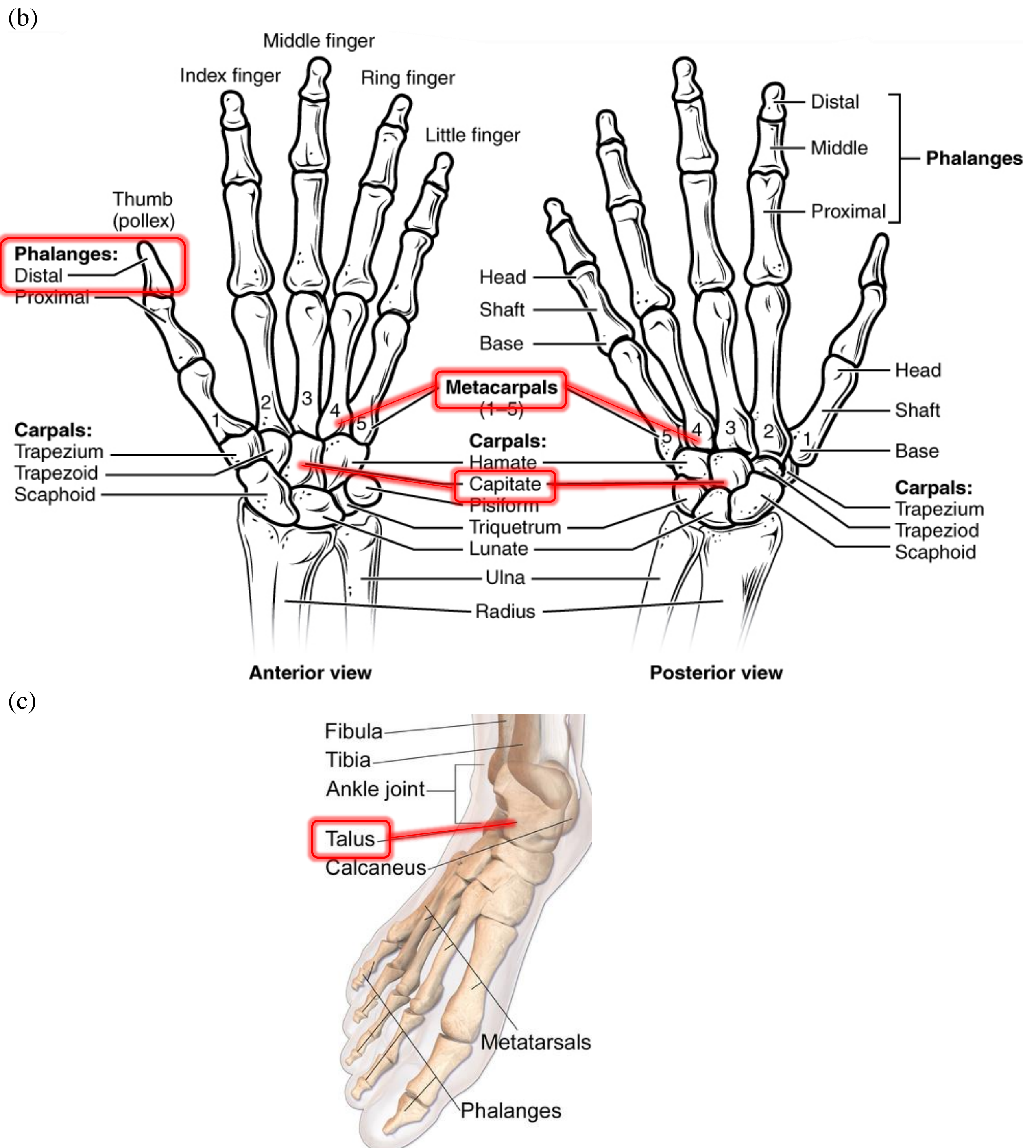


Figure 2 Figures of bone elements that were used in this study with their locations in the skeletal anatomy. (a) The long bones (femur and tibia) and the patella, (b) Small cancellous bones from the hand (1st distal phalange, capitate and metacarpal 4), (c) Small cancellous bones from the foot (talus). All images are in the public domain and redistributable.

Fresh bone samples were used for prior optimisation and then to test for the performance in direct STR analysis from fresh human bones. Casework samples were used to test for feasibility of applying the direct method of STR analysis on casework samples.

2.2 Sample preparation

Each of the collected samples was first cleaned to remove the marrow, residual tissue, and other possible exogenous materials by using a scalpel and sterile distilled water. The bone samples were then soaked in 10% Clorox for five minutes, rinsed with sterile distilled water and followed by soaking in 96% ethanol for 15 minutes following sample preparation method in the study by Hasap *et al*., (2020). The samples were then placed in a sterile fume hood to be air-dried overnight. The cleaned and dried samples were then powdered using a freezer mill (CertiPrep 6750 Freezer Mill SPEX SamplePrep L.L.C., Metuchen, NJ, USA). The powdered bone samples were stored at -20 °C until further processing.

2.3 Optimisation of direct PCR protocol

Initially, a direct protocol where bone powder was directly added to the PCR reaction mix was done during preliminary experiment. 1.0 mg, 1.25 mg, or 1.5 mg of bone powder was added directly into tubes containing reaction mixture for AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific) and Investigator® IDplex Plus Kit (QIAGEN) respectively. Then, a pre-PCR dilution protocol as described by Kitpipit *et al.* (2014), in which amplified the mitochondrial DNA COI region from bones, horns, and antler samples which have similar characteristics and properties with human bones that will be used in this study, was adapted for treating the powdered bone samples before subsequently subjected to PCR amplification. The protocol was first tried with varying volumes of PBS buffer added to varying amount of bone powder in an Eppendorf tube which was then vortexed for one minute to mix (Table 3). The mixture solution was then heated at 98°C for three

minutes. Then, the heated mixture was centrifuged at max speed (14,000g) for one minute and the supernatant was then carried over to the amplification step.

Table 3 The volumes and amounts of PBS buffer and bone powder used in the preliminary optimisation experiment of the developed direct PCR protocol.

Amount of bone powder (mg)	Volume of PBS buffer (µL)
1.00	20 30 40
1.25	20 30 40
1.50	20 30 40
100.0	100 200 250 300 500 1000

2.4 Commercial STR kits performance comparison

Half-volume reaction was done for the PCR reaction following the respective STR kits' manufacturer's recommendation. From the same supernatant, 2.0 µL was added to the PCR reaction mixture in which two different commercial STR typing kits were tested. For Identifiler® Plus (Thermo Fisher Scientific), each reaction consists of 5.0 µL of AmpFlSTR® Identifiler® Plus Master Mix, 2.5 µL of AmpFlSTR® Identifiler® Plus Primer Set and 3.0 µL of PCR-grade water. As for IDplex Plus, each reaction consists of 3.75 µL of Fast Reaction mix, 1.25 µL of Primer Mix and 5.5 µL of PCR-grade water. The reaction mixes were prepared in batches according to number of reactions required to ensure consistency. PCR reactions were performed according to recommended conditions using T100™ Bio-Rad Thermal Cycler (Bio-Rad, California, United States) with reaction conditions as shown in Table 4.

Table 4 The reaction conditions used for PCR amplification of DNA from the resulting supernatant following the respective commercial STR kits' manufacturer's recommendation including the approximate total reaction time for each kit on T100™ Bio-Rad Thermal Cycler (Bio-Rad).

	STR kit	
Thermal steps	**AmpFlSTR® Identifiler® Plus PCR Amplification Kit**	**Investigator® IDplex Plus Kit**
Initial denaturation	95°C, 11 minutes	95°C, 5 minutes
Denaturation	94°C, 20 seconds	96°C, 10 seconds
Annealing	59°C, 3 minutes	61°C, 2 minutes
Final extension	60°C, 10 minutes	-
Cycle	28	30
Total reaction time	~ 2 hours 30 minutes	~ 1 hour 30 minutes

From the two STR kits studied, the better performing STR kit in terms of overall peak height and also the number of alleles was selected for use for the rest of the experiments.

2.5 Direct PCR from different bone elements and from casework samples

For each bone element (femur, 1st distal phalange of the hand, capitate, patella, metacarpal 4, talus, and tibia), 15 samples were used and analysed for studying their performance in direct STR typing. The optimal condition (i.e. the volume of PBS buffer used) and treatment from the optimisation part was applied to the bone powder. One hundred milligrams of bone powder was incubated in 300 µL of PBS at 98°C for three minutes. DNA amplification and STR analysis was done using DNA from the resulting supernatant, amplified with the IDplex Plus Kit based on result from the STR kits performance comparison experiment. For bone element samples from the same individual, any full profiles were cross-checked for concordance, and subsequently the 'true' alleles to be called. This profile was then used as the reference profile for samples from that individual. If no full profile was obtained from any of the bone element samples for that individual, reference STR profile was generated using extracted DNA from femur bone (following Interpol's recommendation). The number of concordant alleles and median peak height was taken into account for the success rate for each sample. Any presence of allele drop-ins and drop-outs were also noted.

A total of 19 individual real casework samples (Table 2) that were requested and obtained from Forensic Medicine and Toxicology unit, Department of Pathology, Faculty of Medicine, Prince of Songkla University, were subjected to treatment conditions from the optimisation experiment that gave the best results in terms of number of alleles and peak height. One hundred milligrams of bone powder was incubated in 300 µL of PBS at 98°C for three minutes. Amplification was done using the IDplex Plus Kit. Alongside with that, DNA extraction was also done (using PSU-BEK bone extraction protocol: (Hasap *et al.*, 2020)) on the casework samples. This was done to test for viability of the samples for attributing the success or failure of direct STR typing from those samples to the nature of the samples (e.g. DNA concentration and DNA degradation) and not due to any possible human and technical error. In brief, for the PSU-BEK bone extraction, 0.5 g bone powder was incubated in 1046 µL of lysis buffer containing 950 µL of 0.5 M EDTA, 50 µL of 1% of sodium N-lauryl sarcosinate, 14 µL of 1 M DTT, and 32 µL of 20 mg/mL proteinase K. The mixture was shaken at 1100 rpm and incubated at 56°C for two hours. After incubation, the solution was

concentrated with 30-kDA Amicon Ultra-0.5 centrifugal filter (Sigma Aldrich, Missouri, United States) and the retentate was then purified using E.Z.N.A.® Cycle Pure Kit (Omega Biotek, GA, USA) following manufacturer's protocol, obtaining a 30 µL eluate of extracted DNA.

2.6 Fragments separation and STR analysis

Product fragments from PCR amplicons were subjected to separation and detection using ABI Prism 310 Genetic Analyser (Thermo Fisher Scientific) following the respective STR kits' recommended protocol for pre-separation treatments (Table 5).

Table 5 The pre-separation treatment and reagents used prior to capillary electrophoresis for fragments separation following the respective commercial STR kits' manufacturer's recommendation.

<table>
<tr><th>STR kit</th><th>Reagent</th><th>Volume (µL)</th><th></th></tr>
<tr><td rowspan="3">AmpFlSTR® Identifiler® Plus PCR Amplification Kit</td><td>GeneScan™ 500 LIZ® Size Standard</td><td>0.5</td><td rowspan="6">Heat at 95°C for 3 minutes, then place on ice for 3 minutes</td></tr>
<tr><td>Hi-Di™ Formamide</td><td>24.5</td></tr>
<tr><td>PCR product/ allelic ladder</td><td>1.5</td></tr>
<tr><td rowspan="3">Investigator® IDplex Plus Kit</td><td>DNA Size Standard 550 (BTO)</td><td>0.5</td></tr>
<tr><td>Hi-Di™ Formamide</td><td>12.0</td></tr>
<tr><td>PCR product/ allelic ladder</td><td>1.0</td></tr>
</table>

A 47 cm/50 µm capillary was used along with POP-4™ polymer and 10x Genetic Analyser buffer with EDTA for the electrophoresis with manufacturers' recommended injection and electrophoresis conditions as shown in Table 6.

Table 6 The injection and electrophoresis conditions following the respective commercial STR kits' manufacturer's recommendation.

Component	Settings	
	AmpFlSTR® Identifiler® Plus PCR Amplification Kit	**Investigator® IDplex Plus Kit**
Module file	GS STR POP4 (1 mL) G5 v2.md5	GS STR POP-4 (1 mL) G5
Matrix file	Standard Set DS-33	Matrix BT5
Size standard	GeneScan™ 500 LIZ® Size Standard	SST-BTO_60-550 bp
Injection time (s)	5	5
Injection voltage (kV)	15	15
Run voltage (kV)	15	15
Run temperature (°C)	60	60
Run time (min)	28	28

Raw data or the electropherograms were imported into GeneMapper® ID Software 3.2.1 for analysis and interpretation. The peak amplitude threshold (cut-off value) was set at 50 RFU. Single-peak locus with peak height of over 150 RFU was called as homozygous based on internal validation. Concordance of alleles were examined for samples of different bone elements from the same individual. STR profile generation from extracted DNA was done if non-concordant alleles were found from the profiles generated by direct PCR method for confirmation.

This study opted to follow guidelines for the Australian DNA database as it is one of most straightforward and easiest to understand and apply. Currently, there is no Thai criteria, but the Thai forensic police are familiar with the Australian system due to the strong ties of the police of both countries. The current Australian policy states that a minimum of 18 alleles is required for a profile to be eligible to be uploaded to the NCIDD, with analysis of the samples being done within an accredited facility (Wilson-Wilde *et al*., 2017; Wilson-Wilde & Pitman, 2017). This criterion was adopted for this study for considering usefulness of the STR profiles generated.

2.7 Statistical analysis

Statistical difference was investigated and compared for the number of alleles and peak heights obtained. IBM SPSS Statistics Software (New York, United States of America) was used for performing the statistical analysis on the resulting numbers of alleles and peak heights: Kruskal–Wallis one-way ANOVA test for comparison from the aspect of bone elements, and Mann–Whitney U test for comparison from the aspect of bone structure and post-mortem interval (PMI). Dunn's Test of Multiple Comparisons Using Rank Sums was done as post hoc analysis for determining the more significant pair (stochastic dominance). Statistical significance for all tests were declared when the p-value is lower than 0.05.

CHAPTER 3

Results and Discussion

This study aimed to optimise treatment for STR typing from human bones without prior DNA extraction. The performance of two different commercial STR kits for direct STR analysis were compared. The performance of various bone elements (femur, 1st distal phalange of the hand, capitate, patella, metacarpal 4, talus, and tibia) for direct STR analysis were also assessed. Finally, the feasibility of applying the developed direct method of STR analysis on some casework samples were also studied.

3.1 Development of a direct PCR method for STR analysis from bones

In this section, an optimised direct PCR method for amplification of DNA from human bones for STR analysis was successfully developed. Preliminary experiments using bone powder (same samples used in the optimisation experiment) added directly into PCR reactions (1.0 mg, 1.25 mg, 1.5 mg), and using various volumes of PBS buffer (20 µL, 30 µL, 40 µL) with varied amount of bone powder (1.0 mg, 1.25 mg, 1.5 mg) (Table 3) resulted in no or very low partial profiles (Table 7), which suggests that either there was insufficient amount of DNA for successful amplification or too much inhibitor which inhibited the amplification reaction. One hundred mg bone powder in different amounts of PBS buffer (Table 3) was then tried and the supernatant was used for subsequent DNA amplification by PCR, giving high partial profile (median of 25.5 to 28.5 alleles) with a median peak height of 184.5 to 433.5 RFU for Identifiler® Plus Kit, and 14 to 31 alleles and 96.5 to 979.5 RFU for IDplex Plus Kit (n=2 for each kit at each of the six different volumes of PBS).

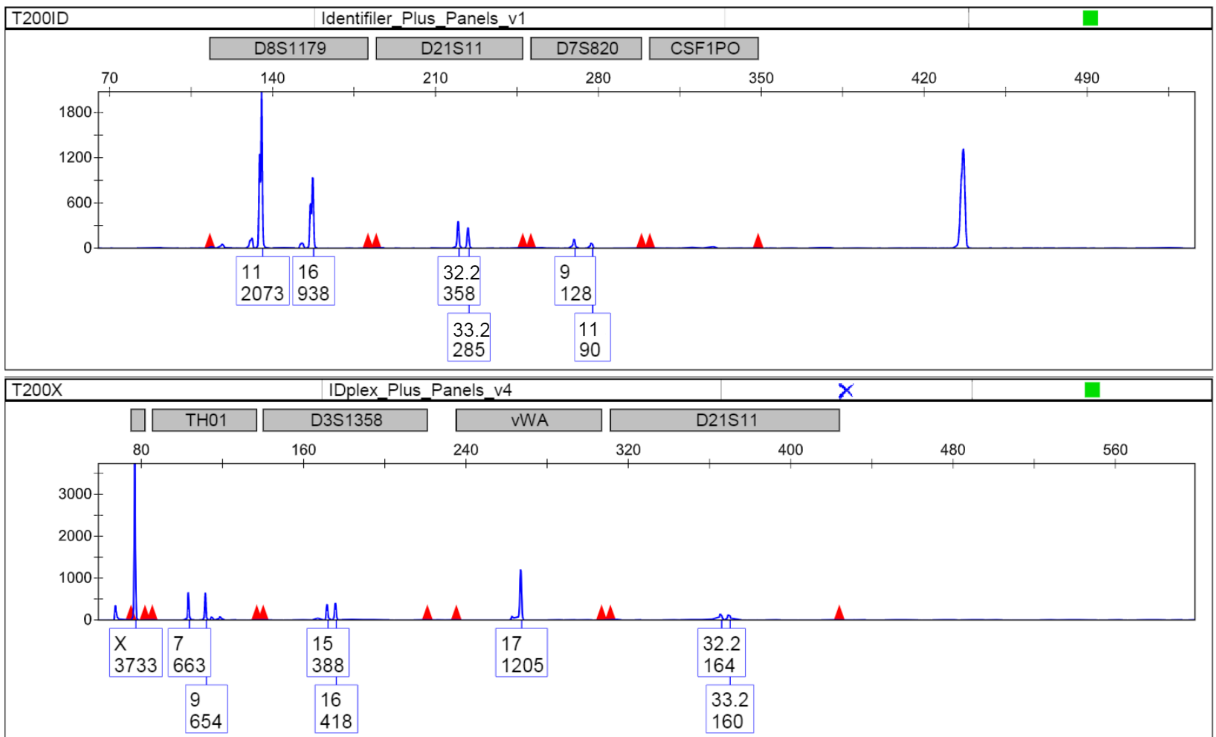
Table 7 The amounts of bone powder, volumes of PBS buffer, and the resulting number of alleles from the preliminary experiments.

Amount of bone powder (mg)	Volume of PBS buffer (µL)	No. of allele
1.00	0 (direct-direct protocol)	0
	20	7
	30	9
	40	11
1.25	0 (direct-direct protocol)	0
	20	0
	30	1
	40	4
1.50	0 (direct-direct protocol)	0
	20	0
	30	0
	40	2

The general trend from the amplifications in the optimisation experiment was an increasing median number of alleles and peak height obtained from supernatant of PBS volume 100 µL to 300 µL for Identifiler® Plus and IDplex Plus (Figure 3 & Table 8). A downward trend was seen starting from PBS volume of 500 µL to 1000 µL for amplification results of both kits. It was deduced that the increased volume of PBS buffer used may have diluted the DNA released from the lysis of bone powder too much. The concentration of DNA per µL of supernatant may have been lower than optimal for ideal amplification. Three hundred microlitres of PBS buffer was determined to be the optimal volume in which 100 mg of bone powder was mixed and heated at 98°C for three minutes (Table 8) as it yielded the highest median number of allele and peak height.

(a)

(b)



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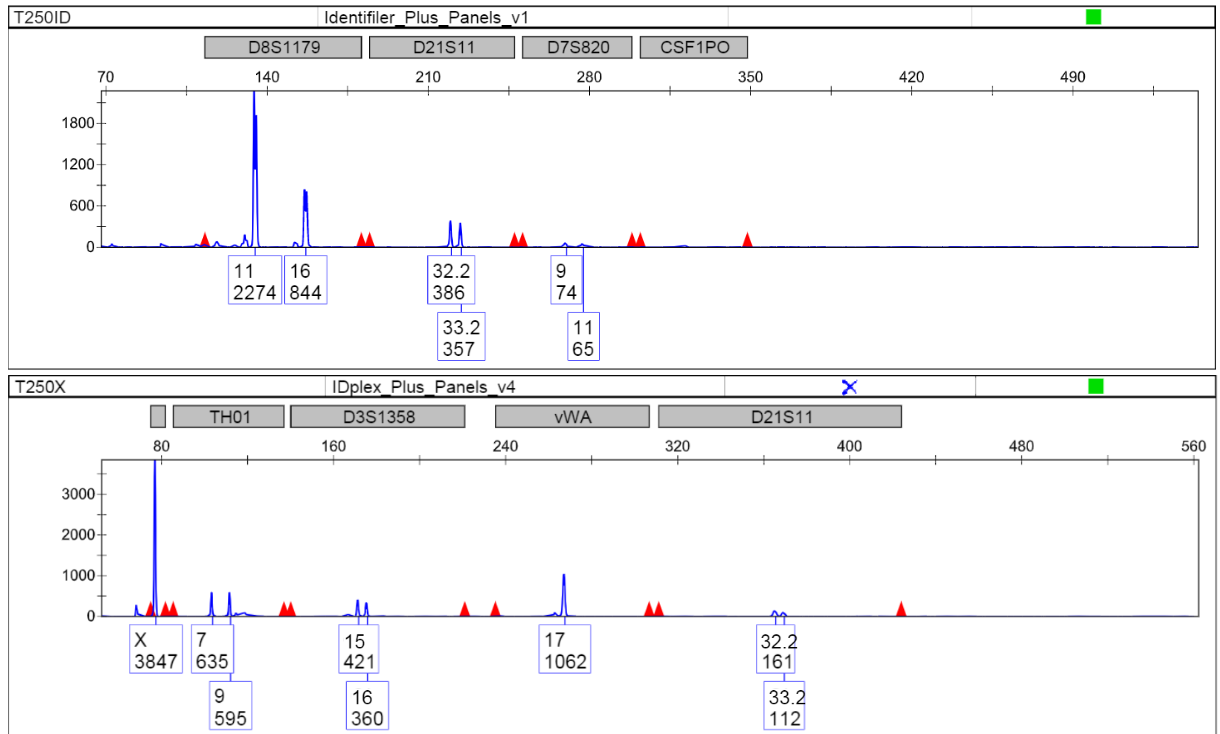

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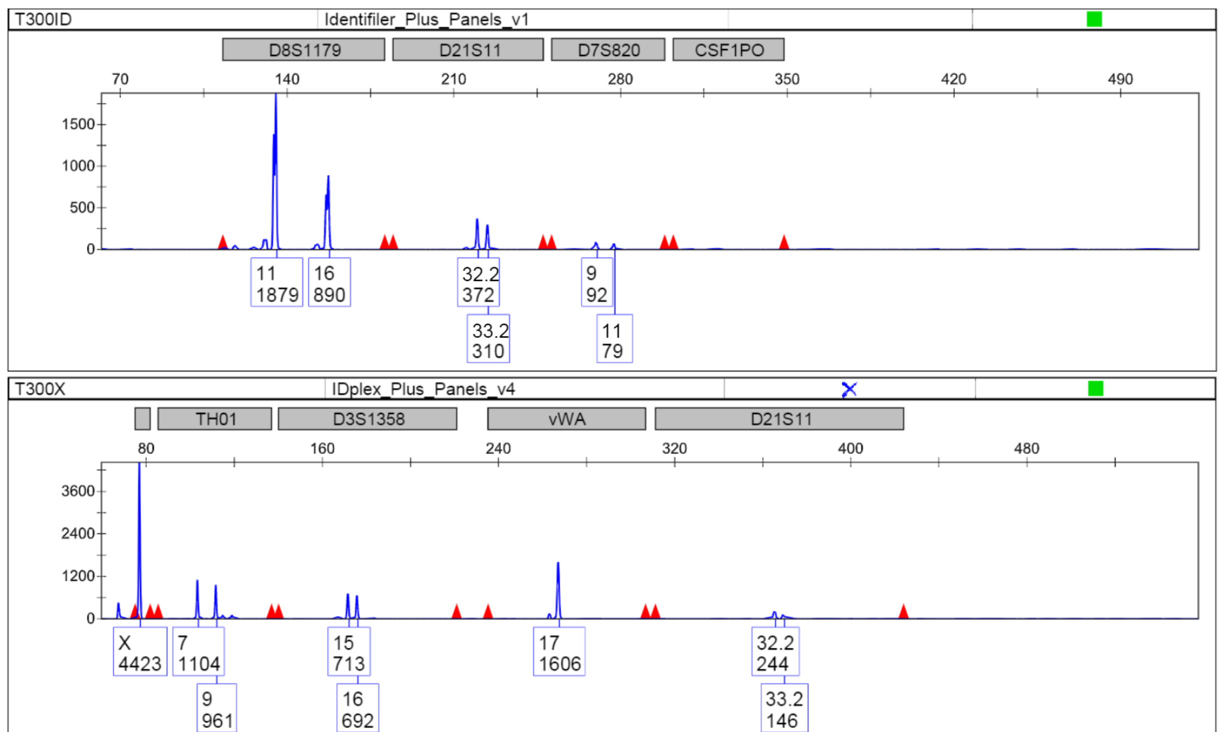

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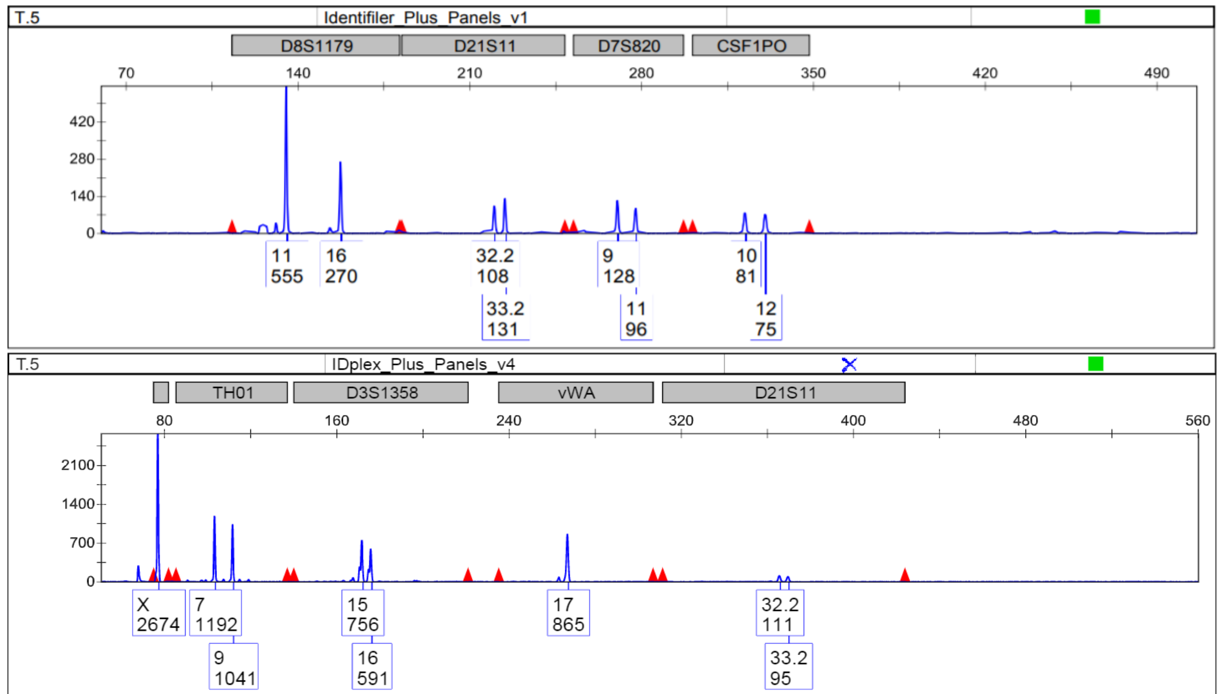

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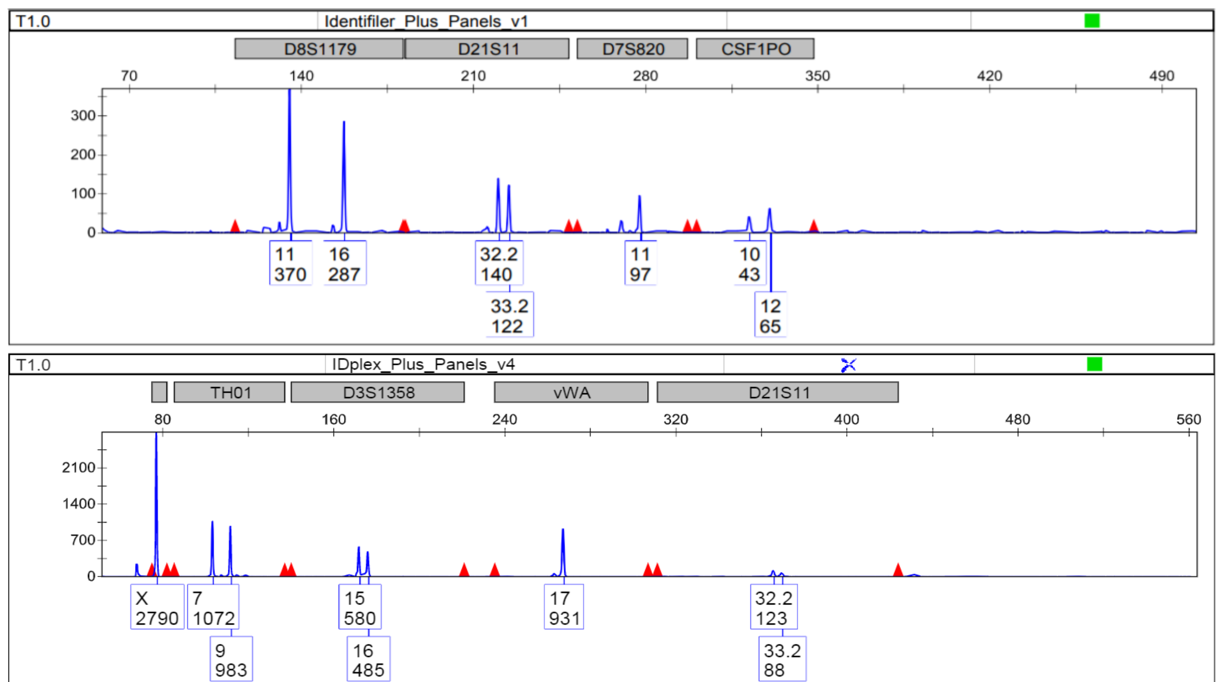


Figure 3 Electropherogram showing the 6-FAM dye portion of the STR profile generated for Identifiler® Plus Kit and IDplex Plus kit using supernatant from 100 mg of bone powder incubated with various volumes of PBS buffer: (a) 100 µL, (b) 200 µL, (c) 250 µL, (d) 300 µL, (e) 500 µL, (f) 1000 µL.

Table 8 The median number of alleles and the median peak height, with IQR, obtained from the optimisation by variation of volume of PBS buffer added to 100 mg of bone powder. The supernatants were subsequently amplified using AmpFlSTR® Identifiler® Plus PCR Amplification Kit and Investigator® IDplex Plus Kit. Two amplifications (one tibia sample and one femur sample) were performed for each PBS volume and kit.

PBS volume (µL)	**STR kit**			
	Identifiler® Plus		**IDplex Plus**	
	No. of alleles	**Median peak height (RFU)**	**No of alleles**	**Median peak height (RFU)**
100	27.0±1.5	242.5±38.8	28.0±0.0	402.5±78.8
200	26.5±1.5	353.0±31.5	31.0±1.0	670.5± 5.8
250	27.0±0.0	433.5±45.8	30.5±0.5	830.0±62.0
300	25.5±2.5	368.0± 2.0	31.0±1.0	979.5±11.3
500	28.5±3.5	187.5±35.8	14.0±2.5	96.5±12.8
1000	26.0±4.0	184.5±19.3	21.5±2.3	100.0± 7.0

Fresh bones were used in the optimisation process due to its higher DNA concentration and intact DNA as compared to aged samples (Mundorff & Davoren, 2014; Turingan *et al.*, 2019). This was to ensure that the concept of the application of direct PCR on human bones is feasible. In normal extractions, the multiple or prolonged decalcification step causes loss of DNA through damage to the DNA (Żołędziewska, Gronkiewicz, & Dobosz, 2002) or decrease in yield (Jakubowska *et al.*, 2012; Schwark *et al.*, 2011). The STR typing success was attributed to the pre-PCR dilution protocol described by Kitpipit *et al.* (2014): the dilution step that incorporated the use of PBS buffer led to the dilution of PCR inhibitors (particularly calcium which bones predominantly is made up of) (Kitpipit *et al.*, 2014a; Thongjued *et al.*, 2019), helped maintain pH of the subsequent PCR (pH of PBS buffer: 7.4), and also possibly diluted the DNA concentration to an optimal range for PCR, as overloading of DNA adversely affect rate of PCR (Linacre & Tobe, 2013). Bones generally are made up 70% of calcium as hydroxyapatite along with calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide and calcium citrate (Loreille *et al.*, 2007) whereby this

calcium acts as a competitive PCR inhibitor with Mg^{2+} cofactor leading to *Taq* polymerase inhibition (Mccord, Pionzio, & Thompson, 2015). Hence, the referenced dilution protocol as described by Kitpipit *et al.* (2014) which was also used here may have also diluted the calcium to a range that did not exert effect significant enough to affect the amplification of DNA from the supernatant. The heating step also denatured proteins and enzymes, which may potentially disrupt DNA integrity or the PCR process (Grevelding, Kampkötter, Hollmann, Schäfer, & Kunz, 1996).

"Ski-slope" effect was also observed in most of the profiles. A "ski slope" effect is when significantly higher signal was observed for smaller molecular size STR markers (left side of the electropherogram) and gradually decreased as the loci's molecular size increase. This observation was due to compromised PCR amplification and attributed to three causes: degraded DNA, low concentration of intact DNA, and inefficient PCR amplification due to inhibitory effect on the polymerase (Butler, 2014). The observation of this "ski-slope" effect in this study could be caused by the two latter reasons. Lysis of the bone powder by heating may not be enough to release more intact DNA within and bound to the bone cells, hence amplification rate, and thus the signal, was lower for high molecular size markers compared to lower molecular size markers. The presence of calcium from bones may also have exerted inhibitory effect on the amplification reaction.

Some signals may be observed alongside with the true allele signals. These are termed as artifacts and originate from biological or instrumental factors. No allelic drop-ins were observed from any of the electropherograms, which indicated no gross or trace DNA contamination. Stochastic effects that cause elevated stutter can also result in allelic drop-ins (Butler, 2014). Other than that, several artifact peaks were seen in the sample electropherograms (Figure 3). Some peaks were due to dye blobs (e.g. plateau peaks before the loci frame of D8S1179 in Figure 3 (a)) which are caused by signals from residual unincorporated dye terminators (Butler, 2014). Some off-scale peaks were also observed from the electropherograms generated. Preferential strand amplification or too much DNA may have caused this. The overloading of DNA may also have caused either stutters or split peaks that can be seen in some of the

electropherograms, with examples with small peaks before the main peaks for loci D8S1179 (Figure 3). Split peaks happens when inefficient polymerase activity occur hence causing incomplete adenylation and could be overcome by prolonging the extension time of the PCR condition (Butler, 2014). Significant-sized minor peaks that could be observed from the electropherograms may also be due to pull-ups and is caused by old matrix standards for the spectrum calibration of the instrument (e.g. uncalled peak at D7S820 in Figure 3(a)). Overlaps of fluorescent colours in the spectrum could not be properly resolved hence causing the overlaps to be called as peaks (Butler, 2014).

DNA quantification would be one of the best predictors on how much samples would be needed or how much DNA should be added to a reaction mix prior to PCR amplification. Generally, samples of lower amount of DNA would lead to STR profiles with fewer loci and alleles, although not necessarily. DNA integrity (i.e. degradation) is also a main factor in determining the success of producing STR profile from samples. However, this information would require a prior DNA extraction step to be performed. DNA extraction from bones are tedious. Initial decalcification and incubation step takes hours to days (two hours PrepFiler® BTA forensic DNA extraction kit; overnight incubation AFDIL casework protocol and total demineralisation protocol (Jakubowska *et al.*, 2012; Loreille *et al.*, 2007). Direct PCR has been gaining favour for application in myriad fields for its lower cost and time consumption (Ambers *et al.*, 2018; Kitpipit *et al.*, 2014a; Thanakiatkrai *et al.*, 2019). Extraction also introduces loss of DNA of up to 76% (Ottens *et al.*, 2013; Templeton *et al.*, 2013).

The developed method poses much convenience when compared to conventional PCR where the sample preparation step (i.e. DNA extraction from bone samples) is rather tedious. A direct PCR feasibility study by Gausterer, Fichtinger, and Stein (2010) had investigated various enzymes and treatments on human bone powder. They amplified the hypervariable region 1 (HV-1) region using different polymerase with direct addition of bone powder, and also supernatant from their dilution protocol (bone powder was incubated in TE buffer supplemented with DNARelease® Additive (Finnzymes, Finland). The supernatant was also amplified using PowerPlex® S5 kit

(Promega) and yielded nine alleles out of 10 (amelogenin included) with a median peak height of 432 RFU. This study confirmed that direct PCR for bone samples was indeed possible, while only using the widely available PBS buffer and with a larger number of loci (16 loci instead of five).

3.2 Commercial STR kits performance comparison

The samples used for optimisation, at the same time, were also subjected to experiment on the performance comparison of two different commercial STR kits: AmpFlSTR® Identifiler® Plus PCR Amplification Kit and Investigator® IDplex Plus Kit. Results from the optimisation study in Table 8 show that IDplex generally performed better in terms of the number of alleles obtained and the median peak height of the generated STR profiles. When 300 µL of PBS buffer was used, DNA amplified using IDplex Plus gave profiles with median allele number of 31 and median peak height of 980 RFU, as compared with 25.5 alleles with median peak height of 368 RFU for Identifiler® Plus. Similar trend was also observed when PBS buffer of volume 100 µL, 200 µL and 250 µL was used. This finding was in parallel to that by Mattayat *et al.* (2016). They found that the two STR kits performed similarly in most aspects, though IDplex Plus kit has higher tolerance towards inhibitors and has statistically higher peak height (Wilcoxon signed-rank test $p < 0.001$). One hundred percent concordance was obtained when the two kits were compared, in both the study by Mattayat *et al*. (2006) and this study.

The results also showed that IDplex generally performed better in four of the six volumes of PBS buffer used in the optimisation study, but it was worth noting that Identifiler® Plus performed consistently in terms of number of alleles obtained for all six volumes of PBS buffer used. This may be attributed to the higher amplification sensitivity of the Kit's chemistry even at lower DNA quantities (i.e. higher volumes of PBS buffer), thus giving flexibility in terms of DNA input range. Although based on the developmental validation reports of Identifiler® Plus and IDplex Plus (Applied Biosystems, 2015; QIAGEN, 2012), both kits reported consistent full profile generation could be obtained from DNA of amount lowest similarly at 125 pg at standard

conditions. Identifiler® Plus Kit and IDplex Plus Kit performed similarly in terms of number of alleles detected from Mattayat *et al*. (2016) (27.1 vs 26.4, Wilcoxon signed-rank test p=0.601). This contradiction with the results of this study may be due to the fact that control DNA was used during the developmental studies and Mattayat *et al.*'s comparison as compared to 'crude' DNA in the supernatant from the developed direct PCR protocol.

Commercial STR kits had undergone rigorous optimisation and validation to make them robust and sensitive in addressing the increasing acceptance and usage range of STR profiling for human identification and individualisation (Butler, 2015; Cavalcanti *et al.*, 2015; Wang *et al*., 2012). Identifiler Plus Kit and IDplex Plus Kit use AmpliTaq Gold DNA Polymerase and HotStarTaq Plus DNA Polymerase, respectively (Applied Biosystems, 2015; QIAGEN, 2012). The difference in their individual proprietary reaction buffer chemistry and composition may also contribute to their difference in performance, and thus suitability of application in different situations and sample types. Overall, based on the result of this comparison, IDplex Plus was chosen for the subsequent experiments for direct STR analysis from human bones.

3.3 Direct PCR from different bone elements

3.3.1 Compact vs. cancellous bones

A total of 105 different bone elements (femur, metacarpal 4, talus, capitate, patella, 1st distal phalange, and tibia) were subjected to direct STR typing, using 100 mg bone powder and 300 uL PBS. Amplification was done using IDplex Plus. The number of alleles in the resulting STR profile from the 105 samples of seven elements of the bone tested varied and partiality of the profile is shown in Table 9, ranging from eight alleles (one sample) to full profiles (45 samples). Overall, 92.4% of the samples tested (97 out of 105, median=31) resulted in high partial (17 to 31 alleles) to full profiles (all 32 alleles), which was high considering no DNA extraction step was involved. Also, no allelic drop-ins were observed from any of the electropherograms observed. The results were first analysed from the aspect of bone structure: compact (femur and tibia) vs. cancellous (metacarpal 4, talus, capitate, patella, 1st distal phalange), with the raw data and descriptive statistics for number of alleles observed and peak height presented in boxplots (Figure 4). By number of alleles observed from the STR profiles generated by direct PCR, compact bones have a median of 30±7 and cancellous bones have a median of 31±5.5. For peak height, compact bones have a median of 490±1216 RFU while cancellous bones have a median of 543±1478 RFU. Independent-Samples Mann-Whitney U Test showed no statistically significant difference in terms of number of alleles obtained (p-value=0.564) but the peak height obtained is significantly better when amplified from cancellous bones than from compact bones (p-value=0.033).

Table 9 The number of profiles with no profile, low partial profile, high partial profile, and full profile that were generated by direct STR typing on the powdered bone samples. Null signifies that no signal was observed from any allele, low partial profile signifies signal from one up to 16 alleles were observed, high partial 17 up to 31, and full profile signifies a complete 32-allele profile was obtained.

Partiality of profile	Null	Low	High	Full
Number of profiles	0 (0%)	8 (8%)	52 (49%)	45 (43%)

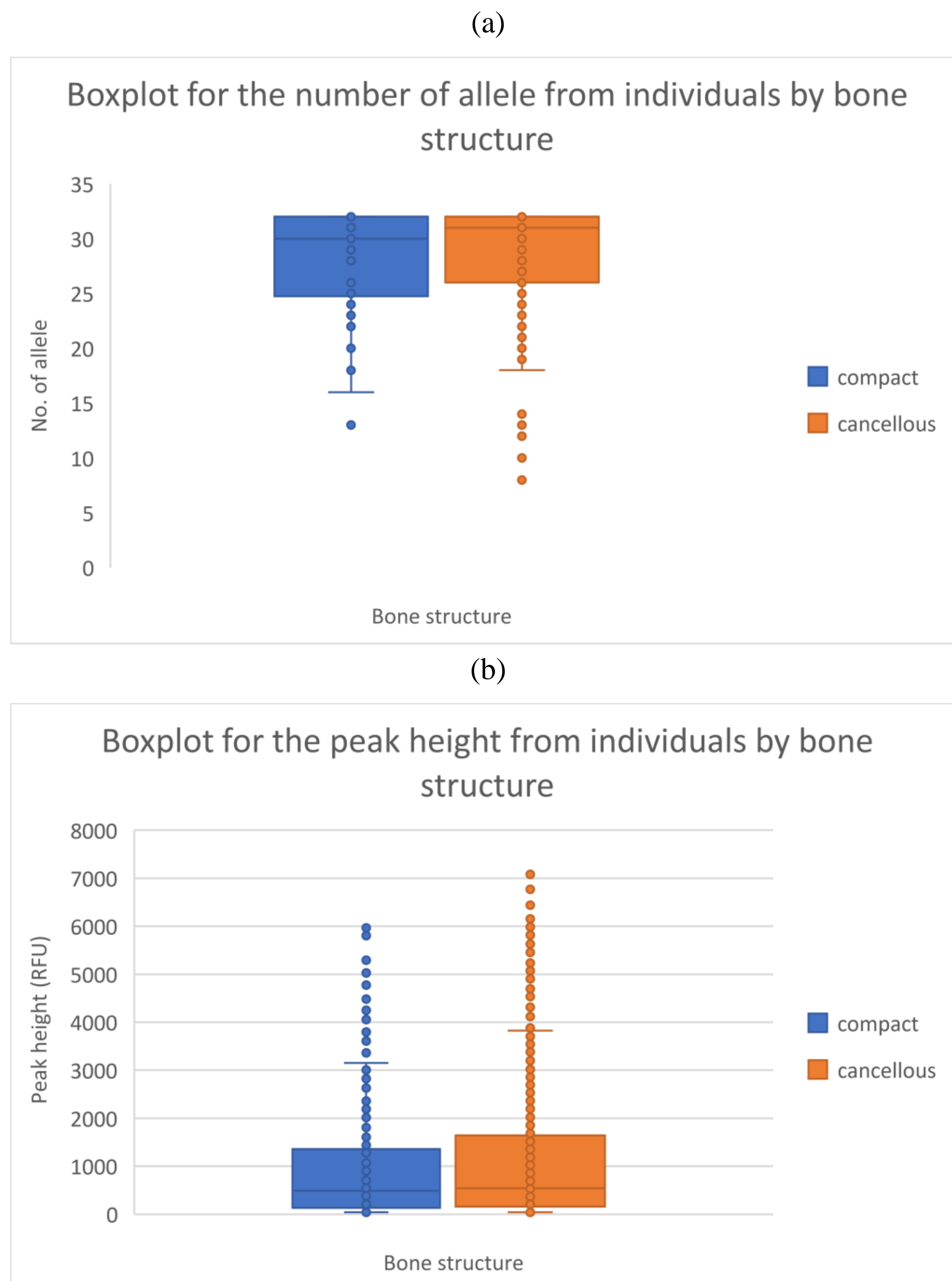


Figure 4 Boxplots showing (a) number of alleles and (b) the median peak height obtained comparing compact bones and cancellous bones using direct STR analysis. The box shows the first and third quartiles of the data set, and the whiskers representing the lower and upper extreme values, with each dot representing one datum. The horizontal line shows the median of the data set.

The results were next analysed from the aspect of different bone elements (Figure 5). By number of allele observed, 1st distal phalange ranked top with median of 32 and IQR of 1.0, patella and tibia 32±5.5, capitate 31±4, talus 30±3.5, femur 30±7.5, and metacarpal 4 with median of 27±8.5. By median peak height, the bone elements ranked slightly differently with 1st distal phalange had highest median, followed by patella, capitate, talus, tibia, femur, and metacarpal 4. There is no statistical difference based on Independent-Samples Kruskal-Wallis test for number of alleles (*p*-value=0.061). The peak heights, however, showed significant difference (*p*-value=0.000).

(a)

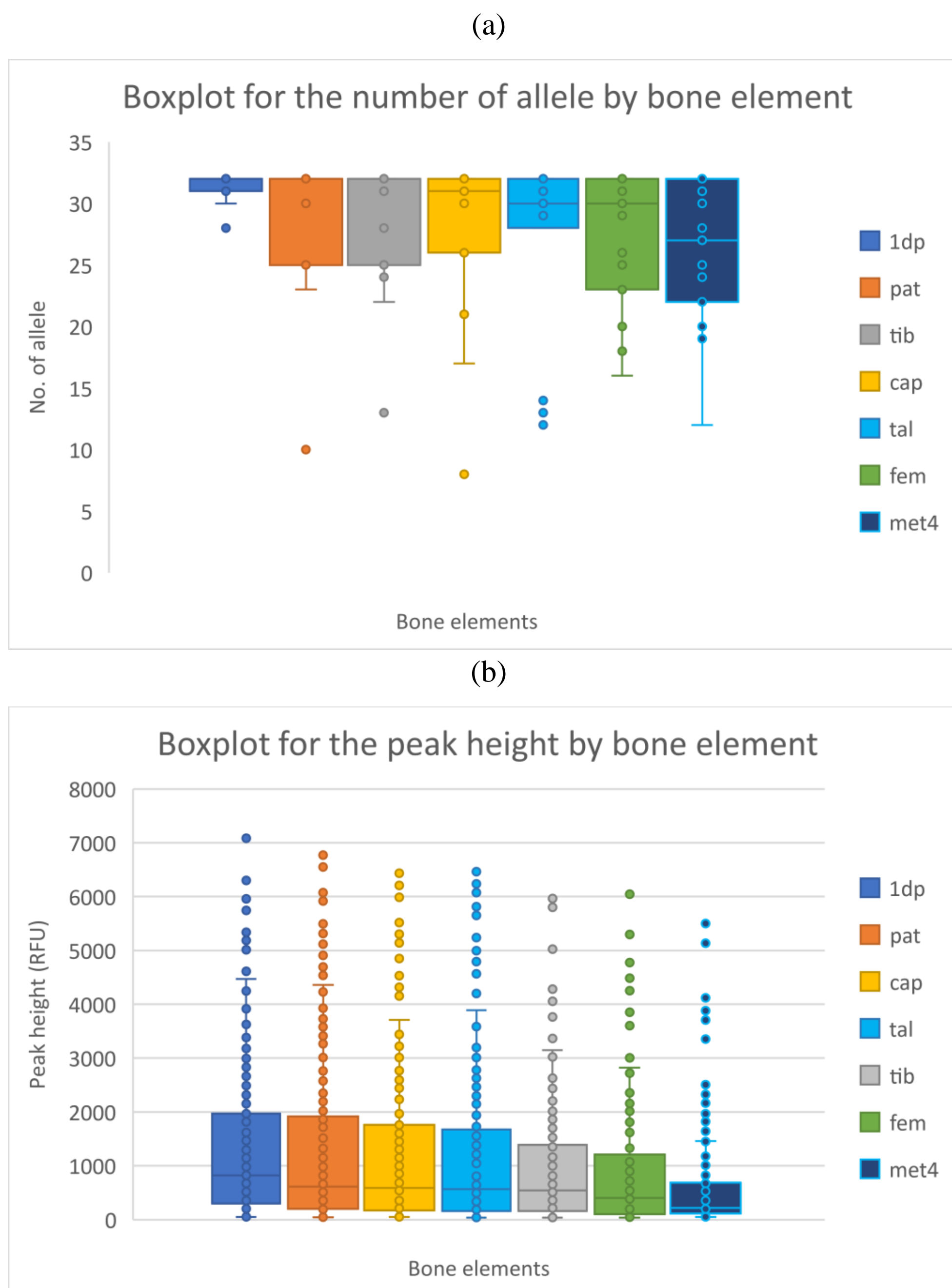


Figure 5 Boxplots showing (a) number of alleles and (b) the median peak height obtained comparing different bone elements using direct STR analysis. The box shows the first and third quartiles of the data set, and the whiskers representing the lower and upper extreme values, with each dot representing one datum. The horizontal line shows the median of the data set.

The bone elements choices used in this study was based on findings from previous studies in which 1st distal phalange, capitate, metacarpal 4, talus, and patella yielded high quantity of DNA (Hasap *et al.*, 2020; Mundorff & Davoren, 2014; Mundorff *et al.*, 2013). Tibia and femur was also included as the long bones of the leg are conventionally-preferred and recommended for collection and STR analysis from human bones for individualisation and identification (Examiners, 2010; INTERPOL, 2018; Mundorff & Davoren, 2014; Mundorff *et al.*, 2009; National Institute of Justice (U.S.). Technical Working Group for Mass Fatality Forensic Identification (NCJ 199758), 2005; National Library of Medicine *et al.*, 2018).

Different bone elements have been proven to differ in terms of DNA content due to various underlying molecular taphonomic factors (Mundorff & Davoren, 2014; Mundorff, Bartelink, & Mar-Cash, 2009), with the results in this present study being consistent with the mentioned works. However, the difference was observed only in the median peak height obtained from STR amplification of different bone elements samples, in which this was dependent on the concentration of DNA. The developed method involved heating of bone powder in PBS buffer at 98°C, lysing the cells and releasing DNA into the supernatant. Since fresh bone samples were used, the quality of DNA liberated may be intact enough and the quantity high enough to be optimally amplified with all markers, hence having no difference in the number of alleles generated from samples of different bone elements.

The significant difference in peak height obtained from direct STR analysis of cancellous bones and compact bones could be crucial for proper allele calling (Albinsson, Hedman, & Ansell, 2011; Leney, 2006). Cancellous bones are less dense than compact bones, are highly vascular, and are made up primarily of trabecula elements groupings (Marks & Odgren, 2002; Mundorff, Davoren, & Weitz, 2013). This tissue element is composed of dense collagenous tissue in which it is theorised where DNA is bound to (Campos *et al.*, 2012). Hence, they could contain more DNA. However, these small bones tend to have lower survival rate than large, compact bones due to decay or animal predation. Compact bones are more efficient in protecting DNA against external degradation due to more protective effect from hydroxyapatite

molecules (Alaeddini *et al*., 2010; Campos *et al*., 2012; Miloš *et al*., 2007; Mundorff *et al*., 2009; Pokines & Symes, 2013; Turingan *et al*., 2019). Past studies have found that age of bone affects DNA yield in a decreasing rate and it differs depending on bone elements (Alonso *et al*., 2001; Amory *et al*., 2012; Loreille *et al*., 2007; Parsons & Haglund, 2005).

If available, the proper selection of best bone elements for DNA analysis during sample or evidence collection is crucial for increased amplification success rate. This in turn should reduce reanalysis and cut down on unnecessary costs and time. Small cancellous bones (1st distal phalange, metacarpal 4, talus, capitate) may be sampled in tandem with direct STR analysis, especially in recent cases and where small bones are available for retrieval (Hasap *et al*., 2020; Mundorff & Davoren, 2014). Cancellous bones could be collected and prepared with just a disposable scalpel rather than a bone saw, in which extra time and precaution needs to be taken for contamination and injury prevention.

The developed direct PCR protocol for STR typing from fresh human bones yielded similar performance from all seven bone elements investigated, indicating that the developed protocol is suitable for all of those bone elements. There is no need for varying the conditions with different bone types, which is convenient for forensic DNA analysts. The protocol is robust, rapid, and easy to be applied, requiring only brief (three minutes) heating of bone powder in PBS buffer that is easily sourced and cost-friendly.

3.3.2 Post-mortem interval

By number of alleles observed from the STR profiles generated by direct PCR using samples of different PMI, bone samples of two days PMI has a median of 32 and IQR of 2 as compared to 28±7 for five days PMI. By median peak height however, the median peak heights were similar for bones collected from cadavers of two days PMI and five days PMI (571±1472 RFU and 567±1405 RFU respectively). Statistically, bone samples that were collected from cadavers with two days and five days PMI showed that the median number of alleles (p-value=0.000), but not the median peak height (p-value=0.559), differ significantly based on Independent-Samples Mann-Whitney U Test (Figure 6).

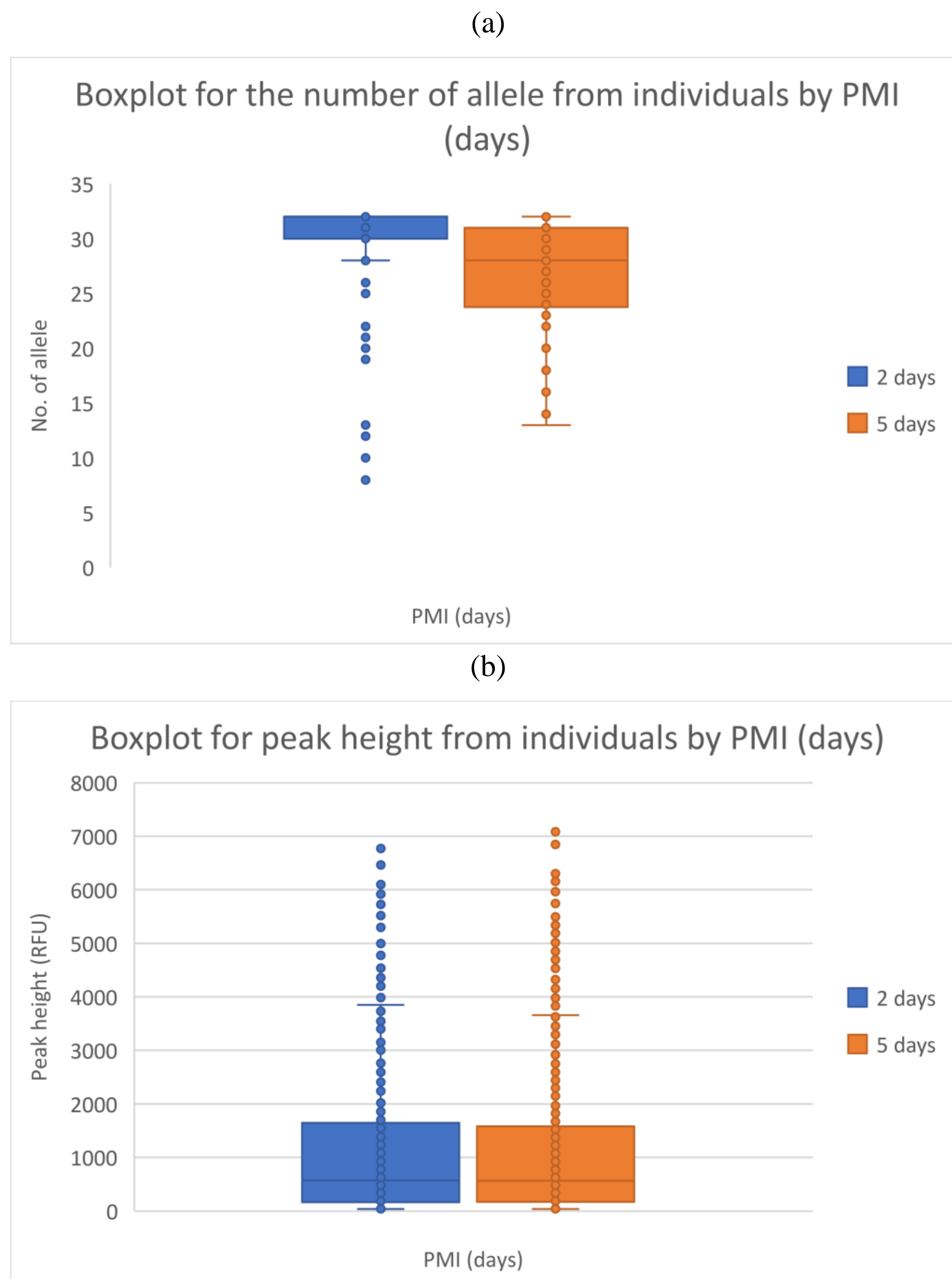


Figure 6 Boxplots showing (a) number of alleles and (b) the median peak height obtained comparing bone collections from cadavers of two days and five days PMI using direct STR analysis. The box shows the first and third quartiles of the data set, and the whiskers representing the lower and upper extreme values, with each dot representing one datum. The horizontal line shows the median of the data set.

DNA degrades at different rates depending on circumstances it is exposed to more than it varies interpersonally (Perry, Bass, Riggsby, & Sirotkin, 1988). The significant difference in the number of alleles obtained from the STR profiles generated using bones of two days and five days PMI may be attributed due to this. The bone remains were collected from cadavers after skilled personnel had performed medical practices on them. As such, they have been exposed to air-conditioned environment (~25°C) while procedures were performed. The timeframe for the PMI was considered starting from death of the donor, cadaver processing by relevant authority, medical practices, until day of sample collections. Therefore, the difference in the number of alleles was assumed to be due to natural autodegradation of DNA post-mortem with no extreme exposure. A study by Ogata *et al*. (1990) analysed the degradation of DNA in muscle samples and found that the yield of DNA decreased from 0.357 mg DNA/g crude tissue at one day PMI to 0.309 mg DNA/g crude tissue at two to three days PMI. Similar findings were also obtained in studies by Bär *et al*. (1988), who analysed post-mortem DNA yield in brain cortex, lymph node, psoas muscle, blood, and spleen, and by Mansour *et al*. (2019) in dental samples. Hence, these finding could serve to fill the gap of knowledge as no study had been done on DNA degradation in bones for PMI of less than a week.

Overall, results from bone elements study are consistent with results from previous studies related to DNA preservation and STR analysis success rates from different bone elements. Although only seven elements of the bones were tested in this study, the findings were considered remarkable as almost no studies have been done on applying direct PCR to human bones for STR analysis. Following entry criteria of Australia National Criminal Investigative DNA Database (NCIDD) (Templeton *et al*., 2015; Wilson-Wilde & Pitman, 2017), a profile with at least 12 alleles (amelogenin inclusive) is considered informative. The current Australian policy also states that a minimum of 18 alleles is required for a profile to be eligible to be uploaded to the NCIDD, with analysis of the samples being done within an accredited facility (Wilson-Wilde *et al*., 2017; Wilson-Wilde & Pitman, 2017). Hence, this direct method of amplification from fresh bone samples was considered to be successfully developed with majority of the bone samples giving profiles that were informative (99/105 i.e.

94.3%). Furthermore, following the current Australian policy, which is also the criterion adopted in this study, a high 91.4% (96/105) of the total profiles generated through the developed direct PCR protocol qualifies for upload to the database. The protocol is feasible and practical in terms of sample amount (100 mg), reagents used (only PBS buffer), and also sample processing procedure (three minutes heating at 98°C).

The developed method also has its limitations. As no DNA extraction was performed, DNA concentration and subsequently the DNA input from the resulting solution could not be estimated for optimal amplification. Too low input may cause inefficient amplification or low amplification product, and overloading of DNA can adversely affect rate of PCR (Linacre & Tobe, 2013). However, the resulting solution could be used for reamplification. In cases of failure to amplify, further dilution of the resulting supernatant, or varying the input volume for amplification could be done to yield better result. A past study on the effect of supernatant storage on amplification success done with wildlife bone samples showed that the supernatant was viable for up to four months (Kitpipit *et al*, 2014a). This was not investigated in this study, but suggested that the supernatant may be reusable for subsequent amplifications.

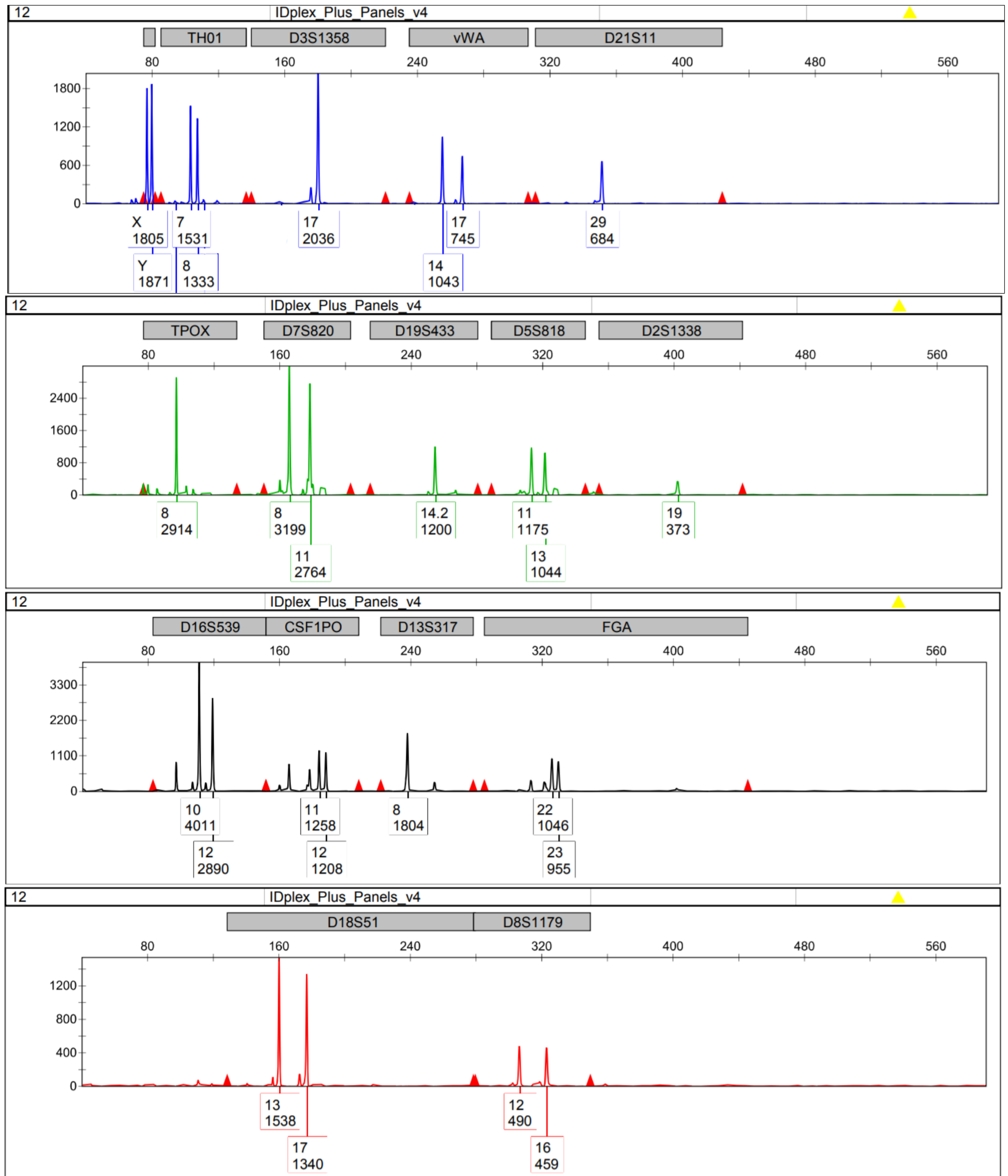
3.4 Feasibility of direct PCR on casework samples

Powdered bones of 19 individual real casework samples were subjected to optimised treatment conditions (100 mg bone powder in 300 µL PBS buffer) and amplified using IDplex Plus. The results are shown in Table 10, with electropherogram from two of the casework samples (one with highest and one lowest number of alleles obtained) shown in Figure 7. The results obtained were of low success rates and profile quality. Only two of 19 samples gave high-partial profiles (25 and 28 alleles). Low-partial profiles were obtained from five of the samples, and the rest (12 samples) did not result in any profile. To determine the samples' DNA quality and quantity, DNA was also extracted using Hasap *et al.*'s extraction protocol (Hasap *et al.*, 2020) and subjected to conventional STR typing (Table 10). DNA of varying concentrations (0.001 ng/µL to 172.335 ng/µL) were obtained by extraction and almost all samples yielded high partial to full STR profiles. DNA concentration did not seem to be dependent on the PMI alone. This could be seen from sample E54091 (172.335 ng/µL and full STR profile), which were collected from burnt skeletal remains of 7 years PMI (longest PMI), with the burning assumed to be only of the flesh although the exact incidence was not known). This suggests that exposure conditions and thus preservation conditions of the bones may have exerted a more significant effect on DNA yield (Latham & Miller, 2019; Soler *et al.*, 2011) compared to PMI. The factor of bone elements for the casework samples were assumed to have minimal effect on the DNA preservation as all of the bone samples were compact bones.

Table 10 The number of alleles and median peak height obtained from direct STR analysis using the optimised developed protocol. The samples were amplified with the better performing Investigator® IDplex Plus Kit. DNA extraction and subsequently STR analysis was also performed on the same samples. N/A denotes not available which means no data was available.

Sample ID	Bone element	PMI	Number of allele (direct PCR)	Median peak height (RFU)	Concentration of extracted DNA (ng/µL)	Number of allele (extracted DNA)
E60045	Femur	< 1 year	0	-	0.527	32
E60033	Humerus	< 1 year	0	-	0.039	25
E60046	Humerus	< 1 year	2	64	1.278	20
E60081	Humerus	< 1 year	0	-	0.219	32
E60082	Humerus	< 1 year	0	-	0.071	27
E60025	Sternum	< 1 year	0	-	0.049	21
E58014	Femur	3 years	0	-	0.001	20
E58015	Femur	3 years	0	-	0.364	32
E58011	Humerus	3 years	25	127	31.752	32
E57041	Femur	4 years	0	-	0.114	32
E57089	Femur	4 years	0	-	0.079	26
E57095	Tibia	4 years	8	64	9.177	28
E57092	Humerus	4 years	0	-	0.004	18
E57071	Sternum	4 years	2	54	1.495	32
E57036	Tibia	4 years	5	82	7.277	32
E57009	Femur	4 years	2	66	4.130	31
E56112	Femur	5 years	0	-	0.023	16
E56004	Unknown	5 years	0	-	0.228	30
E54091	Humerus	7 years	28	1296	172.335	32

(a)



(b)

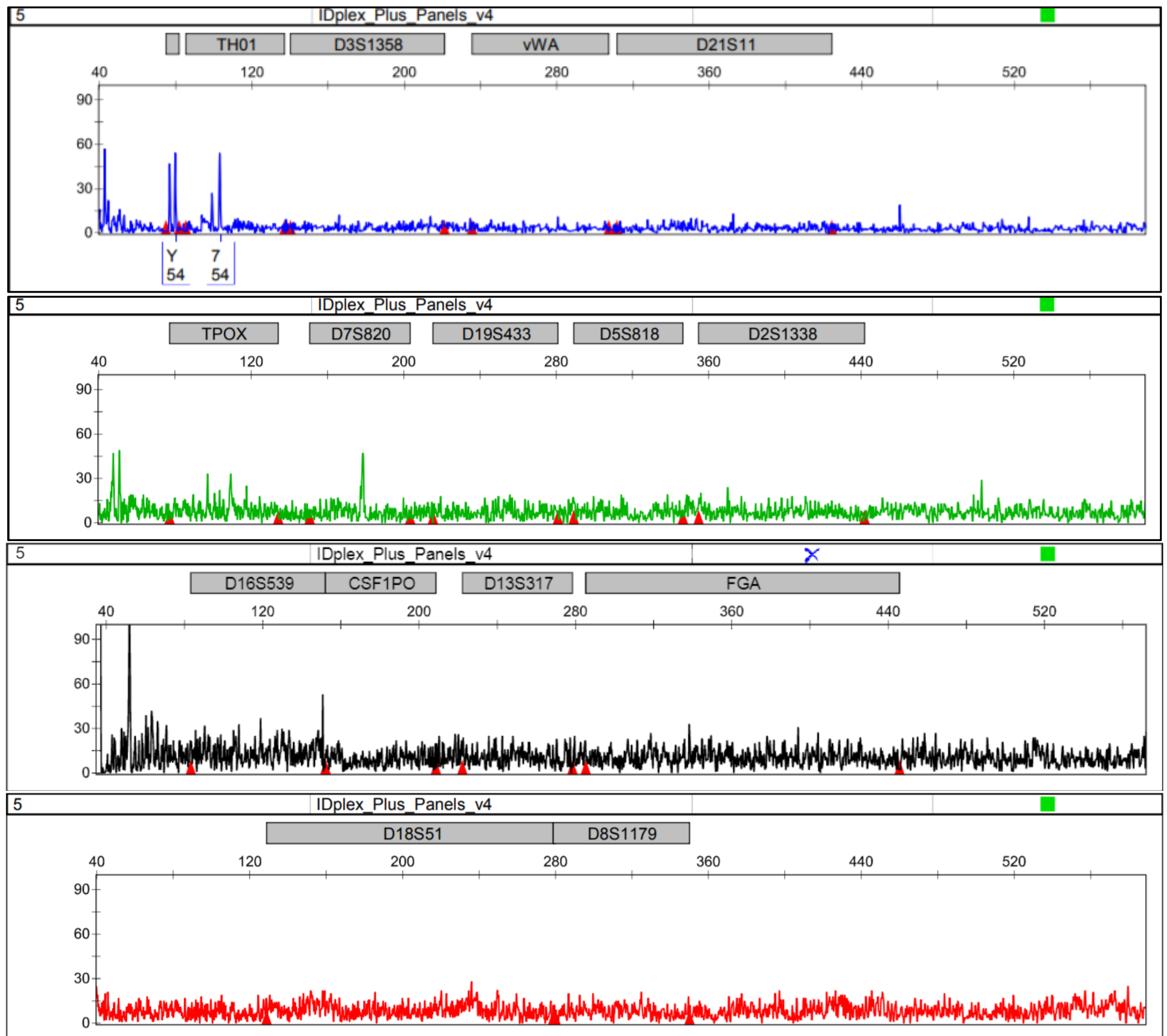


Figure 7 Electropherogram of the STR profile generated using the developed direct PCR protocol from casework samples (a) of ID E54091 which obtained 28 alleles, and of ID E57071 which contained two alleles from AMELY and TH01.

This discrepancy in direct STR and conventional STR result was not surprising due to the type of samples examined. It could be observed that the numbers of allele from the STR profiles obtained through direct PCR correlate with the concentration of DNA that was obtained through extraction. For example, casework sample E54091 with DNA concentration of 172.335 ng/µL, gave a high partial STR profile with 28 alleles and had a median peak height of 1296 RFU with direct PCR. Samples with single digit DNA concentration (e.g. sample E57036: 7.277 ng/µL) and below (e.g. sample E60082: 0.07 ng/µL) gave low partial to no profile. These casework bones had been exposed to various environmental conditions, including but not limited to, being buried in the ground for certain periods of time, reduced to skeletonized remains for individuals, etc. In contrast, bone samples used in the optimisation study and bone elements study were fresh bone samples. They differ in terms of exposure conditions, post-mortem interval, and thus directly have an effect on the integrity, amount, and recoverability of DNA from within the bone (Alonso *et al.*, 2001; Miloš *et al.*, 2007; Perry *et al.*, 1988; Rothe & Nagy, 2016). Exposure conditions and timeframe since occurrence of the criminal act till recovery of the sample affect the concentration and degradability of DNA in the bone (Mundorff & Davoren, 2014; Turingan *et al.*, 2019). Increasing the PCR cycle may increase probability of amplification in cases of low template DNA or concentration. However, this would in turn increase stochastic interference and complexity in interpretation of results due to occurrence of artifacts such as allelic drop-ins (Gill *et al.*, 2000; Jobling & Gill, 2004).

Another factor could lay within the nature of the samples analysed. As had been discussed, bones are predominantly comprised of calcium and that calcium is a PCR inhibitor. The low DNA concentration led to a high calcium to DNA ratio in the resulting pre-PCR solution that was used in the amplification reaction. Further dilution of the resulting pre-PCR solution and also increasing the volume of PBS buffer added to the bone powder prior to heating in order to reduce the inhibitory effect of calcium did not improve the results (not shown). DNA from the bone powder could possibly have been reduced even further from an already low concentration (as seen from results on quantification of extracted DNA) thus leading to failure to amplify. All the casework samples obtained were compact bones. Cancellous bones may yield different results

based on findings from past studies and also this study whereby cancellous bones yielded more DNA and subsequently higher typing success rates. But unfortunately, they could not be obtained for this study due to conventional sample collection preference by the authorities.

The fact that high partial and full profiles were obtainable with DNA extraction means that the DNA within these casework samples are still viable for analysis, even though direct PCR was not successful. Further optimisation of treatment and protocol, such as increased PCR cycle or concentrating the pre-PCR solution, is required for casework samples. Nevertheless, this direct PCR protocol for human bones at its current stage may possibly be adapted for mitochondrial DNA analysis with promising results due to its higher number of copies per cell (Rooney *et al.*, 2015) as compared with nuclear DNA. The drawback of that approach is that mtDNA analysis does not have the uniqueness for individualisation (Budowle *et al.*, 2005).

As emphasis, cancellous bones should be considered for bone collection in cases involving human remains. Based on past studies, cancellous bones had consistently yielded higher DNA amounts and subsequently higher peak height generated, as compared to compact bones. This would allow easier profile interpretation and beneficial in individualisation especially for disaster victim identification. Cancellous bones could be collected and prepared with just a disposable scalpel rather than a bone saw, in which extra time and precaution needs to be taken for contamination and injury prevention. However, case-by-case consideration should be done with compact bones as collection preference instead in cases involving remains with long PMI or exposed to extreme conditions due to their better degree of DNA preservation and resistance to bone degradation.

CHAPTER 4
Conclusion

This research successfully developed and applied the direct PCR protocol for STR typing from fresh human bones samples. Also, the performance for direct STR analysis from human bones of AmpFlSTR® Identifiler® Plus PCR Amplification kit and Investigator® IDplex Plus STR Amplification Kit was also assessed and compared. A practical amount of 100 mg of powdered bone powder heated in 300 µL of PBS buffer poses convenience and beneficial in sample handling, procedure, and time and material consumption as compared to protocols requiring DNA extraction.

Seven elements of the bones were tested with direct PCR in this study. The developed direct PCR could be employed on various bone elements, in which it was applied (femur, 1st distal phalange of the hand, capitate, patella, metacarpal 4, talus, and tibia) with promising results of high partial to full profiles (97/105 samples or 92.4%). These profiles are considered informative following entry criteria of Australia National Criminal Investigative DNA Database (NCIDD), with 91.4% of the profiles uploadable to the database according to Australian policy. This protocol is believed to be expandable to other bone elements (e.g. ribs, vertebrae, and tarsal). The findings were considered remarkable as almost no studies have been done on applying direct PCR to human bones for STR analysis.

The protocol was also feasible to be applied to casework samples though was not promising at its current stage. Cancellous bones could be tried to see if they are more suitable for direct STR typing. Further studies on optimisation of treatment and protocol for casework samples are needed. Tooth samples which contain more DNA per gram may also be considered and studied further to expand the types of samples with similar nature for applying direct PCR for STR typing, which would contribute to the improvement of the overall workflow for DNA analysis from human bones, especially in disasters, other than to reduce overall cost and time consumption.

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APPENDIX

Appendix A

AL-001

หนังสือแจ้งผล
การพิจารณาโครงร่างการวิจัย

สำนักงานจริยธรรมการวิจัยในมนุษย์
15 ถ.กาญจนวณิชย์
ต.คอหงส์ อ.หาดใหญ่ จ.สงขลา 90110

วันที่ 3 เมษายน พ.ศ. 2563

เรื่อง การแจ้งผลการพิจารณาด้านจริยธรรมการวิจัยในมนุษย์
เรียน Cheng Ho Phua

ตามที่ท่านได้ยื่นเสนอโครงร่างการวิจัยเพื่อรับพิจารณาจริยธรรมการวิจัยในมนุษย์ เรื่อง Direct STR Typing from Human Bones หมายเลขสำคัญโครงการ REC.63-143-19-5 นั้น

คณะกรรมการจริยธรรมการวิจัยในมนุษย์ ได้ทบทวนโครงร่างการวิจัย ผลการพิจารณา คือ โครงการเข้าข่ายยกเว้นการพิจารณาจริยธรรมการวิจัยในมนุษย์ (Exempt determination) จึงออกใบรับทราบ เมื่อวันที่ วันที่ 3 เมษายน พ.ศ. 2563

ทั้งนี้ ได้บรรจุในวาระการประชุมคณะกรรมการพิจารณาจริยธรรม ครั้งที่ 10/2563 วาระ 3.3 เพื่อรับทราบต่อไป

จึงเรียนมาเพื่อโปรดทราบ

ลงชื่อ

(รศ.นพ.บุญสิน ตั้งตระกูลวนิช)

ประธานคณะกรรมการจริยธรรมการวิจัยในมนุษย์
คณะแพทยศาสตร์ มหาวิทยาลัยสงขลานครินทร์

หมายเหตุ
- ท่านไม่ต้องรายงานความก้าวหน้าต่อคณะกรรมการจริยธรรมการวิจัยและไม่ต้องต่ออายุโครงการ แต่ยังคงต้องรายงานความก้าวหน้าต่อแหล่งทุนวิจัย
- ท่านยังคงต้องส่งรายงานสรุปผลการวิจัยแก่คณะกรรมการจริยธรรมการวิจัย เมื่อดำเนินการวิจัยเสร็จสิ้น

คณะกรรมการจริยธรรมการวิจัยในมนุษย์
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List of Publication

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