Direct PCR in Forensic Casework

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STATEMENT OF AUTHORSHIP

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All work contributing to this thesis, including published work, was conducted under institutional ethics approval of the Victorian Institute of Forensic Medicine (VIFM RAC037/17).

Chapters or published work included in this thesis are:

- Chapter 1: Introduction

- Chapter 2: Amplification kit determination

- Chapter 3: PM bloodstain samples

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- Chapter 4: AM buccal samples

- Chapter 5: Toenail samples

- Chapter 6: Discussion

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LIST OF ABBREVIATIONS

ADVIC   ANZPAA Disaster Victim identification Committee

AM   Ante Mortem

CE   Capillary Electrophoresis

DVI   Disaster Victim Identification

FTA   Flinders Technology Associates

HID   Human Identification

Interpol   International Police Organization

ISFG   International Society of Forensic Genetics

MBL   Molecular Biology Laboratory

PCR   Polymerase Chain Reaction

PHR   Peak Height Ratio

PM   Post-Mortem

RFU   Relative Fluorescence Unit

RFLP   Restriction Fragment Length Polymorphism

SWGDAM   Scientific Working Group DNA Analysis Methods

STR   Short Tandem Repeat

VIFM   Victorian Institute of Forensic Medicine

ABSTRACT

Forensic DNA analysis is used to assist in the identification of deceased persons during coronial investigations, Disaster Victim Identification (DVI) events, as well as cold case and missing person investigations. For identification purposes, DNA profiles produced from Post-Mortem (PM) samples are compared to DNA profiles generated from Ante Mortem (AM) reference samples containing DNA from either the deceased or a biological relative. Routinely, the process to generate DNA profiles consists of four steps: DNA extraction, DNA quantification, amplification of Short Tandem Repeats (STR) using PCR, followed by capillary electrophoresis (CE). The main disadvantage of this process is the time it takes to complete. Direct PCR is a method for DNA profiling that bypasses the DNA extraction and quantification steps, therefore reducing processing time. Direct PCR kits are STR multiplex kits designed to perform direct PCR of AM blood and buccal samples collected on swab or paper substrates (known as databasing, good quality samples). For PM samples, however, there have been few applications of direct PCR analysis for forensic casework in the literature.

Based on findings from a preliminary study, and the desire to improve (timewise) the current DNA analysis pipeline, the aim of this study was to develop a method that utilises direct PCR to generate DNA profiles for identification purposes for both AM and PM samples. To this end, modifications to the manufacturer's recommended direct PCR protocol were trialled for PM bloodstain and AM buccal samples. With the aim of determining the conditions (such as input amount and cycle number) that would decrease reaction inhibition for bloodstains – as observed in the preliminary study. Furthermore, as toenail samples are the second most common PM sample type received by the laboratory, significant progress was made towards the investigation of conditions suitable for the direct PCR of toenail samples.

The conditions determined for the direct PCR of PM bloodstains and AM buccal swabs samples will be utilised by the Molecular Biology Laboratory (MBL), at the Victorian Institute of Forensic Medicine, to validate their use in casework. Furthermore, the conditions analysed for toenails will be further tested by the MBL to determine if any can be validated for casework.

1. CHAPTER 1: Introduction

1.1 Overview of forensic for DNA identification

1.1.1 Forensic DNA analysis

Forensic DNA is a field of science that utilises DNA analysis methods to assist in the investigation of criminal or civil matters (1). Samples from a crime scene or a deceased person can be analysed for different purposes such as to identify the perpetrator of a crime or identify a deceased person (2). The identification of a deceased person can assist in missing persons cases (3), cold case investigations (4), as well as disaster victim identification (DVI) (5). The samples used for forensic DNA analysis are often compromised with regards to their DNA quality and quantity, characterized by their low DNA yields and even degradation of the DNA. Samples can become compromised as a result of, for example, a long decomposition period or exposure to environmental conditions (such as moisture, extreme temperatures, and soils). Low yield and degradation status of DNA samples could result in incomplete (or partial) DNA profiles, making the analysis and interpretation of the data more difficult.

DNA analysis methods used for DNA profiling have evolved since Professor Sir Alec Jeffreys first exploited genetic variation in human DNA to develop the first method for DNA profiling – known as Restriction Fragment Length Polymorphism (RFLP) (1). Over the years, DNA technology has evolved culminating in a technique known as Short Tandem Repeat (STR) analysis that is at present routinely used in forensic laboratories to produce nuclear DNA profiles (here in referred to a DNA profile) (6). Like RFLP, STR analysis also exploits genetic variation in humans, focusing on short repeat units that are targeted using Polymerase Chain Reaction (PCR) amplification in multiplex reactions (1, 6). The subsequent analysis of the fragments generated gives rise to the STR (DNA) profile for that individual or sample (6).

1.1.2 <u>Forensic DNA analysis for human identification</u>

Forensic DNA analysis is commonly used for human identification, specifically when the deceased is not visually identifiable. Human identification is achieved by comparisons of the DNA profile from the deceased to the DNA profile from the presumed victim or a biological relative (5, 7). This comparative DNA analysis is also applied to identify human remains in different disaster scenarios (5, 7-10), from natural disasters such as; earthquakes (11), fires (9, 12), tsunamis (13, 14), hurricanes (15, 16), or non-natural disasters such as; terrorist attacks (17) and plane accidents (18). In these cases, processes and protocols for DNA analysis differ between laboratories. However, these are usually undertaken by following the International Police Organization (Interpol) DVI guidelines (19), the recommendations from the International Society for Forensic Genetics (ISFG) (20), Scientific Working Group on DNA Analysis Methods (SWGDAM), and/or ANZPAA Disaster Victim identification Committee (ADVIC) (21, 22).

For human identification purposes, different samples are used for analysis, and these differ depending on the circumstance and state of the body (20). If a body is not decomposed, a blood sample is usually collected if available (20), while if there is decomposition (and depending on the state of remains) samples include bones (20) and toenails (23). For AM samples, buccal samples from a family member are usually the preferred sample type. In most instances these are collected from a first-degree relative and ideally from more than one family member to increase the statistical strength of the DNA match (20). Items owned by the presumed deceased such as a toothbrush, hairbrushes, razors, and others, can be used as the AM sample and will be directly compared to the profile of the deceased (20). Forensic laboratories will have validated methods for the processing of samples routinely submitted for DNA analysis.

1.2   Standard DNA analysis process

The process of DNA profiling (also known as DNA genotyping) is conducted at a laboratory equipped for DNA testing. Once the sample arrives at the laboratory for analysis, the standard analysis pipeline for generating DNA profiles is routinely performed via a process which consists of several steps. These steps are sampling (if required), DNA extraction, DNA quantification, DNA

amplification of STR markers, capillary electrophoresis (CE), profile interpretation and reporting (24) (Figure 1.1).

The DNA extraction step is performed to recover the DNA from the nucleus of the cell (25). DNA extraction is achieved by separating the DNA from other cellular material, such as proteins and membranes (25). Importantly, the DNA extraction step enables the removal of inhibitors that can hinder downstream DNA analysis processes (25). Once the DNA is extracted, the next step is to perform DNA quantification to determine the yield of DNA. By quantifying the extracted product, the quantity of DNA is determined and based on the DNA amount, this can be diluted, or its concentration can be increased, to input the ideal amount of DNA into the STR amplification as determined by internal validation (25). This process is used to avoid excessive DNA input into the PCR, as this can result in undesirable overamplification effects in the resulting DNA profile by generating extra peaks, minus A peaks, split peaks, and off-scale data (25). Additionally, by quantifying the DNA, a low amount of DNA input into the PCR can be avoided (25). Low DNA input in the PCR can result in allele drop-out, peak height imbalance and partial profiles. Furthermore, depending on the quantification method used, the presence of PCR inhibitors as well as the level of DNA degradation in the samples can be estimated. Non ideal input amounts, PCR inhibitors and DNA degradation are often detrimental to the PCR process and can impact the ability to obtain a full STR profile.

The next step in the DNA analysis pipeline requires the amplification (using PCR) of the STR loci being analysed. Multiplex STR amplification kits contain PCR reagents to prepare a master mix to which DNA is added for PCR amplification (26). In Australia, the requirements are for DNA profiling to be conducted on a core set of 18 STR markers (or loci) (27). Several commercially available DNA profiling kits meet this criterion, which have been validated for Human Identification (HID) analysis pipelines. These kits vary in reagents and final composition of STR markers tested (18 core loci plus additional markers). The kits that are used in the various forensic laboratories are based on that laboratory's preference.

Amplification of the target regions in the extracted product is by PCR, generating hundreds of millions of copies of a target DNA sequence (6). PCR consists of a change in the temperature for certain number of cycles. The temperature and times for each step in each cycle varies between

amplification kits. In brief, each cycle consist of a denaturation step , a primer annealing step , followed by an extension step culminating in the creation of new DNA strands of the target sequences (6). Commercial STR profiling kits, such as Globalfiler™ PCR amplification kit, add an additional adenosine nucleotide to the 3' end of the PCR product. A final extended extension step so that this adenylation is completed in full is often performed (6).

Following amplification, CE is conducted on a genetic analyser. By performing CE, the analysis or visualisation of the PCR product is achieved by separation and detection of the STR alleles (6). To visualize the DNA profiles, files generated from the genetic analyser will be imported into a genotyping software that utilises allelic ladders and size standards to generate genotypes. A scientist would analyse the DNA profiles by following the laboratorie's guidelines, and finally reporting the case.

1.3 Issues to consider in the standard DNA analysis process for identification

The DNA analysis is well established and routinely used in forensic laboratories. As these strive for continuous improvement, there are several issues to consider from the perspective of using DNA for human identification applications. These include time of processing, contamination risks, and DNA loss. These are described in more detail below.

1.3.1 Time of processing

The main disadvantage of the standard process is the length of the time it takes to produce a DNA profile. The processing time will vary depending on which sample type is being profiled, which is routinely two days for some sample types (24) (Figure 1). Although, the time can change between laboratories due to different methodologies used.

From an identification point of view, waiting for the identification process to be completed is stressful for families; whether it be to identify a close family member, a missing relative, or a deceased loved one during a mass disaster event. Also, the length of time can often result in added stress for loved ones since the deceased cannot be released for burial until the identification process is completed (28). Similarly, the time it takes to receive results from DNA profiling can also

be an issue during criminal investigations; as there is a risk that the perpetrator will reoffend while investigator(s) wait for the DNA evidence (29). Furthermore, the time it takes to generate scientific evidence can be an issue for investigators as government laws or convention limit the time an individual can be detained (29, 30).

1.3.2 Contamination risks and DNA loss

A major risk to DNA profiling is contamination, with the resulting DNA profiling attributed to a contaminant rather than the sample being analysed. External contamination by environmental DNA, or cross contamination during wash methods and tube changing, can occur in process with multiple steps were includes handling the sample.

During the standard processing pipeline there is also the risk of sample (DNA) loss which primarily occurs during the purification steps of a DNA extraction protocol (24). During the extraction step, DNA is isolated and purified from the cell's nucleus with any proteins and any other cellular components that could inhibit the PCR removed. Common DNA purification methods, such as magnetic bead and column-based purification, have been reported to result in DNA loss; with various studies reporting DNA losses of 20 – 90% (31-36). DNA loss during extraction presents an issue during forensic DNA profiling as it could result in partial or no DNA profiles from the sample.

Improvements in the DNA profiling process could benefit everyone who is waiting for a profile result, by reducing the duration of the process. Similarly, increasing the probability of producing a reportable profile, through minimising DNA loss and contamination risks, would also be beneficial. Hence, by removing the extraction and quantification steps with a direct PCR protocol, the issues of sample processing time, contamination, and DNA loss could potentially be minimised.

1.4   Direct PCR method

Direct PCR is a method where biological samples are amplified without first extracting and quantifying the DNA (37). In a direct PCR workflow, once a sample arrives to the laboratory, sample preparation with a direct PCR amplification kit would be undertaken, followed by amplification and

subsequent separation and detection by CE, concluding with data analysis (37) (Figure 1). The issues described above for the standard method may be mitigated with a direct PCR method as there is no extraction and quantification steps.

The main advantages of direct PCR are the reduction in processing time as well as costs resulting from the bypassing the extraction and the quantification steps (Figure 1). Different laboratories have reduced genotyping time and costs by applying direct PCR following the recommended protocol of a commercially available STR kit (37-40). Direct PCR may also decrease the possibility of contaminating the sample; as well as decreasing possible DNA loss.

1.4.1 Commercial kits for direct PCR

Different commercial amplification kits have been developmentally validated by the manufacturers to perform direct PCR on AM blood samples to omit the extraction and the quantification steps (41-43). The GlobalFiler^{TM} Express PCR amplification kit (hereafter referred to as GlobalFiler Express) is one of a number of commercial amplification kits explicitly developed to be used for direct PCR (41). The GlobalFiler Express kit was developed to perform direct PCR amplification for: (i) AM reference bloodstains and buccal samples collected on treated paper, (ii) AM reference bloodstains and buccal samples on untreated paper and prepared with Prep-n-Go^{TM} Buffer, and (iii) AM buccal swabs treated with Prep-n-Go^{TM} buffer (41). The GlobalFiler Express kit utilizes 6-dye fluorescent chemistry to amplify 21 autosomal STR loci, 1 Y-STR, 1 Y chromosome indel, and a sex determination marker (Amelogenin) (41, 44). Developmental and internal validations of the GlobalFiler Express kit have demonstrated the successful direct PCR of AM blood and buccal samples, when following the manufacturer's recommendations (39, 44).

1.4.2 Direct PCR- AM samples

Living donor samples (AM) are usually buccal or blood samples (45). These biological samples are collected from living persons suspected of a crime, families of an unidentified deceased, or research volunteers (45). Living donor samples serve as a good source because their level of

undegraded DNA is higher than post-mortem (PM) samples, meaning they have high levels of reproducibility (46).

Buccal samples

The non-invasive nature of the collection process, as well as the fact that these samples can be collected by nonmedical workers with minimal training, makes buccal samples the primary reference sample used in forensic DNA analysis. Additionally, this sample type allows for the cost-effective collection of large numbers of samples. In forensics, buccal samples are collected for different purposed, usually from living donors. Buccal cell samples are commonly used to analyse DNA for different biological purposes (46-49). This includes missing persons investigations, where buccal samples are collected from relatives of the missing person as part of the AM data collection (50), as well to assist in the identification of deceased persons reported to the Coroner, where buccal samples from a family member are used for kinship comparison with the deceased to assist in the identification process (51). Buccal samples are also collected from individuals suspected of a crime, for comparison to criminal database; and also from those persons wishing to undertake kinship comparison for parentage or sibship testing (52). On mass buccal samples are also collected from volunteers to assist in the development of population databases to assist in statistical analysis (46, 53).

Direct PCR performed with AM buccal samples has been shown to generate complete DNA profiles with high success rates, and a shorter processing time than standard DNA profiling protocols (54-57). Although buccal samples can be collected and utilised differently, the most common ones are buccal swabs or buccal cells on FTA paper. The GlobalFiler Express kit, the STR amplification kit chosen for this project, is designed to be used with AM buccal samples or more specifically, buccal swabs or buccal cells on treated or untreated paper (41). Different validation studies have also demonstrated the successful performance of this kit when using AM buccal samples (39, 44).

1.4.3 <u>Challenges faced when performing direct PCR in forensic DNA analysis</u>

Despite all the advantages that direct PCR has, the performance of this method has disadvantages that can negatively impact the quality of the DNA profile. The disadvantages faced when performing direct PCR, which are not common to the standard method, are caused by not performing the extraction and the quantification steps before STR amplification.

The extraction step is conducted on the sample to recover the DNA from the cell, and to purify the extracted DNA from other cellular components, thus reducing the presence of PCR inhibitors. Therefore, by not performing this step during the direct PCR method, inhibitors are not removed from the sample prior to PCR. The manufacturer of the GlobalFiler Express kit suggests that for AM buccal and blood samples, substrates such as treated paper (e.g. NUCLEIC-CARD^{TM} or Whatman^{TM} FTA^{TM} cards), untreated paper (e.g. 903 paper or Bode Buccal DNA Collector^{TM}), and swabs just for buccal samples (e.g. FLOQSwabs^{TM} or cotton swabs) are used (41). FTA (Flinders Technology Associates) cards contain filter paper with reagents that allow the cell to lyse and release the DNA from the cell matrix (58) as well as preserves the DNA integrity at room temperature (59, 60). Collecting the sample on FTA cards eliminates the need for lysis buffers to be used in conjunction with a direct amplification protocol. However, some samples cannot be collected on these substrates, such as nails, which is a PM sample used for DNA identification of decomposed remains (23).

PCR inhibitors are present in biological samples and can vary depending on the sample type. They can impact negatively on the quality of DNA profiles by, depending on the inhibition level, increasing peak height imbalance and producing allele drop-out. Ambers *et al*. tested the MicroFLOQ Direct swab, a substrate designed for direct PCR (61). When the manufacturer's protocol was followed for bloodstain samples, however, incomplete profiles were obtained, and inhibition was suspected (61). Therefore, Ambers *et al.* tested the swab by amplifying the positive control DNA (007), to determine if the source of the inhibition was from the swab or the specimen. This yielded complete DNA profiles, demonstrating that the inhibition was caused by the bloodstain sample and not by the substrate. However, by diluting the sample before amplification, results improved as inhibitors were diluted (61). This describes that substrates designed for direct PCR may not mitigate PCR inhibitors, and therefore, modifications from the manufacturer

recommended protocols may need to be applied, such as a diluting the sample or decreasing the input amount into the PCR.

Inhibition is not an issue limited only to blood samples, with Aboud *et al.* demonstrating a decrease of 50 % in the peak height of buccal specimens when the individual consumed coffee or alcohol one minute before collecting the sample, indicating that the sample contains PCR inhibitors which are not removed by the reagents added before amplification (30). Additionally, Park *et al.*, demonstrated in 2008, that 30% of buccal samples amplified using direct PCR, failed to show complete profiles, while 100% of the samples produced complete profiles when amplified with the standard method (60). This demonstrated that amplification kits designed for use in direct PCR are susceptible to PCR inhibitors resulting in incomplete DNA profiles. During direct PCR, there is no opportunity (unlike the standard process) to determine how much DNA is present, and importantly if any inhibitors are present, prior to amplification.

Additionally, in the standard method, the extracted product is then quantified. By not conducting the quantification step in the direct PCR method, the optimal amount of DNA for amplification cannot be added to the PCR as the concentration of the sample is not known. There is a reliance on sufficient DNA being released from the substrate for the amplification to result in acceptable profiling outcomes. All sample types, and especially PM samples, vary in the yields of DNA that can be recovered (62). Without quantifying and therefore adding the optimal amount of DNA in the PCR, the risk is that DNA profiles may exhibit signs characteristic of insufficient or excessive DNA input. When insufficient DNA amount is added to the PCR, this may result in profiles with low peak heights, allele drop-out, and peak height imbalance (63, 64). In contrast, excessive DNA input into the PCR may result in profiles with incomplete adenylation (manifested as minus A peaks) and peaks with fluorescence beyond the detection limits of CE instrumentation. As such, a non-ideal starting DNA amount in the PCR can result in the profile generated not meeting reporting requirements. Hence, a method to perform direct PCR must be developed for each sample type independently, by determining the optimal sample input amount and cycle number that produces acceptable quality profiles with majority of the samples. Furthermore, as DNA amount will differ even within the same sample type, it is expected that even when the optimal input amount and cycle number is determined for a sample type, some samples may still result in profiles

characteristic of low or high DNA input. The proportion of samples which fall under this category needs to be assessed during method development.

The majority of commercial amplification kits are designed to perform direct PCR with AM reference samples (41, 65, 66). This represents a gap for forensic DNA profiling, as PM samples require DNA profiling as well, and ideally PM and AM samples would utilise the same method to save cost and reduce processing time. In the literature, however, are only a few applications of direct PCR with PM samples. PM samples face the challenge during direct PCR of containing (i) lower levels of DNA, or (ii) degraded DNA, or (iii) having higher compounds that inhibit the PCR (67) than AM samples. This can be caused by the decomposition of the body and/or the circumstances surrounding death. Poor specimen condition can be managed in the standard process through the extraction and quantification steps, where the DNA sample can be purified, and its concentration determined.

Since there is no manufacturer that recommends a method to perform direct PCR on PM samples, a method would need to be developed for each sample type to determine if direct PCR can replace the standard DNA profiling workflow for coronial identification casework. Some laboratories apply direct PCR on the majority of their samples, while still applying the standard method to other sample types (37). Not being able to process all sample types together, reduces the direct PCR advantage of decreasing processing time.

PM Blood

Blood is a body fluid that contains cellular and non-cellular components. Cellular components in blood comprise of (i) erythrocytes (red blood cells), (ii) leucocytes (white blood cells), and (iii) thrombocytes (platelets) (68). In contrast, the non-cellular component in the blood is the plasma, which contains serum and fibrinogen (68). While erythrocytes can be utilized in forensic serology as a source of non-DNA markers, leucocytes are the only cells in the blood that contain a nucleus (68). Hence from a DNA typing point of view, leucocytes are the source of DNA from blood samples (68).

As mentioned, commercial amplification kits designed for direct PCR are validated for use with AM bloodstains, with the manufactures providing protocols for AM buccal or blood samples (41). However, for forensic identification purposes, there is a need to also be able to process PM samples, such as blood, from the deceased that requires identification. In the literature, there are few applications of direct PCR using PM blood samples. In 2014, Hall and Roy, tested nine commercial PCR amplification kits using a 1.2 mm punch of PM bloodstains on FTA cards and illustrated that complete DNA profiles were generated from PM bloodstains with six out of the nine amplification kits tested (57). These blood compounds are routinely removed in the standard method during the DNA extraction step. The application of direct PCR with PM samples faces the challenge of compounds that are a presence in the sample, and that can inhibit the PCR, which could result in an incomplete DNA profile.

PM Toenails

Keratinous tissues such as nails are utilised as sources for DNA profiling because the DNA is somewhat protected within these tissues from DNA degradation caused by the decomposition process (69). During the standard profiling method, DNA is released from the keratin structure during the DNA extraction step. Therefore, not having an extraction step could be a challenge when performing direct PCR with toenails, with respect to not releasing the DNA from the nail sample. In 2014, Ottens *et al.* applied direct PCR on AM fingernail clippings using the AmpFLISTR^{TM} NGM^{TM} PCR amplification kit (70), where it was reported that direct PCR was a successful method for fingernails as it produced data reportable to the Australian National Criminal DNA database (70). It should be noted, however, that just 17 out of 40 samples (42.5%) yielded complete profiles. Such a low success rate would not lend itself as a suitable replacement to standard analysis workflow for this sample type in routine identification casework.

1.5   Purpose of the study

The Molecular Biology Laboratory (MBL) at the Victorian Institute of Forensic Medicine (VIFM) performs DNA testing for identification purposes. To decrease profiling time and costs, the MBL aimed to evaluate if a direct PCR workflow could be suitable as a replacement for a standard profiling workflow for routine DNA identification casework (Figure 1.1). The purpose of this study

was to develop a method to perform direct PCR to generate DNA profiles for identification casework samples at the MBL. Method development was performed for AM buccal FTA samples, PM bloodstain samples, and toenail samples (from volunteers).

This study evaluated the performance of direct PCR using AM and PM samples (received at MBL for routine identification casework) to produce DNA profiles, with method development geared towards the generation of STR profiles that meet the laboratory requirements. Method development was performed independently for each sample type as these differed in the amount of DNA and PCR inhibitors present. This study aimed to utilise the same amplification kit and amplification cycle number for common samples received by the laboratory (AM buccal and PM blood samples) which, if successful, would result in time and costs savings. Furthermore, the study aimed to commence preliminary examinations for the direct PCR of toenail samples – a sample type expected to be challenging and not previously reported – using volunteer samples.

To develop the method for each sample type, the study needed to consider the following: (i) the amplification kit to be used; (ii) if treatments (pre – and post-PCR) were needed, (iii) amount of input into the PCR, and (iv) PCR cycle number. Ideally, no pre-treatment would be needed for direct PCR, but as the extraction step is removed, it was expected that some PM samples (such as toenails) would require a treatment before amplification to release the DNA for amplification. When considering a treatment regime, this was developed based on conditions that would still reduce processing time and costs compared to the standard workflow. Also, to consider replacing the standard method with the direct PCR, this should meet the assessment criteria of approx. 90% of samples tested producing complete DNA profiles and 75% with an average peak height between 1,200 and 14,000 RFU.

(A)
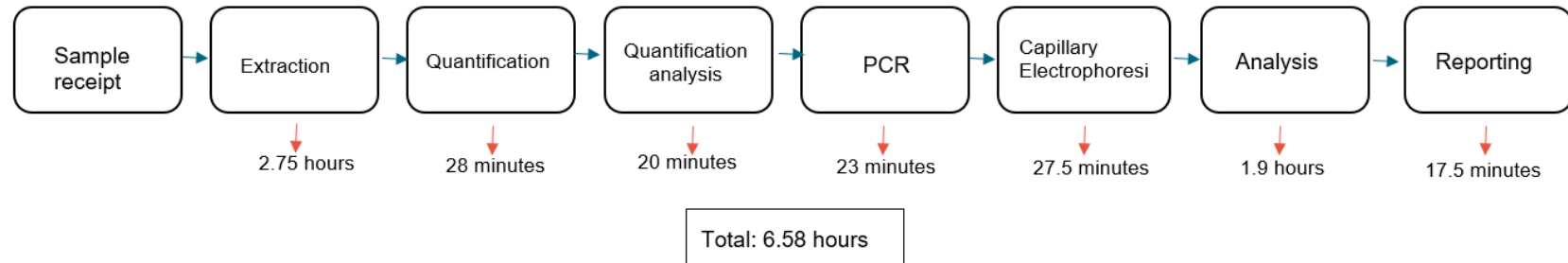
(B)
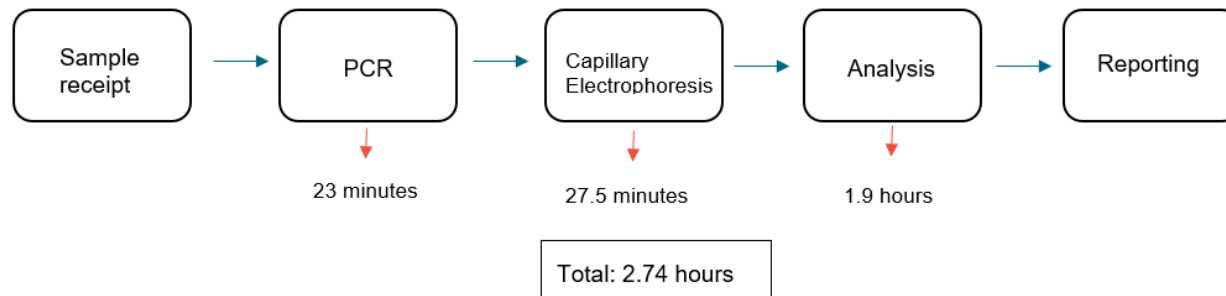
Figure 1.1. DNA analysis workflow. Steps conducted once a sample arrives to the laboratory until is processed by following (A) the standard method currently performed at MBL, or (B) a direct PCR method.

2. CHAPTER 2: Amplification kit determination

2.1 Introduction

There are several commercially available kits for the amplification of STRs. These kits are primarily designed for the amplification of DNA extracts of known quantity that have been extracted from biological samples. Similarly, there are commercial kits designed for the direct PCR of biological samples, which do not require prior DNA extraction of the sample. Both types of kits, standard and direct PCR, have advantages and disadvantages. Commercial kits used for standard amplification, such as the GlobalFiler™ PCR Amplification kit (hereafter referred to as the GlobalFiler kit), are designed to amplify samples in larger reaction volumes (in comparison to direct PCR kits) and to withstand a level of inhibition and degradation of the input material and have been validated for the amplification of PM samples (71). Their use for the direct PCR of PM samples, however, was not their intended purpose and would require further validation studies. Although direct PCR kits, like the GlobalFiler Express kit, have been designed for the direct PCR of AM bloodstains and buccal swab samples (41) with demonstrated success in profiling these sample types (39, 44), their use for PM samples is not well established (72). Any application of the GlobalFiler Express kit for the direct PCR of PM samples, such as bloodstains, would require internal validation.

In a preliminary study, the MBL previously looked at the use of the GlobalFiler Express kit for the direct PCR of PM bloodstains and reference AM buccal samples on FTA/Copan Cards, to test the feasibility of its use with these sample types that are received for casework. In brief, using the manufacturer's recommended protocol for AM bloodstains (41), 1.2 mm punches from PM bloodstain samples were amplified at 25, 26, and 27 PCR cycles. Similarly, using the manufacturer's recommended protocol (41), 1.2 mm punches from buccal swab samples were amplified at 26, 27, and 28 PCR cycles. The results from this preliminary study indicated that the GlobalFiler Express kit may not be suitable for the direct PCR of PM bloodstains, as high levels of inhibition were observed in profiles ((72), unpublished data). Furthermore, the AM buccal samples analysed as part of this preliminary study yielded DNA profiles of insufficient peak height based on the acceptance criteria set for the study. As a result of these findings ((72), unpublished data), the MBL sought to evaluate

both the GlobalFiler kit and the GlobalFiler Express kit, for the direct amplification of PM bloodstains and AM buccal samples.

The GlobalFiler kit is currently in use for casework in the MBL, therefore the use of the GlobalFiler Express kit for the analysis of casework samples (if demonstrated to work) would complement the laboratory's current DNA analysis workflow – maintaining the same STR regions analysed and downstream DNA profiling analysis. The first step in the evaluation of direct PCR in this study was to compare the performance of direct PCR using the GlobalFiler and the GlobalFiler Express kits with PM bloodstain and AM buccal samples, and to evaluate how the resulting DNA profiles met the acceptance criteria set for this method development, by comparing between the kits used, the profile completeness, average peak height, peak height balance, inhibition ratio, and minus A peaks.

In this chapter, a comparison of direct PCR using the two kits (GlobalFiler and GlobalFiler Express kits) with the same set of PM bloodstains and AM buccal samples is described. Based on the results of the preliminary study, examining sample input size (0.5 mm or 1.2 mm punch) and amplification cycles (25 or 28 cycles) was also performed. The aim of this experiment was to determine which kit (GlobalFiler or GlobalFiler Express) performed better based on the DNA profile acceptance criteria set by the laboratory, which would inform further experiments to optimise cycle number and use with other sample types.

2.2   Materials and Methods

2.2.1   Samples

Twenty-six PM bloodstain samples and twenty-six AM buccal FTA samples, both on Whatman^{TM} FTA^{TM} cards (GE Healthcare) and Copan NUCLEIC-CARD^{TM} (Copan, CA), were available for this research project. PM bloodstain samples were prepared by mortuary staff following admission of the deceased to the VIFM and were received by the MBL as part of coronial identification casework. For AM buccal samples, 16 samples were self-collected (the individual swabbed their own cheek and then pressed the swab on the card), while the remaining 10 samples were received by the MBL

following collection by police officers from various jurisdictions throughout Australia on behalf of the VIFM for identification purposes. Both PM bloodstains and AM buccal samples were de-identified at the completion of the identification process for the purposes of this research project. All PM bloodstains and AM buccal samples were stored in sealed bags at room temperature before and during the study.

2.2.2   Sample selection and preparation

Eight PM bloodstains and eight AM buccal FTA samples were selected for this experiment from the samples allocated to the project. Sample selection for this experiment was based on results previously obtained by the MBL ((72), unpublished data). Selected PM bloodstain and AM buccal samples included a representative combination of those that had previously yielded incomplete, overamplified and ideal profiles (Table 2.1).

Table 2.1. Kit comparison – Sample selection criteria. Details of reasons for sample selection from the available 26 samples based on profiling results from the preliminary study.

Selection Criteria	Samples	
	PM bloodstains	AM buccal FTA
No alleles detected	7 & 23	-
Allelic drop-out	6	-
Sign of insufficient DNA input	-	20 & 23
Sign of excessive DNA input	19 & 25	11
Profile ski-slope	12	17
Acceptable Quality	13 & 18	1, 4, 10 & 13

Using a Whatman^{TM} Harris micro punch of 0.5 mm size (GE Healthcare), PM bloodstain samples were punched on top of a Harris cutting mat (GE Healthcare), and the punch was subsequently added to a labelled tube. AM buccal samples were punched using a Whatman^{TM} Harris micro punch of 1.2 mm size (GE Healthcare) on top of a Harris cutting mat. For both sample types, after punching

a sample, the micro punch was cleaned with 70% (v/v) ethanol, dried using a Kimwipe, and a blank FTA card punched before being used to punch a new sample.

2.2.3   Direct PCR

Direct PCR was performed for all samples using both the GlobalFiler™ Express PCR Amplification kit (Life Technologies, Australia) and the GlobalFiler™ PCR Amplification kit (Life Technologies, Australia). In brief, amplification reactions were performed in DNA-free 0.2 mL strip tubes with each individual tube containing one punch of the respective sample and the master mix from the corresponding kit (Table 2.2). A positive and negative control were prepared for both the GlobalFiler Express and the GlobalFiler amplifications. The positive control 007 DNA provided with each kit was used at the concentration specified by the respective manufacturers (41, 73), while the negative control consisted of the master mix for the respective kit and a blank punch. The PCR tubes were briefly centrifuged to ensure the punches were suspended in the reaction mix at the bottom of the tube.

Table 2.2. Kit comparison – PCR master mixes. Volume of individual master mix components and final master mix volume of a single PCR reaction for the Globalfiler Express (GFE) and Globalfiler (GF) kits.

Reaction components	Reagent volume (µL)	
	GFE	GF
Master Mix	6	7.5
Primer Set	6	2.5
Low TE Buffer	3	15
Total Volume	15	25

The eight PM bloodstain samples (0.5 mm punch) were amplified using both the GlobalFiler and GlobalFiler Express kits. The eight AM buccal FTA samples (1.2 mm punch) were amplified using the GlobalFiler kit. During a preliminary study previously conducted by the MBL, the same eight AM buccal FTA samples had been amplified using the GlobalFiler Express kit under the same conditions, with the resulting data used for comparison in this chapter.

Samples were amplified on an Applied Biosystems™ Veriti™ 96-well thermal cycler (Thermo Fisher Scientific, Australia). PM bloodstain samples were amplified for 25 cycles, while AM buccal FTA samples were amplified for 28 cycles. The PCR conditions followed the respective manufacturer's recommended cycling protocol (41, 73) (Table 2.3).

Table 2.3. Kit comparison – PCR protocol. Manufacturer recommended PCR cycling protocols for the GlobalFiler Express (GFE) and GlobalFiler (GF) kits.

	Initial Incubation	*Denature*	*Anneal/Extend*	*Final Extension*	*Final Hold*
	Hold	*Cycle*		*Hold*	*Hold*
GFE	95°C 1 minute	94°C 3 seconds	60°C 30 seconds	60°C 8 minutes	4°C Up to 24 hrs
GF	95°C 1 minute	94°C 10 seconds	59°C 90 seconds	60°C 10 minutes	4°C Up to 24 hrs

2.2.4 <u>Capillary electrophoresis</u>

For CE, the master mix for both the GlobalFiler Express and the GlobalFiler amplifications were set up as described in Table 2.4 as specified by the respective manufacturer protocol (41, 73). Either 1 µL of amplified product or 1 µL of the appropriate allelic ladder [GlobalFiler™ Express Allelic Ladder; or GlobalFiler™ Allelic Ladder at 50% (v/v)] was added to 10 µL of the appropriate master mix. CE was conducted on an Applied Biosystems™ 3500 Genetic Analyser (Thermo Fisher Scientific, Australia) using POP-4™ polymer (Thermo Fisher Scientific, Australia) with 36 cm capillary array. Injection conditions were 1.2 kV for 15 seconds, with run conditions at 13 kV for 1550 seconds.

Table 2.4. Kit comparison – CE master mix. Volume of individual master mix components and final master mix volume for preparation of a single sample for CE for the GlobalFiler Express (GFE) and GlobalFiler (GF) kits.

Reaction component	Reagent volume (µL)	
	GFE	GF
GeneScan^{TM} 600 LIZ^{TM} Size Standard v2.0	0.5	0.4
Hi-Di^{TM} Formamide	9.5	9.6
Total	10	10

2.2.5 Data analysis

Following successful CE, the resulting .fsa files were imported into the genotyping software GeneMapper^{TM} ID-X (v1.6) (Thermo Fisher Scientific, Australia) for analysis. Samples amplified using GlobalFiler Express were analysed using the GlobalFiler Express Panel v1.3.1, while samples amplified using GlobalFiler were analysed using the GlobalFiler Panel v4, applying the manufacturer defined stutter thresholds for each kit. Based on the MBL's validated thresholds for GlobalFiler, the analytical threshold was set at 50 RFU for all analyses.

Sizing quality was examined to check that the size standard was correct. The positive controls were checked to ensure they showed the expected allele call, and the negative controls were checked to ensure there were no alleles present. Known artifacts identified and removed. Additional peaks such as split peaks or 1 bp smaller than the allele peaks (so-called minus A peaks) caused by incomplete adenylation, were recorded and removed. In addition, non-allelic artifact peaks of unknown origin were counted and removed. The resulting DNA profiles obtained were compared to previously obtained DNA profile data for the samples to ensure concordance of the profiling data. Any additional peaks that were due to contamination were removed from the profile. The contamination source was determined, if possible, by checking if it was cross-contamination from

other samples processed, or from the person handling the sample, or from any member from the MBL. Profile completeness was assessed as the number of known autosomal alleles present in the profile, with known homozygotes counted as 2 alleles, expressed as a percentage with the maximum number of possible alleles present being 42.

Following analysis using GeneMapper™ ID-X, peak height data for each profile were recorded in an Excel spreadsheet for further analysis. Peak heights of known homozygote alleles were divided by two to calculate it as two alleles. The peak heights for all allelic peaks identified (homozygous and heterozygous) were recorded and the average peak height calculated for each profile. In addition, the peak height balance was calculated by determining the peak height ratio (low allele/highest allele) for each locus. If a single allele had dropped out at a known heterozygote locus, the analytical threshold value of 50 was used as the peak height of this dropped out allele.

To create a numerical measurement of the ski-slope effect in the profile (assumed to be caused by inhibition in this project) a so-called 'average inhibition ratio' was calculated. The average inhibition ratio was calculated by dividing the height of the lowest molecular weight autosomal allele by the height of the highest molecular weight autosomal allele in each dye channel of the profile, with the average of these ratios forming the final average inhibition ratio (Table 2.5). Where the allele required for the calculation of this ratio had dropped out, the analytical threshold value of 50 was used for calculation, although the ratio was not calculated where both alleles required for calculation of the ratio were absent.

Table 2.5. Kit comparison – inhibition ratio. Loci used in the calculation of average inhibition ratio.

(Peak height of shortest allele) / (Peak height of longest allele)
D3S1358 / TPOX
D8S1179 / D18S51
D2S4411 / FGA
D22S1045 / SE33
D10S1248 / D2S1338

2.3   Results

Experiments using the GlobalFiler and GlobalFiler Express kits were performed to determine the amplification kit to be utilised in subsequent components of this research project. In order to compare the performance of the two kits, eight PM bloodstain samples were tested with both the GlobalFiler and GlobalFiler Express amplification kits, while eight AM buccal FTA samples were amplified using the GlobalFiler kit and these results compared to those previously obtained by the MBL for these samples using the GlobalFiler Express kit (unpublished data).

2.3.1   Profile concordance and completeness

Concordance of the profile data obtained was determined by comparison to the known profiles for the samples analysed. All PM bloodstain samples produced concordant profiles with both kits used. All but one of the AM buccal samples produced concordant profiles using GlobalFiler Express. One sample (sample 17) did not give the expected DNA profile; this was attributed to the processing of an incorrect sample in its place. This sample was removed from any subsequent comparisons between the two kits. Once concordance was established, the completeness of the profiling data (complete, partial or no profile) was noted.

For the PM bloodstain samples, profile completeness for the GlobalFiler and GlobalFiler Express kits were recorded for comparison in Table 2.6. Five (samples 12, 13, 18, 19 and 25) out of eight samples (62.5%) produced complete profiles when amplified with the GlobalFiler kit, while six (samples 6, 12, 13, 18, 19 and 25) out of eight samples (75%) yielded complete profiles when amplified with the GlobalFiler Express kit. In addition, two (samples 7 and 23) out of eight samples (25%) analysed did not yield any profiling data using either kit. One sample (sample 6) produced a complete profile with the GlobalFiler Express kit but produced a partial profile (34 out of 42 alleles) when profiled with the GlobalFiler kit. This sample was the only one to display allelic drop-out.

For the AM buccal samples, profile completeness for the GlobalFiler and GlobalFiler Express kits were recorded for comparison in Table 2.7. All samples produced complete profiles when amplified with both the GlobalFiler kit and the GlobalFiler Express kit.

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2.3.2   Average peak height

The average peak height was calculated for all samples analysed that yielded complete or partial DNA profiles. For PM bloodstain samples, the average peak height from each profile obtained using both the GlobalFiler kit and the GlobalFiler Express kit was recorded for comparison in Table 2.6. Five (samples 6, 12, 13, 18 and 25) out of six samples (83%) that produced allelic data obtained a higher average peak height when amplified with the GlobalFiler Express kit than with the GlobalFiler kit. Sample 19 was the only sample were the profile average peak height was higher when using the GlobalFiler kit (13,388 RFU) compared to the GlobalFiler Express kit (11,304 RFU).

For AM buccal samples, the average peak height from each profile obtained using both the GlobalFiler kit and the GlobalFiler Express kit was recorded for comparison in Table 2.7. Four (samples 1, 10, 11 and 23) out of seven samples (57%) analysed produced higher average peak heights when amplified with the GlobalFiler Express kit compared to the GlobalFiler kit. In contrast, three samples (samples 4, 13 and 20) produced a greater average peak height when amplified with the GlobalFiler kit compared to the GlobalFiler express kit.

2.3.3   Peak height balance

The peak height balance was calculated for all samples analysed that yielded complete or partial DNA profiles. For PM bloodstain samples, the average peak height balance from each profile obtained using both the GlobalFiler kit and the GlobalFiler Express kit was recorded for comparison in Table 2.6. The average peak height balance across all samples amplified using GlobalFiler and GlobalFiler Express was 0.89 ± 0.05 and 0.85 ± 0.06, respectively. The average peak height balance was slightly higher in five (samples 6, 12, 18, 19, and 25) out of six samples (83%) analysed using the GlobalFiler kit compared to the GlobalFiler Express kit although peak height balance was suitably high in all profiles except sample 6.

For AM buccal samples, the average peak height balance from each profile obtained using both the GlobalFiler and the GlobalFiler Express kits were recorded for comparison in Table 2.7. As with PM bloodstains, similar average peak height balances were observed across all AM buccal samples when using the GlobalFiler kit (0.89 ± 0.04) versus the GlobalFiler Express kit (0.88 ± 0.03).

2.3.4   Inhibition ratio

The effect of inhibition, as an average inhibition ratio, was calculated for all samples that yielded any allelic data. For PM bloodstain samples, the inhibition ratio was calculated and recorded for comparison in Table 2.6. Five (samples 6, 13, 18, 19 and 25) out of six samples (83%) analysed had higher average inhibition ratios when using the GlobalFiler kit than when using the GlobalFiler Express kit. Sample 12 was the only sample that showed a higher inhibition ratio when amplified with the GlobalFiler Express kit (4.4) compared to the GlobalFiler kit (4.0). The average inhibition ratio across for all samples was calculated for both kits and demonstrated a higher inhibition ratio with the GlobalFiler kit (3.7 ± 2.4)) compared to the GlobalFiler Express (3.1 ± 2.4).

For AM buccal samples, the average inhibition ratio was calculated and recorded for comparison in Table 2.7. Inhibition was higher in all samples tested using the GlobalFiler kit and the overall average across all samples using the GlobalFiler kit was 3.0 ±1.1 and for the GlobalFiler Express kit was 1.6 ± 0.6.

Table 2.6. Kit comparison – profile attributes for PM bloodstain samples. Profile completeness, average peak height, average peak height balance, and average inhibition ratio obtained from PM bloodstain samples tested with the GlobalFiler (GF) and GlobalFiler Express (GFE) kits. Samples 7 and 23 that did not yield any results were excluded from the table for clarity.

	Profile Completeness (%)		Average peak height (RFU)		Average peak height balance		Average inhibition ratio	
	GF kit	GFE kit	GF kit	GFE kit	GF kit	GFE kit	GF kit	GFE kit
Sample 6	81	100	194 ±157	270 ±214	0.79 ±0.14	0.76 ±0.16	8.1 ±1.4	7.3 ±4.7
Sample 12	100	100	9,195 ±5,178	11,682 ±6,293	0.87 ±0.08	0.83 ±0.11	4.0 ±2.1	4.4 ±2.7
Sample 13	100	100	2,185 ±768	3,437 ±1,149	0.94 ±0.04	0.94 ±0.05	1.3 ±0.4	0.8 ±0.2
Sample 18	100	100	7,817 ±2,588	12,240 ±2,584	0.94 ±0.03	0.88 ±0.03	1.8 ±0.3	1.5 ±0.2
Sample 19	100	100	13,119 ±8,394	11,304 ±6,511	0.89 ±0.06	0.84 ±0.07	3.7 ±2.8	2.9 ±1.0
Sample 25	100	100	10,525 ±6,586	14,421 ±4,503	0.90 ±0.07	0.86 ±0.08	3.2 ±2.7	1.7 ±0.9
Average	-	-	7,217 ±5,059	8,892 ±5,648	0.89 ±0.05	0.85 ±0.06	3.7 ±2.4	3.1 ±2.4

Table 2.7. Kit comparison – profile attributes for AM buccal FTA samples. Profile completeness, average peak height, average peak height balance, and average inhibition ratio obtained from AM buccal FTA samples tested with the GlobalFiler (GF) and GlobalFiler Express (GFE) kits. Sample 17 was excluded from the table. **Note:** Results for the GlobalFiler Express kit were previously obtained by the MBL (unpublished data).

	Profile Completeness (%)		Average peak height (RFU)		Average peak height balance		Average Inhibition ratio	
	GF kit	GFE kit	GF kit	GFE kit	GF kit	GFE kit	GF kit	GFE kit
Sample 1	100	100	3,184 ±1,488	7,073 ± 2,289	0.88 ±0.07	0.90 ± 0.07	2.5 ±0.5	1.5 ±0.4
Sample 4	100	100	6,971 ±3,380	6,841 ±1,878	0.93 ±0.07	0.89 ±0.10	2.7 ±0.9	1.4 ±0.1
Sample 10	100	100	3,329 ±1,408	5,425 ±1,382	0.94 ±0.06	0.83 SD: 0.08	2.3 ±1.0	1.5 ±0.3
Sample 11	100	100	5,069 ±2,816	10,928 ±2,333	0.91 ±0.06	0.90 ±0.06	3.3 ±1.7	1.1 ±0.3
Sample 13	100	100	5,289 ±3,286	4,467 ±1,500	0.90 ±0.04	0.87 ±0.08	2.5 ±1.1	1.6 ±0.6
Sample 20	100	100	1,109 ±562	722 ±236	0.89 ±0.08	0.90 ±0.08	2.1 ±0.4	1.2 ±0.4
Sample 23	100	100	241 ±224	2,412 ±1,218	0.81 ±0.14	0.85 ±0.11	5.3 ±2.7	2.9 ±1.3
Average	-	-	3,599 ±2,384	5,409 ±3344	0.89 ±0.04	0.88 ±0.03	3.0 ±1.1	1.6 ±0.6

2.3.5   Minus A peaks

For all samples that yielded complete or partial DNA profiles, the presence of minus A peaks (as a sign of overamplification) were recorded. These results are compared in Table 2.8. For PM bloodstain samples, two (samples 19 and 25) out of six samples (33%) analysed had minus A peaks in their profiles when amplified with the GlobalFiler kit, compared to only one (sample 19) out of six (17%) samples amplified using the GlobalFiler Express kit (Table 2.8A). For sample 25, minus A peaks (15 in total) were present in the profile only when amplified using the GlobalFiler kit. For sample 19, minus A peaks were present in profiles generated with either amplification kit; however, double the number of minus A peaks were noted when using the GlobalFiler kit (20 in total) compared to the GlobalFiler Express kit (10 in total) (Table 2.8A). Figure 2.1A illustrates two representative examples of the minus A peaks observed in the profile obtained for sample 19 when amplified with the GlobalFiler kit. The peak height ratio of each minus A peak to its respective allelic peak was calculated as a further measure of the degree of overamplification evident in a profile (Table 2.8A). As shown in Table 2.8A, minus A peaks present in the profiles of samples 19 and 25 had higher peak height ratios when the samples were amplified with the GlobalFiler kit than with the GlobalFiler Express kit.

For AM buccal FTA samples, three (samples 11, 13 and 20) out of the seven (43%) samples analysed had minus A peaks in their profiles when amplified with the GlobalFiler kit, compared to no minus A peaks noted in any of the samples when amplified with the GlobalFiler Express kit (Table 2.8B). The peak height ratios of the minus A peak to its respective allelic peak were also calculated and recorded (Table 2.8B). All the minus A peaks present in the profiles of samples 11 and 20 had percentage peak height ratios to their respective allelic peaks of <10%. While minus A peaks present in the profile of sample 13 (4 in total) had percentage peak height ratios to their respective allelic peaks of between 10 and 40% (Table 2.8B). Figure 2.1B illustrates two of the four minus A peaks observed with sample 13 when amplified with the GlobalFiler kit.

Table 2.8. Kit comparison – Overamplification. Number of minus A peaks present in profiles and the percentage of peak height ratio (PHR) of these minus A peaks to the associated allele peak from: A) PM bloodstain samples, and B) AM buccal FTA samples, obtained when amplified with the GlobalFiler (GF) and the GlobalFiler Express (GFE) kits.
Data shown only for samples that displayed minus A peaks.

A)

	Total minus A peaks		Number of alleles with percentage PHR of minus A peak to allelic peak					
			<10%		10-40%		>40%	
	GF	GFE	GF	GFE	GF	GFE	GF	GFE
Sample 19	20	10	0	0	4	10	16	0
Sample 25	15	0	1	0	9	0	5	0
Total	35	10	1	0	13	10	21	0

B)

	Total minus A peaks		Number of alleles with percentage PHR of minus A peak to allelic peak					
			<10%		10-40%		>40%	
	GF	GFE	GF	GFE	GF	GFE	GF	GFE
Sample 11	2	0	2	0	0	0	0	0
Sample 13	4	0	0	0	4	0	0	0
Sample 20	1	0	1	0	0	0	0	0
Total	7	0	3	0	4	0	0	0
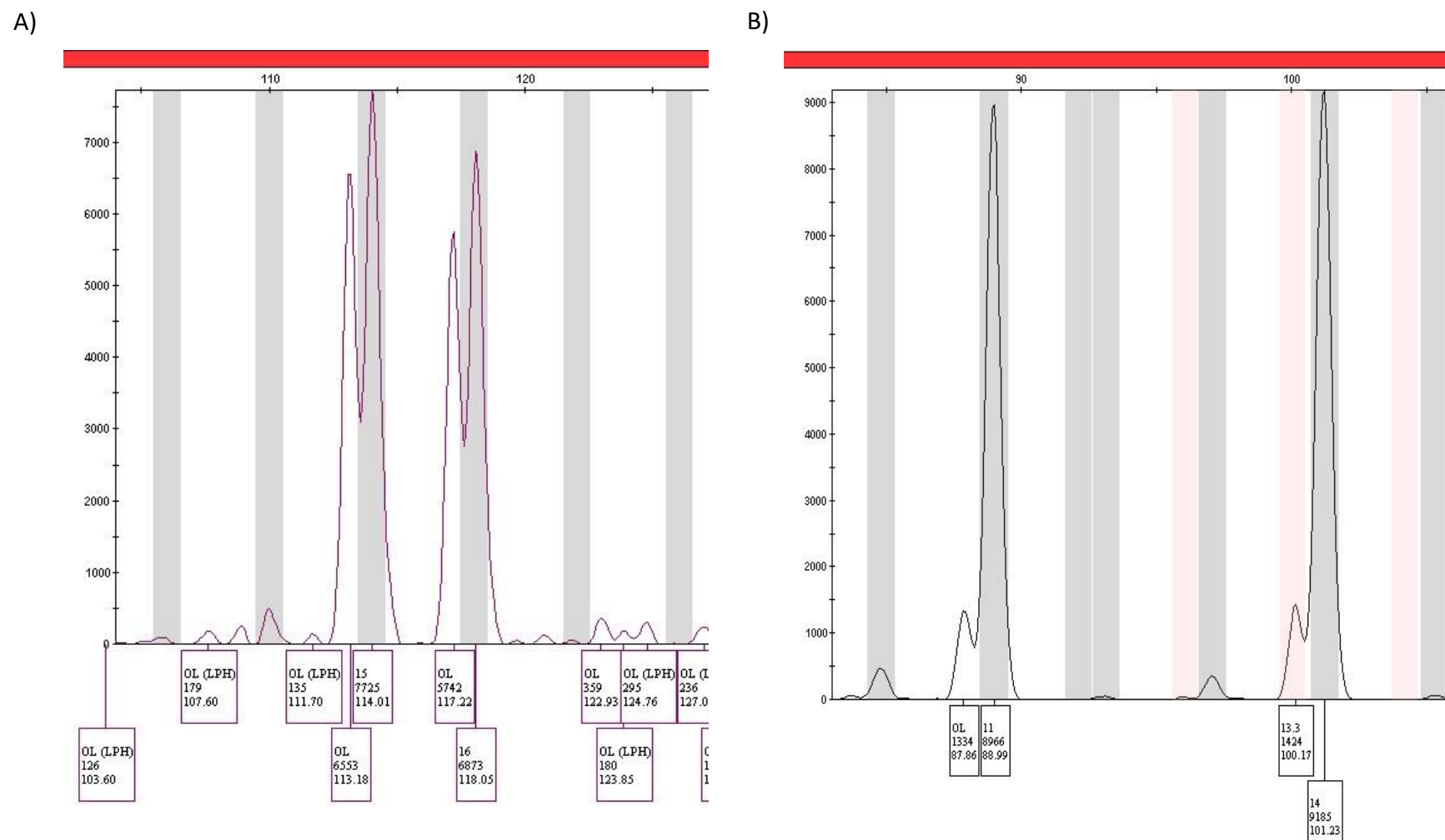
Figure 2.1. Kit comparison – Illustration of minus A peaks. Representation of minus A peaks observed in the DNA profile from PM bloodstain sample 19 (Panel A) and AM buccal FTA sample 13 (Panel B), both amplified using the GlobalFiler kit. In Panel A, minus A peaks have a percentage to allelic peak of 96% and 90%, respectively. In Panel B, minus A peaks have a percentage to allelic peak of 14% and 15%, respectively.

2.4   DISCUSSION

The method development to perform direct PCR for PM identification at the MBL started by determining the amplification kit to be used with all sample types. A comparison between the GlobalFiler kit and the GlobalFiler Express kit was undertaken based on the performance during direct PCR with AM buccal and PM bloodstains FTA samples. This section will discuss the findings from the comparison between these amplification kits, as well as justifying the determination of the amplification kit for all further experiments.

The GlobalFiler Express kit, which was designed to perform direct PCR (as described in section 1.4), was previously tested by the MBL using PM bloodstains and AM buccal FTA samples following the manufacturer's recommended protocols ((72), unpublished data). That study consisted of performing direct PCR on 1.2 mm punch of PM bloodstains and AM buccal samples, respectively, using the GlobalFiler Express kit – testing different cycle numbers for each sample type as recommended by the manufacturer's user guide (41). Based on the data obtained, DNA profiles from PM bloodstain samples demonstrated high inhibition levels when using the GlobalFiler Express kit ((72), unpublished data). This suggested that the direct PCR kit, using the manufacturer's recommended protocol for AM samples, did not perform well with this type of PM sample. This may be expected as the GlobalFiler Express kit was optimized to be used with AM but not with PM bloodstains.

Based on the preliminary study, it was decided to investigate the use of the GlobalFiler kit for direct PCR, as this has a larger reaction volume compared to the GlobalFiler Express kit, which may dilute any PCR inhibitors that may be present in the PM sample. As one of the goals for the MBL is to introduce a direct PCR analysis pipeline that would be complementary to its current workflow, a comparison of GlobalFiler and GlobalFiler Express kits for direct PCR was undertaken. The GlobalFiler kit, currently used at the MBL for casework, is designed to be used with DNA extracts following an extraction and quantification step (73). If the GlobalFiler kit was deemed suitable to perform direct PCR, this would be an advantage over the GlobalFiler Express kit as the kit is already validated at the MBL.

Furthermore, the comparison between the GlobalFiler and GlobalFiler Express was done on sample types that make up the bulk of samples received for coronial casework. Per annum, approximately 65% of PM samples are bloodstains, while all AM samples are buccal samples (Dr D. Hartman personal communication).

Inhibition

Results from the preliminary study conducted at the MBL were the reasons for undertaking this comparison, as they showed high levels of inhibition when performing direct PCR with 1.2 mm punch of PM bloodstains using the GlobalFiler Express kit (72). The purpose of this experiment was to compare the performance between the GlobalFiler kit and the GlobalFiler Express kit. Testing of both kits was performed with a reduced input amount (0.5 mm punch) in an attempt to reduce the input of inhibitors (see Chapter 3 for comparison between the 0.5mm punch and the 1.2 mm punch input amounts). Using a lesser input amount was shown to have a positive effect on reducing inhibition levels (see Chapter 3), thus demonstrating a comparison of the performance of different amplification kits using this input amount was warranted. This section will describe the differences in the observed levels of inhibition from a kit comparison point of view.

As previously mentioned, the GlobalFiler kit was used to perform direct PCR in order to determine if inhibition levels were lower compared to when using the GlobalFiler Express kit. The levels of inhibition of the amplification reaction, as determined from calculated inhibition ratios, were on average higher when amplified with the GlobalFiler kit than with the GlobalFiler Express kit, regardless of sample type. With PM bloodstain samples, inhibition ratios were higher in all samples except one when amplified with the GlobalFiler kit compared to amplified with the GlobalFiler Express kit. Sample 12 was the only sample with an inhibition ratio higher when amplified with the GlobalFiler Express kit than with the GlobalFiler kit; although, similar average inhibition ratios for each kit were observed for this sample. All of the AM buccal FTA samples tested showed a higher inhibition ratio when amplified with the GlobalFiler kit compared to the GlobalFiler Express kit. Based on the data observed, the hypothesis that the GlobalFiler kit could overcome inhibition effects with its larger reaction volume did not hold true, with the GlobalFiler Express kit outperforming the GlobalFiler kit with respect to inhibition.

Higher levels of inhibition with GlobalFiler kit may not be surprising as the kit is designed to be used in a standard process, which includes extraction, purification and a quantification steps (73). Although the kit is designed to withstand a level of inhibitors in the PCR reaction (71), the GlobalFiler kit is intended to be used with purified DNA, diminishing exposure of the PCR reaction to potential inhibitors. On the other hand, the GlobalFiler Express kit is designed to perform direct PCR (41). For the majority of the samples tested in the present experiment, lower inhibition levels were noted when using the GlobalFiler Express kit, which could be due to the optimised buffer stated in the user guide (41) that may tolerate potential inhibitors better than the GlobalFiler kit.

Furthermore, the ability of the GlobalFiler Express kit to tolerate inhibitors was explained by Wang *et al.* in their developmental validation study (39). Wang *et al.*, demonstrated that the GlobalFiler Express kit produced a 'reverse-ski' slope' pattern in each dye channel (increased peak heights as the amplicon size increases) when amplifying the positive control DNA 007 (39). Inhibitors in an amplification reaction tend to produce a ski slope that consists of the peak heights decreasing while the amplicon size increases (78, 79). Therefore, the GlobalFiler Express kit produces the reverse-ski slope to minimize the impacts of a ski slope when inhibitors are present (39). Since the GlobalFiler kit does not produce a reverse-ski slope (39), this may be the reason for the GlobalFiler Express kit showing less inhibition.

From an inhibition point of view, based on the samples analysed, the GlobalFiler Express kit demonstrated greater ability to tolerate inhibitors during direct PCR compared to the GlobalFiler kit. In contrast, the GlobalFiler kit produced profiles with higher inhibition ratio levels for both sample types. Hence for method development, the GlobalFiler Express kit would be suitable for the direct PCR of PM bloodstain and AM buccal samples, able to overcome inhibitors.

Incomplete adenylation

The presence of minus A peaks within the profiles obtained was evaluated as a part of this comparison. Minus A peaks frequently occurs when there is an excessive DNA input into to the PCR and/or there are PCR inhibitions present affecting the PCR mechanism (74). Part of the PCR mechanism consists of the Taq DNA polymerase adding to the 3' end of the PCR product an adenosine nucleotide (74). When an excessive amount of DNA template is present, excessive

amplification occurs and this can result in insufficient adenosine bases within the reaction resulting in this final adenosine addition being incomplete. This results in what is termed as a 'minus A' peak being produced (74). When amplified using the GlobalFiler kit, minus A peaks were seen in resulting profiles regardless of sample type. In contrast, minus A peaks were only seen in a single PM bloodstain sample in all of the profiles generated with the GlobalFiler Express kit.

The PM bloodstain samples that displayed minus A peaks were selected for this experiment based on their previously observed signs of excessive DNA input, so it was not surprising that these samples produced minus A peaks. The GlobalFiler Express kit fared better with only one of the two samples having minus A peaks; with only half the number of minus A peaks observed in comparison to the GlobalFiler kit. Furthermore, in comparison to the GlobalFiler kit, the GlobalFiler Express kit produced no minus A peaks that had a percentage peak height ratio of minus A peak to allelic peak of >40%, with all minus A peaks observed falling between 10-40%. The percentage peak height ratio of minus A peak to allelic peak was classified into three different groups: <10%, 10 - 40%, and >40%. For identification casework, minus A peaks with a ratio to the allele <10% should provide minimal interpretation issues, while a ratio of 10 – 40% may complicate the interpretation of the profile. A profile displaying minus A peaks with a ratio of >40% would not be considered for further analysis. (Zoe Bowman personal communication).

For the profiles obtained from the AM buccal samples, three were shown to produce minus A peaks when amplified with the GlobalFiler kit while none were noted with the GlobalFiler Express kit. One of these samples was selected for this experiment based on its previously observed over-amplification, so it was not surprising to observe minus A peaks with this sample. However, it was perhaps surprising to see minus A peaks in two samples that did not previously behave as high DNA input samples based on the peak heights observed. Furthermore, one of these samples had previously demonstrated insufficient DNA input while the other samples previously produced high quality profiles. The discrepancies in the current observations with those noted previously for these samples may be explained by the sample punching technique, as the punches were made from different locations in the card, with the amount of DNA for the same sample differing depending on the sampling location.

The presence of minus A peaks with direct PCR has been reported previously by Chong *et al*, where direct PCR was performed with AM bloodstains collected with microFLOQ™ Direct swabs, as well as amplification of the samples with the standard method (75). No minus A peaks were produced with any of the profiles obtained with the standard method, while these artefacts were present when using direct PCR (75). They hypothesized that this may have occurred due to excessive DNA input into the PCR when performing direct PCR, as there is less control over the DNA input amount. While, this did not occur in the standard method as the DNA extracts are quantified prior to amplification with the optimal amount added (based on the manufacturer's recommendation) to the reaction.

Gouveia *et al*., performed direct PCR on eight AM bloodstains and buccal samples on FTA cards using the GlobalFiler kit (76). Different input amounts were tested in that experiment – 0.5, 0.75, 1, and 1.2 mm punches (76). Results demonstrated when the input amount was equal or greater than 0.75 mm punch, minus A peaks were produced – although the number of samples or total of minus A peaks was not specified. However, with the input amount of 0.5 mm punch, no minus A peaks were produced. This differed to the findings of this experiment, as minus A peaks were noted when using an input of 0.5 mm punch with both sample types with the GlobalFiler kit. This may be the result of our study using PM bloodstains, for example, or as noted above due to difference in template amount available for direct PCR even when the same punch size is used.

Hence for method development, the GlobalFiler Express kit would be suitable for the direct PCR of PM bloodstain and AM buccal samples, as it seems less prone to incomplete adenylation compared to the GlobalFiler kit.

Profile Properties

When looking at the properties of the DNA profiles (profile completeness, average peak height balance; and average peak height balance) both kits tested had similar outcomes.

For the PM bloodstain samples, two did not yield results with either of the kits tested. This is not likely to be due to the kit used, but rather as a result of the samples themselves which is further explored in Chapter 3. A higher number of samples produced complete profiles when amplified with the GlobalFiler Express kit than when using the GlobalFiler kit, with one sample producing a complete profile when amplified with the GlobalFiler Express kit, but only a partial profile when

amplified with the GlobalFiler kit. For the AM buccal samples, all produced complete profiles regardless of the kit used.

For PM bloodstain samples, the average peak height was higher for all samples when amplified with the GlobalFiler Express kit except for one sample which was higher when using the GlobalFiler kit. The overall average peak height obtained with the GlobalFiler Express kit was higher compared to the GlobalFiler kit, this suggests that the GlobalFiler Express kit is likely to produce profiles with slightly higher peak heights than with the GlobalFiler kit. Additionally, most of the AM buccal FTA samples displayed a higher average peak height with the GlobalFiler Express kit compared to using GlobalFiler.

Conclusions

A preliminary study conducted by the MBL, suggested that the GlobalFiler Express kit was not suitable for the direct PCR of PM bloodstain samples – a sample type that is routinely received for casework. Hence, the purpose of this study was to compare the performance of the GlobalFiler and GlobalFiler Express kits for the direct PCR of PM bloodstains and AM buccal samples, with the view of selecting an amplification kit to be utilized in subsequent experiments for this research project. In this study, for AM buccal swab and PM bloodstains samples received by the laboratory, less inhibition, higher average peak heights, and fewer minus A peaks were observed in the profiles obtained when amplified with the GlobalFiler Express kit compared with the GlobalFiler kit. Based on these finding, the GlobalFiler Express kit was chosen as an amplification kit for method development of this research project for the sample types of bloodstains (see chapter 3), buccal samples (see chapter 4), and toenails (see chapter 5).

3. CHAPTER 3: PM Bloodstain samples

3.1   Introduction

Blood is a common sample type used in DNA typing for human identification (77) and can be an AM or PM sample. PM bloodstain samples are routinely received for DNA profiling for identification purposes. PM bloodstain samples are usually obtained from a deceased showing minimal signs of decomposition and are therefore expected to yield fairly good quality DNA. Nevertheless, PM bloodstain samples are not as pristine sample, from a DNA point of view, compared to AM bloodstain samples – the sample type for which GlobalFiler Express was developmentally validated. PM bloodstain samples, when available and suitable, are the first choice of sample type for use at the MBL (Zoe Bowman, personal communication). Furthermore, the International Society for Forensic Genetics (ISFG) recommends during a DVI, the collection of blood on FTA card or swab as the first-choice of sample type if the body is not decomposed or partially decomposed (20). As a routine sample type received in the laboratory for DNA profiling, it would therefore be beneficial to consider the application of direct PCR for PM bloodstain samples in order to improve reporting turn-around times.

The challenges faced when performing direct PCR (as discussed in section 1.4.4) are somewhat augmented when considering PM blood as a sample type, due to diminishing DNA quality and quantity as a result of PM changes. The concentration of calcium and magnesium ions increase in serum PM (78), and these components have demonstrated to inhibit the PCR (79, 80). High levels of inhibition of the reaction could yield incomplete profiles or alleles with low peak heights, resulting in profile data that is not analysable or of low reportable value. Therefore, to perform direct PCR using PM bloodstain samples, method development to overcome these issues is needed.

The GlobalFiler Express kit is validated for AM bloodstain samples by the manufacturer (41). However, it has been shown to yield complete DNA profiles from PM bloodstain samples when modifying the recommended protocol by decreasing the input size amount to a 0.5 mm punch (72). For AM bloodstain samples, the GlobalFiler Express kit's user guide prescribes the use of 1.2 mm punch (41). A previous feasibility study, conducted by the MBL, sought to test the manufacturer's recommendations on PM bloodstain samples ((72), unpublished data). The experiment was

performed using 26 PM bloodstain samples on FTA and Copan Nucleic cards, utilising a 1.2 mm punch, with one punch per sample tested using the GlobalFiler Express kit at the various recommended cycle numbers (25, 26, and 27 cycles) ((72), unpublished data). The findings indicated that all amplifications (regardless of cycle number) showed a high level of inhibition. These results illustrated that further experimental work was required to investigate the feasibility of GlobalFiler Express for the direct PCR of PM bloodstain samples.

In Chapter 2, it was demonstrated that the GlobalFiler Express kit performed better than the GlobalFiler kit for PM bloodstain samples using a limited sample set, although a certain degree of inhibition was still noted. As one of the aims of this research study was to evaluate the direct amplification for PM bloodstain samples, further work was undertaken to assess the 0.5 mm punch size as the optimal sample size for input into the PCR, as well as the optimum PCR cycle number that would be needed to produce acceptable quality DNA profiles.

Based on the results obtained in Chapter 2, the current chapter describes data comparisons (data from Chapter 2 and the previous preliminary study conducted by the MBL) designed to assess a 0.5 mm punch size as the input amount for PM bloodstain samples to reduce the impact of any inhibitors that may be present in this sample type. It was hypothesised that adding a smaller amount of bloodstain sample (smaller punch size) into the PCR would reduce the effects of inhibition as there would be fewer inhibitors added to the reaction. The results obtained using a 0.5 mm punch of sample as an input were compared to those obtained previously by the MBL when using a 1.2 mm punch of sample for amplification.

Amplifications were also conducted using the same input amount (0.5 mm punch) but varying the amplification cycle number, to determine which cycle number tested produced DNA profiles of a suitable quality to use in PM identification casework at the MBL. The aim was to determine if a single cycle number could be used to process both PM bloodstain and AM buccal samples. This has benefits for casework, enabling the PM sample (i.e. bloodstain) to be amplified at the same time as the AM sample (i.e. buccal FTA) for that case, leading to improvements in reporting times and cost savings. Furthermore, amplifications were conducted to evaluate the pre-treatment (heating) of PM bloodstain punch prior to amplification at 25 cycles, or post-treatment (dilution) of the amplified products (at 27, 28 or 29 cycles) prior to CE.

3.2 Materials and Methods

The experiment of the sample input determination used the results (described in Chapter 2) from the DNA profiles obtained from the eight PM bloodstain samples using 0.5 mm punch amplified with the GlobalFiler Express kit (41). These were compared to results from the same eight PM bloodstain samples obtained in the preliminary study conducted at the MBL by using 1.2 mm punch (72) following the GlobalFiler Express recommended protocol (41).

3.2.1 Sample selection and preparation

The 26 PM bloodstain samples available for the experiments performed in this chapter were previously described in Chapter 2 (Section 2.2.1).

PCR cycle number determination: all 26 PM bloodstain samples allocated for this research project were tested. Each sample was prepared as described in section 2.2.2 by punching (with a Whatman™ Harris micro punch) a 0.5 mm punch of the sample (one punch for each cycle tested) and adding it to the amplification tube.

Pre-treatment (heating): six of the available 26 PM bloodstain samples were used in this experiment based on the cycle number experimental data (Table 3.1). To each 0.5 mm punch sample deposited in individual 1.5 ml Eppendorf tubes, 5 µL of low TE buffer was added, then samples were incubated at 99°C using an Eppendorf Thermomixer® (Eppendorf) with shaking at 900 rpm for 10 minutes.

Post-treatment (dilution): six of the 26 PM bloodstain samples were selected for use in this experiment based on the cycle number experimental data, with samples from each average peak height group selected (Table 3.2).

Method verification: all 26 PM bloodstain samples were tested.

Table 3.1. PM Bloodstain – Sample selection for pre-treatment (heating) experiment. Basis of sample selection for the pre-treatment (heating) experiment based on profiling outcomes obtained in the cycle determination experiment at 25 cycles.

Selection Criteria	Bloodstain samples
No alleles detected	7 & 23
Allele drop-out	6
Peak heights lower than 1,200 RFU	21
Peak heights higher than 14,000 RFU	14
Suitable profile quality	4

Table 3.2. PM Bloodstain – Sample selection for post-treatment (dilution) experiment. Basis of sample selection for the post-treatment (dilution) experiment based on profiling outcomes obtained in cycle number determination at 25 cycles.

Average peak height	Sample
Allele drop-out	6
<1,200 RFU	16
1,200 – 14,000 RFU	24, 25
>14,000 RFU	12, 19

3.2.2 Direct amplification

GlobalFiler Express amplification reactions were carried out in DNA-free 0.2 ml strip tubes, with master mix volumes and positive and negative control reactions as specified by the GlobalFiler Express kit manufacturer (41) (section 2.2.3). PCR was performed using an Applied Biosystems™ Veriti™ 96-well thermal cycler (Thermo Fisher Scientific, Australia) following the GlobalFiler Express manufacturer's recommended cycling protocol (41) (section 2.2.3). PCR cycle numbers and any additional modifications are described below.

PCR cycle number determination: each sample was amplified at either 24, 25, and 26 cycles.

Pre-treatment (heating)*:* Master mix was prepared as described in Table 2.2 for GlobalFiler Express, without the addition of the low TE buffer. Following incubation, samples were briefly centrifuged and 3 μL of each eluate was added into individual DNA-free 0.2 mL strip tubes, followed by 12 μL of master mix, then amplified at 25 cycles.

Post-treatment experiment (dilution)*:* each sample was amplified at either 27, 28 and 29.

Method verification: each sample was amplified at 27 cycles.

3.2.3 Capillary electrophoresis

The PCR products were separated and detected using capillary electrophoresis on an Applied Biosystems™ 3500 Genetic Analyser (Thermo Fisher Scientific, Australia) under conditions specified by the manufacturer for the GlobalFiler Express kit (41). Modifications to the manufacturer protocols were made for the following experiments:

Post-treatment (dilution): PCR products were diluted using distilled water in a four-step, two-fold serial dilution. Diluted products were then subjected to capillary electrophoresis as above.

Method verification: each PCR product was diluted 1 in 8 and 1 in 16 using distilled water. Diluted products were then subjected to capillary electrophoresis as above.

3.2.4 Data analysis

Following capillary electrophoresis, DNA profile data was analysed as described in Section 2.2.5 using GeneMapper™ ID-X (v1.6) (Thermo Fisher Scientific).

3.3   Results

Experiments were performed to develop a method for the direct PCR of PM bloodstain samples. The input amount (0.5 vs 1.2 mm punch), amplification cycle number, and pre-and post-PCR treatments were examined.

3.3.1 Sample input determination

A preliminary study conducted by the MBL utilising 1.2 mm punch of PM bloodstain samples demonstrated high levels of inhibition (72). In Chapter 2, an input size of 0.5 mm punch for PM bloodstain samples was tested using a subset of eight PM bloodstain samples under the same amplification conditions, and the results described from a kit comparison perspective. Described here, from a sample input perspective, is a comparison of the data obtained for the same eight PM bloodstain samples when using the 1.2 mm punch (preliminary study) and the 0.5 mm punch (Chapter 2) as the input for the direct PCR. Comparisons based on profile attributes are shown in Table 3.3 and are described below.

Sample input determination - Profile concordance and completeness

All profiles obtained with either 0.5- or 1.2-mm punch input sizes, produced concordant profiles.

Six (samples 6, 12, 13, 18, 19 and 25) out of eight samples (75%) tested produced a complete profile when using the 0.5 mm punch input, while five (samples 12, 13, 18, 19 and 23) out of eight samples (62.5 %) tested produced complete profiles when using the 1.2 mm punch input. No partial profiles were obtained with 0.5 mm punch input, with one sample (sample 6) yielding a partial profile (35 out of 42 alleles) when using a sample input of 1.2 mm punch. No allelic data was obtained for samples 7 and 23 using either the 0.5- or 1.2-mm punch input. Hence, only the six samples that yielded profiling data were further analysed.

Sample input determination - Average peak height

Overall, the average peak height obtained across all six samples was higher when using the 0.5 mm punch input (9,039 ± 5,702 RFU) than when using the 1.2 mm punch input (6,588 ± 5,656 RFU). Four (samples 12, 18, 19 and 25) out of six samples (67%) analysed obtained a higher average peak height when using 0.5 mm punch input than when amplified with the 1.2 mm punch input. In contrast, samples 6 and 13, produced higher average peak heights when amplified with a 1.2 mm punch input compared to using a 0.5 mm punch input.

Sample input determination - Average peak height balance

Average peak height balance was calculated for complete and partial profiles obtained with both input sizes tested, with the values recorded for comparison in Table 3.3. Sample 6 yielded a similar average peak height balance at both inputs tested, but it was higher when using the 1.2 mm punch than when amplifying with the 0.5 mm punch. While the rest of the samples yielded a higher average peak height balance when using the 0.5 mm punch than when amplifying with the 1.2 mm punch.

Sample input determination - Inhibition

All samples tested produced profiles with lower levels of inhibition when amplified with a 0.5 mm sample input compared to 1.2 mm sample input, with samples 12 and 19 in particular exhibiting a marked reduction in inhibition. Furthermore, the overall average inhibition ratio across the six samples was higher with the 1.2 mm punch input (11.4 ± 11.6) than with the 0.5 mm punch input (3.1 ± 2.4) indicating the reduction in sample input to the 0.5 mm punch size appeared to alleviate the high levels of inhibition seen with the 1.2mm punch input.

Sample input determination - Overall findings

Overall, the use of 0.5 mm punch input produced profiles exhibiting lower inhibition levels which also lead to higher average peak heights and an improved average peak height balance. This improvement has resulted in profiles with a more acceptable quality than the profiles produced from the 1.2 mm punch. In terms of individual samples, sample 6 produced a complete profile when using the 0.5 mm punch, but it obtained a partial profile when using the 1.2 mm punch, which could be caused by the higher levels of inhibition showed when amplifying 1.2 mm punch. Additionally, the average peak height in the sample 6 profile was higher when using the 1.2 mm punch, the quality was better in the profile obtained from the 0.5 mm punch as this produced a complete profile and lower inhibition levels. Samples 19 and 25, which were chosen for showing the highest average peak height on the preliminary study, demonstrated an increase of peak heights when using the 0.5 mm punch which may be related to the inhibition levels reducing compared to when amplifying 1.2 mm punch.

In conclusion, the profiles obtained from the 0.5 mm punch demonstrated an improvement on the quality over the profiles obtained from the 1.2 mm punch, which can be deduced to be a result of

reducing inhibition levels. Therefore, the 0.5 mm punch was the input size selected for PM bloodstains to be used for the remainder of the method development.

Table 3.3. PM Bloodstain – Sample input determination. Profile completeness, average peak height, average peak height balance, average inhibition ratio, and standard deviation (±) of averages, obtained from bloodstain samples tested with the input amount of 0.5- and 1.2-mm punch. Samples 7 and 23 did not yield any allelic results and were excluded from the table for clarity.

	Profile completeness (%)		Average peak height (RFU)		Average peak height balance		Average inhibition ratio	
	0.5 mm	1.2 mm	0.5 mm	1.2 mm	0.5 mm	1.2 mm	0.5 mm	1.2 mm
Sample 6	100	83	270 ±214	315 ±310	0.76 ±0.16	0.77 ±0.14	7.3 ±4.6	11.6 ±7.7
Sample 12	100	100	11,682 ±6,293	5,661 ±5,746	0.83 ±0.11	0.71 ±0.16	4.4 ±2.7	30.3 ±43.6
Sample 13	100	100	3,617 ±1,486	6,714 ±1,837	0.94 ±0.05	0.91 ±0.06	0.8 ±0.2	1.4 ±0.3
Sample 18	100	100	12,941 ±4,660	7,319 ±2,823	0.88 ±0.03	0.87 ±0.05	1.5 ±0.2	2.3 ±0.3
Sample 19	100	100	11,304 ±6,511	8,041 ±7,420	0.84 ±0.07	0.76 ±0.10	2.9 ±1.0	11.6 ±4.1
Sample 25	100	100	14,421 ±4,503	11,481 ±5,125	0.86 ±0.08	0.82 ±0.09	1.7 ±0.9	2.7 ±1.7
Average	-	-	9,039 ±5,702	6,588 ±5,656	0.85 ±0.06	0.81 ±0.07	3.1 ±2.4	11.4 ±11.6

3.3.2 PCR cycle number determination - 24, 25, and 26 cycles

Following sample input determination, this experiment was conducted to determine the optimal PCR cycle number to use when performing direct PCR using an input of a 0.5 mm sample punch. Direct PCR at 24, 25, and 26 cycles was performed using the 26 PM bloodstain samples, and results were compared by analysing the performance for each sample at the respective cycle number. To establish if profiling outcomes would be sufficient to consider utilising a direct PCR protocol for routine PM bloodstains, the aim was to determine which (if any) of the cycle numbers tested could achieve >75% of sample profiles with an average peak height between 1,200 and 14,000 RFU, and at least 90% of the samples producing complete profiles.

PCR cycle number determination - Profile concordance and completeness

All profiles obtained following amplification at 24, 25, and 26 cycles produced concordant profiles.

Completeness of profile data (complete, partial, or no results) obtained for each cycle tested, were recorded in Table 3.4. All cycles produced complete profiles in 23 out of 26 (88%) samples analysed. Sample 7 failed to produce any allelic data at any cycle number. Sample 23 yielded a complete profile at 24 cycles but failed to produce any allelic data at the other cycles tested. No result was obtained for Sample 25 at 24 cycles although full profiles were obtained at 25 and 26 cycles. Sample 6 produced partial profiles at all cycles, with 15, 25 and 31 out of 42 alleles present at 24, 25 and 26 cycles, respectively. Overall, the completeness of profile was similar with all cycles tested, and all cycles were close enough to the aim of having at least 90% of the samples producing complete profiles.

PCR cycle number determination - Average peak height

The average peak height of each DNA profile was calculated. The distribution of average profile peak heights for PM bloodstain samples amplified at 24, 25 and 26 cycles are shown in Table 3.4. None of the cycle numbers tested met the aim of having >75% of samples with an ideal average peak height between 1,200 and 14,000 RFU. Nevertheless, both 25 and 26 cycles had the highest percentage of samples (69%) with an average peak height between 1,200 and 14,000 RFU.

Table 3.4 PM Bloodstain – PCR cycle number comparison (24, 25, and 26 cycles). Percentage of the 26 samples that produced a complete, partial, or no profile; as well as the percentage that produced an average peak height of <1,200 RFU, 1,200-14,000 RFU, or >14,000 RFU.

	% Samples			% Average peak height (RFU)			
Cycle Number	No result	Partial profile	Complete profile	No result	<1,200	1,200-14,000	>14,000
24	8	4	88	8	35	54	4
25	8	4	88	8	11	69	11
26	8	4	88	8	4	69	19

PCR cycle number determination - Unknown artifacts

Profile artifacts of unknown origin (hereafter referred to as unknown artifacts) are not ideal in DNA profiles as these can increase the profile analysis time. Hence, it was aimed to determine which cycle would produce the profiles with the smaller number of unknown artifacts. Unknown artifacts present in each profile obtained at 24, 25, and 26 cycles were counted. Figure 3.1 demonstrates the relationship between the number of unknown artifacts and the average peak height obtained for each profile. As the average peak height increased, so did the number of unknown artifacts. Profiles with an average peak height of < 1,200 RFU, had two or fewer unknown artifacts. Profiles that produced an average peak height between 1,200 and 14,000 RFU contained between 0 and 102 unknown artifacts. In contrast, all profiles with an average peak height > 14,000 RFU, had between 85 and 180 unknown artifacts. As seen in Table 3.4, when the cycle number increased, the percentage of samples obtaining an average peak height > 14,000 also increased, which indicates that when the cycle number is higher more profiles will obtain a large number of unknown artifacts.

PCR cycle number determination – Peak height balance and inhibition ratio

The average peak height balance and the average inhibition ratio obtained from the 26 samples at 24, 25 and 26 PCR cycles were compared (Figure 3.2). The median inhibition ratio obtained is similar at 25 and 26 cycles. However, at 26 cycles there is more variability than at 25 cycles. While for the

average peak height balance, the box plot demonstrates acceptable average peak height balance from all cycles tested as these are all higher than 0.8. Nonetheless, comparison between 25 and 26 cycles, illustrates that the average peak height balance obtained at 25 cycles is better quality than at 26 cycles as the 50% of all the data at 25 cycles is higher.

PCR cycle number determination – Overall findings

In conclusion, cycles 25 and 26 are the closest to meeting the criteria of having at least 90% of the samples producing a complete profile and having the average peak height between 1,200 – 14,000 RFU. Cycle 24 was not a choice for determining the optimal cycle number, as it demonstrated a higher percentage of samples producing an average peak height < 1,200 RFU, compared to the other cycles. Based on the assessment criteria, both cycles 25 and 26 would be acceptable to use in a method for casework. Nonetheless, to determine one cycle for the method, the unknown artifacts, the average peak height balance, and the average inhibition ratio were assessed. Cycle 25 was chosen as the preferred cycle number for performing direct PCR with PM bloodstains because it produced fewer samples with a high number of unknown artifacts, as well as a higher average peak height balance, and less variability in the inhibition ratio.
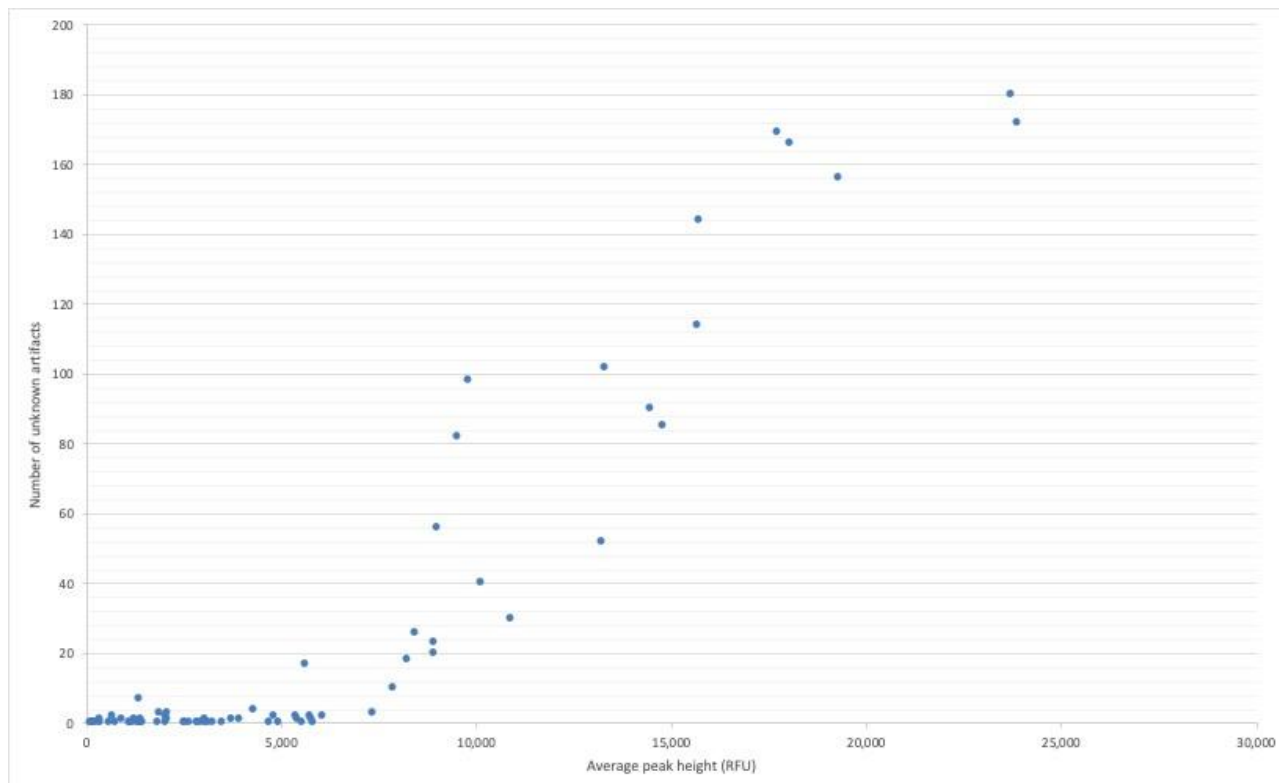
Figure 3.1 PM Bloodstain – Scatter plot of the average peak height vs number of unknown artifacts. Relationship between the number of unknown artifacts and average peak height (RFU) of a profile. Data is for the 26 samples each amplified at 24, 25 and 26 cycles.
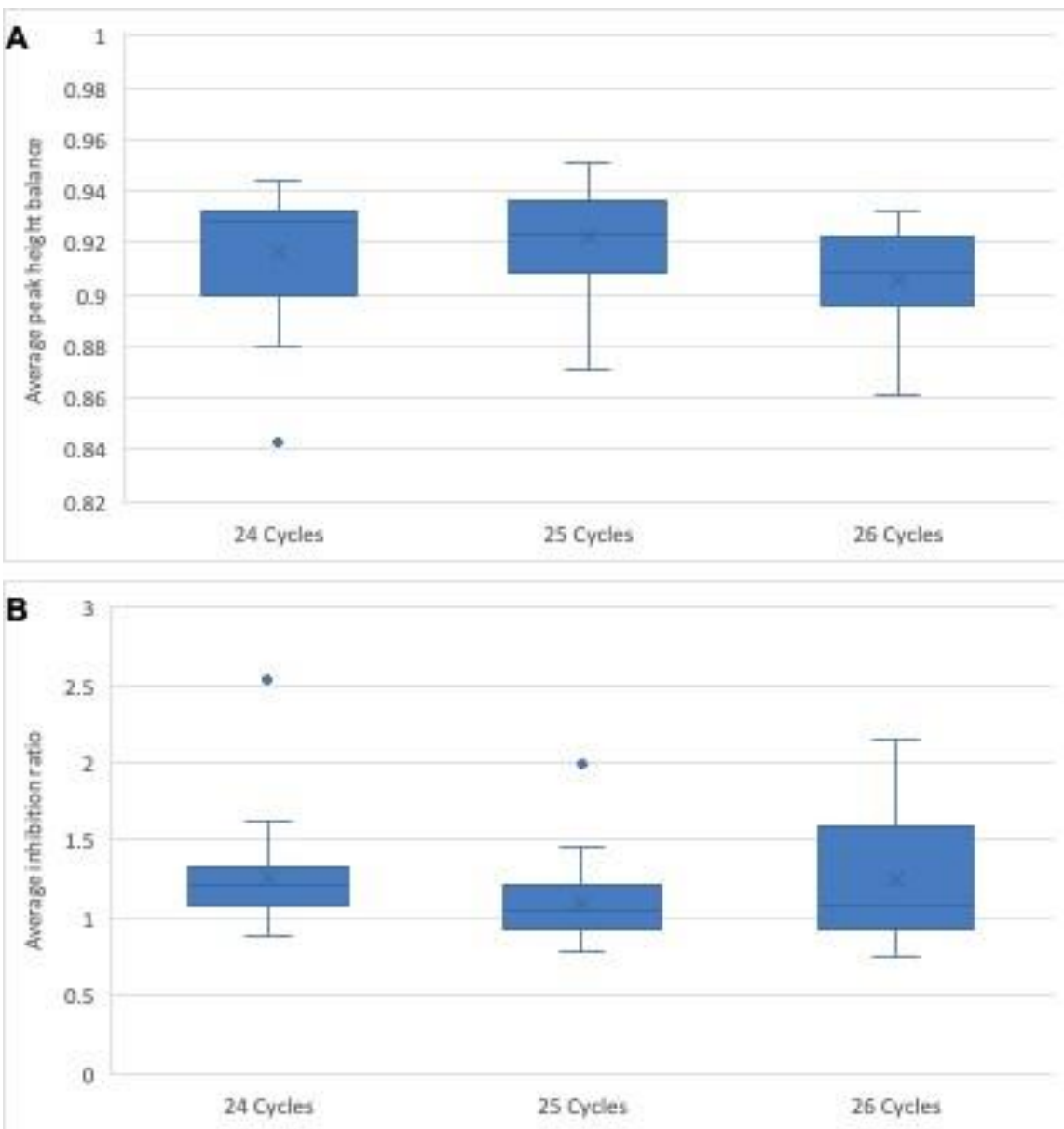
Figure 3.2 PM Bloodstains – PCR cycle number comparison (24, 25, and 26 cycles). Box plots of the (A) average peak height balance and (B) average inhibition ratio obtained from the 26 samples amplified at 24, 25, and 26 PCR cycles.

3.3.3 Pre-treatment (heating) prior to a 25-cycle amplification

Based on the cycle number experiment above, the following experiment was aimed at determining if a heating pre-treatment step prior to amplification at 25 cycles would improve profiling outcomes for samples that produced incomplete profiles (sample 6) or failed to produce any allelic data (samples 7 and 23) when directly amplified at 25 cycles. In addition, samples that had produced profiles with either high peak heights (sample 14), low peak heights (sample 21), or profiles of acceptable quality (sample 4), were also included to assess the impact of the pre-treatment on their performance, with the aim of assessing if a pre-treatment step would be a viable option for improving overall profiling success with direct PCR. The pre-treatment consisted of heating the sample punch in TE buffer at 99°C for 10 minutes and then amplifying the TE buffer solution instead of the actual punch. The results were compared to those previously obtained for these samples amplified at the same conditions (25 cycles) without a pre-treatment (section 3.3.2) and are presented in Table 3.5.

Pre-treatment at 25 cycles - Profile attributes

All profiles obtained when using a heating pre-treatment produced concordant profiles.

Complete profiles were obtained from three (samples 4, 14, and 21) of the six samples (50%) analysed with or without a pre-treatment. Sample 6 gave a partial profile both with and without a pre-treatment. However, for this sample, a more complete profile (83%) was observed with a pre-treatment compared to without pre-treatment (60%). Sample 23, which had previously not yielded a profile, showed an improvement with pre-treatment, producing a profile with 86% completeness. Although sample 23 did not produce a complete profile and peaks heights were low (Figure 3.3A), these were the first direct PCR conditions in which sample 23 had yielded any profile data (Figure 3.3B). Sample 7 failed to yield any profiling data under either set of conditions.

When comparing the outcomes for sample 4, chosen for producing acceptable quality DNA profiles in past experiments, a lower average peak height and average peak height balance were observed with pre-treatment compared to without pre-treatment, which could be the result of having a higher inhibition when performing the pre-treatment.

While, when comparing the outcomes for sample 14, which had previously produced a high average peak height (15,698 ±3,683 RFU) without a pre-treatment, an even higher average peak height of 11,236 ±3,997 RFU was observed with the pre-treatment. The average peak height balance was similar between the pre-treatment and without a pre-treatment, but the average inhibition ratio was higher in the profile obtained from the pre-treatment.

For sample 21 that had previously shown a low peak height (581 ±165 RFU) without the pre-treatment, a similar average peak height of 565 ±169 RFU was observed with the pre-treatment. Even though, there was not much difference between values, the average peak height balance was higher and the inhibition ratio lower in the profile without a pre-treatment compared to the profile with a pre-treatment.

Pre-treatment at 25 cycles – Overall findings

The partial profiles obtained with a pre-treatment from samples 6 and 23 showed an improvement compared to without performing a pre-treatment, by producing a higher number of known alleles. Nonetheless, with the other samples tested, reduced profile quality was observed with inhibition levels being higher with a pre-treatment than without a pre-treatment. Hence, it was decided not to proceed with this pre-treatment, as this did not produce complete profiles in samples 6, 7, and 23, and had a negatively impact in sample 4.

Table 3.5. PM Bloodstain – Pre-treatment (heating) prior to 25 cycle amplification. Comparison of PM bloodstain sample performance with pre-treatment (PT) and with no pre-treatment (NPT) prior to direct PCR at 25 cycles. Sample 7, which did not yield any results with or without a pre-treatment, was excluded from the table for clarity.

	Complete profiles (%)		Average peak height (RFU)		Average Peak height balance		Inhibition ratio	
	PT	*NPT*	*PT*	*NPT*	*PT*	*NPT*	*PT*	*NPT*
Sample 4	100	100	352 ±120	2526 ±895	0.88 ±0.10	0.92 ±0.06	1.3 ±0.4	0.9 ±0.3
Sample 6	83	60	180 ±142	165 ±140	0.78 ±0.14	0.78 ±0.12	7.1 ±3.4	6.2 ±3.4
Sample 14	100	100	11,236 ±3,997	15,698 ±3,683	0.90 ±0.05	0.91 ±0.04	2.4 ±0.7	1.5 ±0.3
Sample 21	100	100	565 ±169	581 ±165	0.84 ±0.13	0.87 ±0.12	1.4 ±0.5	1.0 ±0.3
Sample 23	86	NR	128 ±61	NR	0.80 SD: 0.15	NR	2.7 ±1.4	NR
Average	-	-	2,492 ±4891	4742 ±7375	0.84 ±0.05	0.87 ±0.06	3.0 ±2.3	2.4 ±2.6

NR= No result

A)
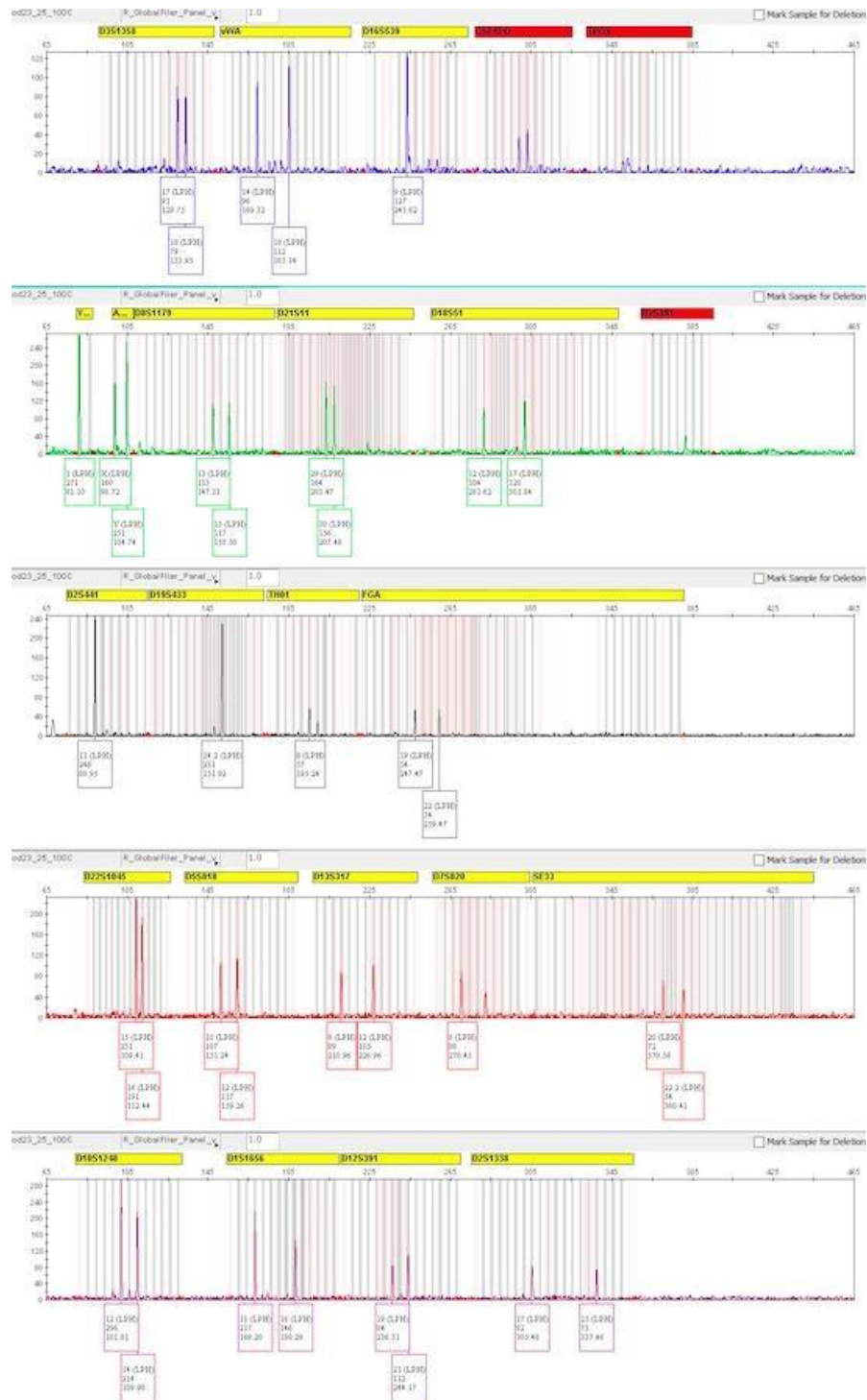
Figure 3.3A. PM Bloodstain – DNA profiles from bloodstain sample 23. (A) shows the DNA profile obtained with a pre-treatment consisting of heating the sample at 99°C for 10 minutes before amplification at 25 cycles.

B)
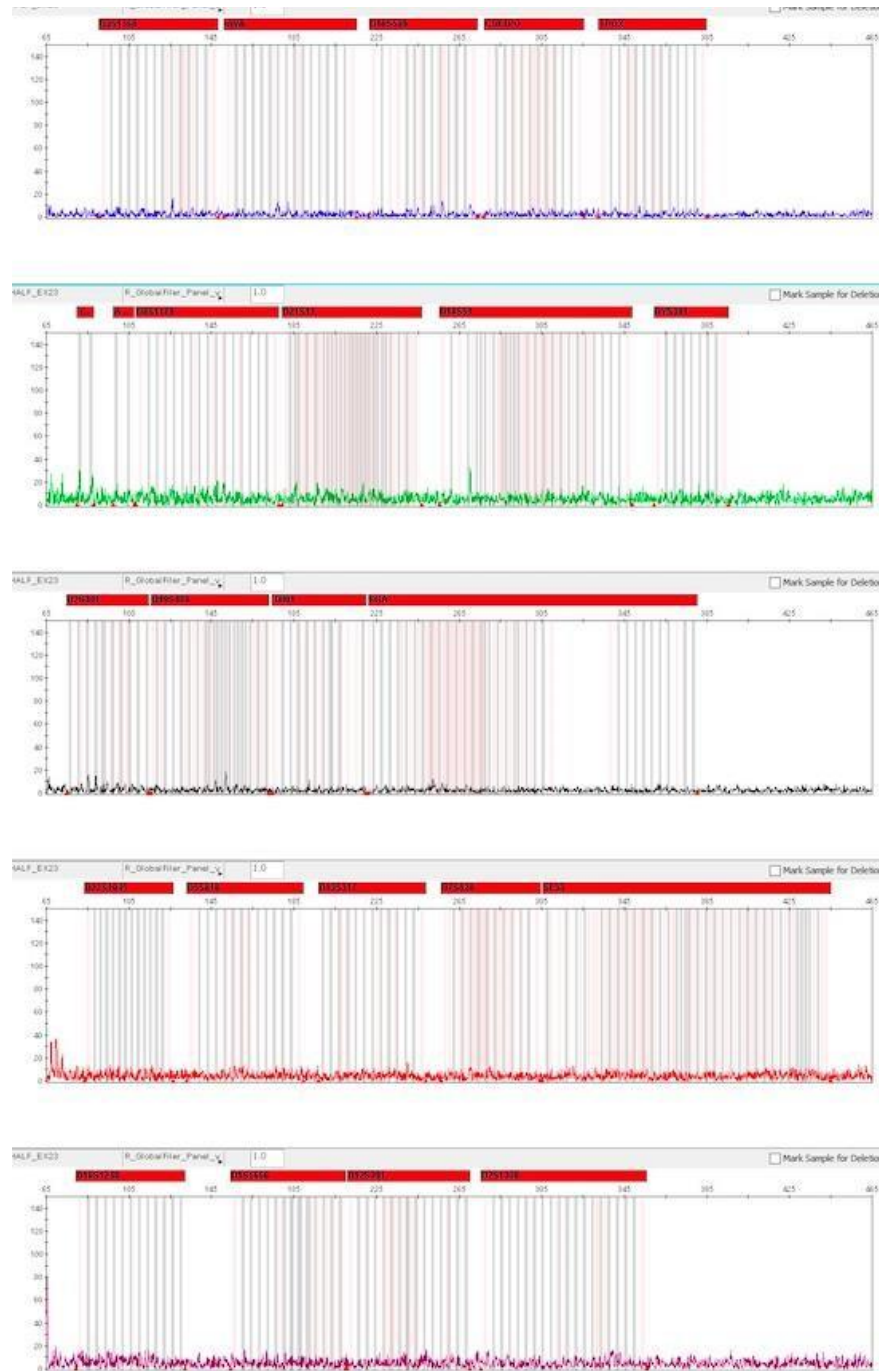
Figure 3.3B. PM Bloodstain – DNA profiles from bloodstain sample 23. (B) shows the profile obtained by direct amplification of a 0.5mm punch at 25 PCR cycles.

3.3.4 Post-treatment (dilution) after a 27, 28 or 29 cycle amplification

Results in section 3.3.2 demonstrated that direct PCR using 0.5 mm punch and amplifying at 25 cycles was optimal for PM bloodstain samples and it is a method that could be suggested for validation at the MBL. As it was a goal to have the same direct PCR cycling conditions for AM buccal FTA samples, 25 cycles were tested with AM buccal samples (see Chapter 4). However, as 25 cycles were found to be unsuitable for AM buccal samples, additional testing of PM bloodstain samples was required at higher cycle numbers – 27, 28 and 29 cycles – to determine if similar conditions for both sample types could be established. This experiment used six PM bloodstain samples based on their performance in previous experiments. In addition, as higher cycle numbers were expected to potentially result in the over-amplification effects, PCR products were diluted according to a four-step, two-fold serial dilution prior to capillary electrophoresis. As sample 6 had previously produced partial profiles under all conditions tested and similar results might have been expected with increasing dilution, this sample was serially diluted only three times. These results are presented in Tables 3.6 and 3.7.

Post-treatment- Profile concordance and completeness

All profiles obtained following post-PCR treatment produced concordant profiles.

As shown in Table 3.6, samples 12, 19, 24 and 25 gave complete profiles under all cycle number and dilution conditions tested. Sample 16 yielded a complete profile under all but one of the conditions tested, with a partial profile obtained with 27 PCR cycles and a 1 in 2 dilution. Sample 16 was chosen for this experiment because it showed low peak heights, so it would not be odd if it produced a partial profile, however, it is unexpected that higher dilutions at the same cycle produced complete profiles. Therefore, it is possible that this partial profile was caused by pipette handling error while preparing for CE. Sample 6 produced a partial profile with all conditions tested.

Post-treatment- Average peak height

As shown by the average peak height distributions in Table 3.6, the majority of the samples produced profiles with average peak heights between 1,200 and 14,000 RFU under all cycle number and dilution conditions tested. However, sample 6 produced an average peak height of

<1,200 RFU at all cycle numbers and dilutions tested, and sample 16 gave an average peak height of <1,200 RFU at 27 cycles with a post-PCR 1 in 2 dilution, consistent with the previously stated assumed pipetting error. Not surprisingly, only the lowest dilution conditions (1 in 2 and 1 in 4) at all three cycle numbers resulted in profiles with average peak heights of >14,000 RFU. These profiles were represented only by samples 12, 19 and 25, which might have been expected to produce higher average peak heights given their previous profiling outcomes that indicated the presence of excess DNA input and thus the reason for which they had been selected for this experiment (Table 3.2).

Post-treatment - Minus A peaks

Signs of overamplification, in the form of minus A peaks, noted for the samples analysed were recorded for comparison in Table 3.7. Sample 6 was the only sample tested that did not produce any minus A peaks. For the remainder of the samples, a greater number of minus A peaks were noted when the cycle number increased, with the greatest number of minus A peaks at the highest cycle number tested (29 cycles). Overall, however, the number of minus A peaks observed at each cycle number tested did not appear to decrease with increasing post-PCR dilution, indicating these peaks representing overamplification were unable to be successfully diluted out prior to capillary electrophoresis.

Minus A peaks were assessed for each sample, as the samples were selected based on specific past performance. Sample 16, previously shown to have insufficient DNA input at 25 cycles, produced minus A peaks in the majority of the cycles and dilutions tested. Of the samples which had previously shown acceptable profile quality, profiles generated from Sample 25 had minus A peaks with all cycles and dilutions tested while the profiles from Sample 24 only had minus A peaks at 29 cycles. Of the samples that had previously shown evidence of excessive DNA input, Sample 19 produced profiles with minus A peaks at all cycles and dilutions tested, while fewer minus A peaks at 27 and 28 cycles were observed in Sample 12 as compared to Sample 19.

Table 3.6. PM Bloodstain –post-direct PCR treatment (dilution) (27, 28 and 29 cycles). Sample's classification based on profile completeness and average peak height for samples amplified at 27, 28 and 29 PCR cycles with various pre-CE dilutions.

		Sample Profile Completeness		Sample Average Peak Height (RFU)		
Cycle #	Dilution	Complete profile	Partial profile	<1,200	1,200 -14,000	>14,000
27	1 in 2	12, 19, 24, 25	6, 16	6, 16	19, 24, 25	12
	1 in 4	12, 16, 19, 24, 25	6	6	12, 16, 19, 24	25
	1 in 8	12, 16, 19 24, 25	6	6	12, 16, 19, 24, 25	
	1 in 16	12, 16, 19, 24, 25			12, 16, 19, 24, 25	
28	1 in 2	12, 16, 19, 24, 25	6	6	12, 16, 24	19, 25
	1 in 4	12, 16, 19, 24, 25	6	6	12, 16, 19, 24, 25	
	1 in 8	12, 16, 19, 24, 25	6	6	12, 16, 19, 24, 25	
	1 in 16	12, 16, 19, 24, 25			12, 16, 19, 24, 25	
29	1 in 2	12, 16, 19, 24, 25	6	6	16, 24	12, 19, 25
	1 in 4	12, 16, 19, 24, 25	6	6	12, 16, 19, 24, 25	
	1 in 8	12, 16, 19, 24, 25	6	6	12, 16, 19, 24, 25	
	1 in 16	12, 16, 19, 24, 25			12, 16, 19, 24, 25	

Table 3.7. PM Bloodstain –post-direct PCR treatment (dilution) (27,28 and 29 cycles) – minus A peaks. The number of minus A peaks obtained for each sample when amplified at 27, 28 or 29 cycles followed by a twofold serial dilution before CE.

Cycle #	Dilution	Total	# of minus A peaks					
			Sample 6	Sample 12	Sample 16	Sample 19	Sample 24	Sample 25
27	1 in 2	19	0	3	0	13	0	3
	1 in 4	28	0	0	9	15	0	4
	1 in 8	26	0	2	10	13	0	1
	1 in 16	24		2	6	15	0	1
28	1 in 2	37	0	6	3	19	0	9
	1 in 4	34	0	7	0	19	0	8
	1 in 8	35	0	6	2	17	0	10
	1 in 16	32		6	0	18	0	8
29	1 in 2	65	0	14	5	18	9	19
	1 in 4	59	0	14	4	18	7	16
	1 in 8	69	0	14	9	18	9	19
	1 in 16	65		14	7	18	9	17

Method verification- Profile concordance and completeness

All profiles obtained when diluting the amplification products 1 in 8 or 1 in 16 produced concordant profiles.

Completeness of profile is recorded in Table 3.8. A slightly higher number of complete profiles were obtained with the 1 in 8 dilution compared to the 1 in 16 dilution, with 23 (88%) and 22 (85%) complete profile obtained, respectively. Sample 6 produced partial profiles with both

dilutions, however, more alleles were recovered with the 1 in 8 dilution (22 out of 42 alleles) compared with the 1 in 16 dilution (9 out of 42 alleles). The 1 in 8 dilution resulted in no allelic data for two samples (7 and 23), while the 1 in 16 dilution resulted in no data for three samples (7, 21, and 23).

Method verification- Average peak height

Average peak heights obtained are displayed in Table 3.8. Under both dilution conditions, the majority of samples produced profiles with an average peak height within the desired range (1,200 – 14,000 RFU). For the 1 in 8 dilution, 20 out of 26 samples (77%) tested produced profiles with an average peak height between 1,200 and 14,000 RFU, while for the 1 in 16 dilution the same result was seen for 19 out of 26 samples (73%). Four of the 26 (15%) samples tested produced profiles with average peak heights of <1,200 RFU under both dilution conditions. Of the two conditions tested, the 1 in 8 dilution at 27 cycles met the criterion of having at least 75% of the samples producing profiles with an average peak height range between 1,200 RFU and 14,000 RFU. At this dilution, sample 21 produced a complete profile with an average peak height range of <1,200 RFU, while it did not yield a profile with the 1 in 16 dilution.

Method verification- Average peak height balance and average inhibition ratio

The average peak height balance and average inhibition ratio were calculated for all 26 samples with both post-PCR dilutions 1 in 8 and 1 in 16, and values were displayed in box plots (Figure 3.4) to determine if 27 cycles have a negative impact on these profile parameters. Minimal difference was observed with the average peak height balance between tested dilutions and both dilutions showed an acceptably high average peak height balance. Minimal difference was also observed between the dilutions for the inhibition ratio, with this parameter also displaying acceptable results. It was expected to obtain minimal difference between the dilutions as these parameters are not affected by dilution, however, it was confirmed that amplification at 27 cycles of PM bloodstains, do not impact negatively on the profile, as no peak height imbalance or high inhibition levels were achieved.

Table 3.8. PM Bloodstain – Profile attributes for all 26 samples amplified at 27 cycles with post-treatment (dilution). The percentage of samples with complete, partial, or no results and the percentage of samples producing an average peak height of <1,200 RFU, or between 1,200 to 14,000 RFU, or >14,000 RFU.

	% Samples						
	Completeness of profile			*Average peak height (RFU)*			
Dilutions	*No results*	*Partial*	*Complete*	*No results*	*<1,200*	*1,200 – 14,000*	*> 14,000*
1 in 8	8	4	88	8	15	77	0
1 in 16	11	4	85	11	15	73	0
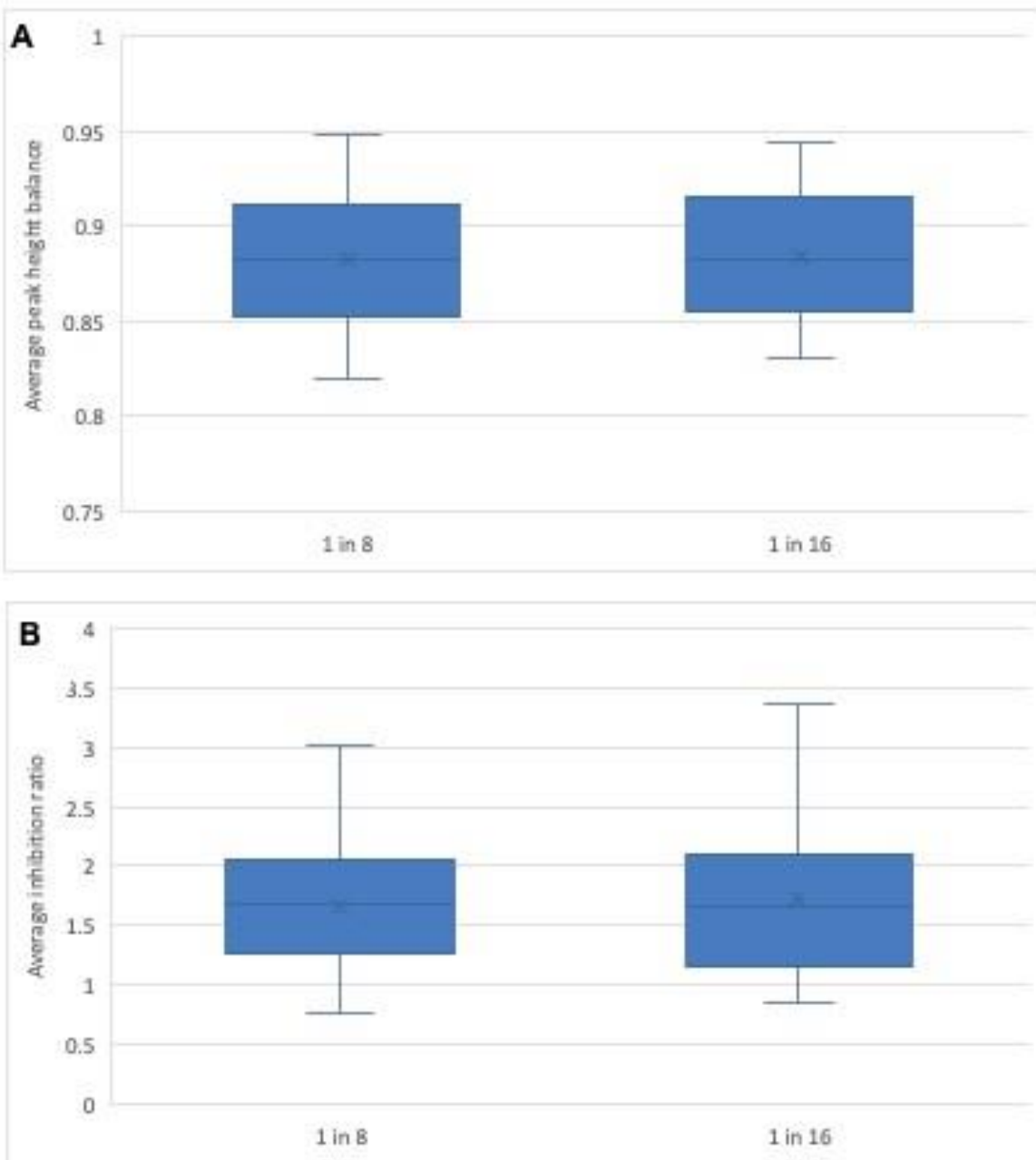
Figure 3.4. PM Bloodstains – Peak height balance and inhibition ratio. Box plots of the (A) average peak height balance and (B) average inhibition ratio obtain from the profiles of all 26 samples amplified at 27 cycles with a post- PCR dilution 1 in 8 or 1 in 16.

<u>PM bloodstains- overall findings</u>

A preliminary study of direct PCR with PM bloodstains using the GlobalFiler Express kit demonstrated high inhibition levels when following the manufacturer's protocol. This study demonstrated that by decreasing the input amount into the PCR to a 0.5 mm punch, inhibition levels decreased, and the quality of the profile improved compared to when amplifying with a 1.2 mm punch. Also, the method of performing direct PCR with 0.5 mm punch at 25 cycles was determined to meet acceptability criteria. Nonetheless, a higher cycle number was tested for PM bloodstains, as 25 cycles was insufficient for buccal samples and it would be ideal for the laboratory to have the same PCR cycle number for both of these sample types. It was demonstrated that the method of amplifying 0.5 mm punch of PM bloodstains at 27 cycles and conducting a post PCR dilution of 1 in 8, is acceptable based on profile completeness, average peak height, peak height balance, and inhibition ratio. Nonetheless, in order to conclude on the suitability of the method, assessment of the impact of incomplete adenylation on the profiles needs to be assessed.

3.4   Discussion

Input amount determination

The GlobalFiler Express kit recommends performing direct amplification of AM blood samples by adding a 1.2 mm punch to the PCR reaction (41). However, preliminary study conducted at the MBL demonstrated that the manufacturer recommended protocol produced profiles indicating high levels of inhibition, resulting in DNA profiles that did not meet the reporting requirements of the laboratory (72). Different studies have demonstrated that some blood component levels, such as lactic acid levels, formic acid, calcium, magnesium, and potassium, change after death (78, 81). Their increase in PM blood samples could be the reason inhibition of the amplification reaction when using PM samples was observed.

To test whether the observed inhibition from the preliminary study could be reduced, a smaller input amount than what is recommended by the manufacturer for AM bloodstain samples was tested. The 0.5 mm punch sample size was tested to determine if profiles generated exhibited

less inhibition when compared to the 1.2 mm punch sizes, by hypothesising that by adding less amount of sample into the PCR this would also mean adding less PCR inhibitors. Results indicated the reduced sample input amount resulted in less inhibition of the amplification reaction which confirmed that sample amount is directly related to the number of inhibitors present.

Despite adding less sample into the PCR, the peak heights were higher. This may be because inhibition is lower which results in a more efficiently amplification; it is known that inhibition negatively impacts peak heights in DNA profiles (82, 83). Our findings infer an increased presence of inhibitors in the PCR reaction when using a 1.2 mm punch compared to a 0.5 mm punch. The presence of inhibitors is further supported by the finding that although less sample (and presumably DNA template) was added into the direct PCR, 0.5 mm versus 1.2 mm punch, 83% of that produced results had a higher average peak height when using the 0.5 mm punch.

In literature, it has been demonstrated that diluting blood samples results in reducing inhibition levels by improving the quality of profiles (60, 84, 85). This project took a different approach to reduction of inhibitors into the direct PCR reaction, which would require less sample handling than dilution. Nevertheless, both methods seek to reduce the concentration of inhibitory substances within the reaction for which we were successful. Hence, based on the DNA profiles observed, it was decided that an input amount of 0.5 mm punch size would be used in subsequent method development experiments for performing direct PCR with PM bloodstain samples.

Cycle number determination

The GlobalFiler Express kit recommends the amplification of AM bloodstain samples at 25, 26, and 27 cycles in order to determine the optimal cycle number (41). During the preliminary study conducted at the MBL using 1.2 mm punch samples, high peak heights were observed for the lower molecular weight loci when samples were amplified at 27 cycles which indicated that a lower cycle number may be required for this sample type. Based on these findings, the optimal cycle number for an input of 0.5 mm punch was tested at 24, 25, and 26 cycles.

In the MBL, the most suitable cycle number would be the one that met the first assessment criterion of at least 90% of the samples yielding a complete profile. Based on the results obtained,

this criterion was not met by any of the three cycles tested; however, all cycles did yield complete profiles in 88% of the samples analysed. For all the cycles tested, the same three of the 26 samples analysed did not produce a complete profile. The second assessment criterion would be for a cycle number that resulted in greater than 75% of the samples analysed having an average peak height between 1,200 and 14,000 RFU as profiles obtained within this range are of, generally, acceptable quality for casework. To this end, results were analysed in groups depending on their average peak height, in order to see which cycle number met this assessment criterion. Unfortunately, none of the three cycle numbers tested (cycles 24, 25, and 26) met this criterion. This inability to produce enough profiles within the desired range could be a result of a variability in white cell counts inherent to PM blood samples. White cells could decrease (86, 87) or increase (88, 89) due to the cause of the unexpected death which are common in PM identification casework. As such, it is unlikely that it would be possible to implement a method which strictly fits the original criterion. Based on the results obtained, both 25 and 26 cycles resulted in 69% of the samples yielding a profile average peak height between 1,200 and 14,000 RFU.

To further assist in selecting a cycle number for the direct PCR of PM bloodstains, an analysis of unknown artifacts was undertaken. A higher number of DNA profiles at 26 cycles produced an average peak height higher than 14,000 RFU compared to profiles obtained with 25 cycles. As such, it is likely that 26 cycles would have greater number of these unknown artifacts. It was noted that the number of unknown artifacts was higher as the average peak height increased. It was illustrated that profiles with an average peak height >14,000 RFU also contained between 85 and 180 unknown artifacts. At cycle 25, two out of 26 samples (8%) had an average peak height >14,000 RFU, while at cycle 26 five out of 26 samples (19%) had an average peak height >14,000 RFU. This would infer that at 26 cycles there is greater possibility to obtain profiles with at least 80 unknown artifacts. Following the evaluation of unknown artifacts at each cycle, 25 cycles was selected as it would be expected to result in fewer unknown artifacts when undertaking direct PCR of PM bloodstain samples. This would in turn, decrease the analysis time required to analyse profiling data to remove unknown artifacts, and reduce the likelihood of the requirement for rework to be undertaken.

Pre-treatment (heating) at cycle 25

From the 26 PM bloodstain samples allocated for this research, three were noted not to perform as well as others (in terms of profile completeness and peak heights) in all profiling attempts. As these samples are typical of the type received for routine identification casework, it could indicate that perhaps 12% of the samples that arrive for casework might behave this way when subjected to direct PCR. Initially it was believed that inhibition was responsible for the sample failures, however additional testing (outside the scope of this project) with a different amplification kit containing quality sensors indicated that no inhibition was present in the amplification reaction with these samples (unpublished data). It could be that the reason the three samples did not produce a complete profile in the majority of conditions tested was because the DNA was not being released from the card. Hence, the inclusion of a heating pre-treatment was performed, expecting that this incubation would improve the release of DNA into the TE buffer. It was decided to trial a pre-treatment heating step prior amplification, to see if more DNA template could be released, as Watherston *et al.* demonstrated improvement in amplification outcomes (90). In addition, other samples (including ones with low and high peak heights, and one that was of suitable quality) were also analysed to see if the pre-treatment had any impact on these samples, and to ensure that any improvements noted for the three samples that yielded poor results did not come at the expense of other samples, so that the pre-treatment could be reasonably applied to all samples. The pre-treatment consisted of heating the samples at 99°C for 10 minutes in 10 µL of TE, and then amplifying the eluate (rather than the punch sample itself).

Following pre-treatment, two of the three samples that had previously shown poor results did not show marked improvement, while the other sample produced a full profile, albeit with low peak heights. However, the sample that previously produced profiles of suitable quality and average peak height showed lower peak heights when the pre-treatment was performed, which could be a result of an increase in inhibition levels or a result of less DNA. However, inhibition ratio was higher in all profiles obtained with a pre-treatment than without a pre-treatment. This illustrates that the heating process is releasing more sample from the card, which is resulting in a higher addition of PCR inhibitors into the PCR.

As the pre-treatment heating step did not improve the profiles obtained for samples 6 and 7, which previously performed poorly, and it did not improve the profiles for the additional samples analysed, no further testing was undertaken to try to improve on the results obtained when a heating pre-treatment was included. Furthermore, as the inclusion of a pre-treatment added an additional step in the direct PCR analysis pipeline (adding both additional sample handling steps and additional processing time), it would need to demonstrate significant improvement for it to be considered as part of the methodology development, which was not the case.

Post-treatment (dilution) at cycle 27,28 and 29 cycles

Based on the results, the direct PCR of PM bloodstain samples using a 0.5 mm punch and 25 cycles of amplification meets acceptability criteria set out for a method to be validated for casework. For AM buccal FTA samples, however, direct PCR using 25 cycles of amplification was insufficient to generate profiles to meet the laboratory requirements (Chapter 4). As one of the overall objectives of this research project was to determine if a direct PCR protocol using the same cycle number could be developed for PM bloodstain and AM buccal FTA samples, it was decided to test the performance of higher cycle numbers with PM bloodstain samples in order to explore whether the same PCR cycle number could be achieved for PM bloodstains as for AM buccal FTA samples.

It was decided to test PM bloodstain samples at 27, 28 and 29 cycles. However, as it was expected that these cycles would result in overamplification leading to sub-optimal DNA profiles, dilutions of the PCR products were performed prior to CE, to hopefully dilute out the effects of overamplification. For each PCR cycle number, a two-fold dilution series of the PCR product was performed prior to CE followed by assessment of the DNA profiling data to see if it met the assessment criteria of at least 90% of samples producing complete profiles and at least 75% of profiles with average peak heights between 1,200 and 14,000 RFU. A similar approach, by diluting the blood sample, demonstrated an improvement of profile quality in the literature (60, 74). Although a dilution step would add time to the direct PCR analysis pipeline, this is not expected to be significant. As an additional benefit of this method, samples with lower DNA input which may exhibit drop out with this method could be re-injected with CE without dilution to possibly

recover full profiles. Therefore, this would reduce time and cost, avoiding reamplification or rework of the entire process in these cases.

Results from the six samples tested at cycles 27, 28, and 29 with subsequent serial dilution, demonstrated that the 1 in 8 and 1 in 16 dilution conditions resulted in profiles within the ideal average peak height range of between 1,200 and 14,000 RFU for all samples. Cycles 28 and cycle 29 were discounted as possible PCR cycle numbers for this method, as profiles resulting from amplifications at these cycle numbers showed a greater number of minus A peaks – especially at cycle 29, which resulted in minus A peaks in profiles from all samples. Cycle 27 also resulted in minus A peaks, but these were fewer in number compared to cycle 29.

The conditions of 27 cycles with subsequent 1 in 8 and 1 in 16 dilution were selected for further analysis using all 26 PM bloodstain samples. Under these conditions, the 1 in 8 dilution produced a higher number of samples with complete profiles than did the 1 in 16 dilution. Furthermore, a greater number of samples with profile average peak heights between 1,200 and 14,000 RFU were obtained with the 1 in 8 dilution compared to the 1 in 16 dilution. One of the samples analysed showed a complete profile (with an average peak height <1,200 RFU) with the 1 in 8 dilution, but did not produce any results with the 1 in 16 dilution This may indicate that the 1 in 16 dilution is too high for low-DNA or degraded samples and can result in undetectable allelic data.

Based on profile completeness and average peak height obtained from the 26 samples tested, the method of amplifying 0.5 mm punch of PM bloodstains at 27 cycles followed by a 1 in 8 dilution prior to capillary electrophoresis, was proposed to be acceptable method to perform direct PCR as, if buccal samples are successful at 27 cycles (see chapter 4), it would allow to have the same amplification conditions for both sample types. However, in order to further substantiate this proposition, further analysis of the data to assess the impact of incomplete adenylation on profile reportability would be required. It is expected that the assessment of these parameters will further assist in determining if these direct PCR conditions for PM bloodstains samples should be validated by the MBL for application to identification casework.

4. CHAPTER 4: AM Buccal samples

4.1 Introduction

Buccal samples are one of the most common samples processed as a reference sample for DNA analysis at MBL. As such, it would be ideal to implement a direct PCR methodology for this sample type to save processing time and costs.

Preliminary work was conducted at the MBL using the GlobalFiler Express kit following the manufacturer's recommended sampling protocol for buccal cells on treated paper at 26, 27, and 28 PCR cycles (unpublished data). However, this resulted in peak heights lower than expected for several of samples tested, likely as a result of insufficient DNA input into the PCR (unpublished data).

One of the overall objectives of this study was to explore whether it would be possible to establish the same PCR cycling protocol for PM bloodstains and AM buccal samples as both sample types are commonly processed together for identification purposes. Hence being able to process both buccal samples and bloodstains according to the same method, or at least the same amplification conditions, would allow for simultaneous processing of PM and AM reference samples belonging to the same case, saving both processing time and reagent costs.

In Chapter 3, a method for the direct PCR of PM bloodstain samples was developed. Given the issues with PCR inhibition seen with these bloodstain samples, it was hypothesised that PM bloodstain samples would prove more challenging to successfully adjust the DNA input amount to a fixed cycle number. As such, it was decided to first determine the cycle number for PM bloodstains and attempt to modify the buccal sample input amount to allow for the buccal samples to be successfully profiled with this cycle number. As seen in Chapter 3, 25 PCR cycles were determined to be suitable for PM bloodstains.

Based on the preliminary study for AM buccal FTA samples demonstrating that an input of 1.2 mm punch was insufficient for acceptable peak heights at 26 and 27 PCR cycles, an increased input amount would be required to successfully utilise 25 PCR cycles for direct amplification of AM buccal FTA samples. If unsuccessful, a higher cycle number will be tested for PM bloodstains and based on that outcome, additional cycle numbers or method modifications will be tested for AM

buccal FTA samples. Consequently, the method development for performing direct PCR of AM buccal FTA samples with the GlobalFiler Express kit and with the same cycle number as for PM bloodstains, will be presented in this chapter.

4.2   Materials and methods

4.2.1 <u>Sample selection and preparation</u>

AM buccal FTA samples available for the experiments performed in this chapter were previously described in Chapter 2 (Section 2.2.1). The 1.2 mm punch size was used for all experiments. Protocols used for sampling with the 1.2 mm punch and cleaning between samples was performed as outlined in section 2.2.2.

Direct input amount – 25 cycles: This experiment tested sample inputs of 2 and 3 punches. Five samples were selected for this experiment which had previously shown low allelic peak heights at 26 cycles in the preliminary experiment conducted at the MBL (unpublished data). Samples and number of punches added to the PCR reaction are shown in Table 4.1.

Table 4.1. AM Buccal – Sample selection. The samples and number of punches used for direct amplification at 25 cycles.

	Buccal FTA samples
2 punches	17, 18, 19, 20, 21
3 punches	17, 18, 19, 21, 22

Pre-treatment with incubation at room temperature - at 25 cycles: Samples 19 and 21 were used in this experiment. Five and ten punches of each sample were added to 10 µL and 15 µL of low TE buffer, respectively, vortexed and incubated at room temperature for 10 minutes.

Pre-treatment with incubation at room temperature - at 27 cycles: Samples 5, 7, 12, 18, 19 and 21 were selected for this experiment. Samples 19 and 21 were chosen because these two samples

demonstrated consistent effects of insufficient DNA input previously at 25 cycles. The other samples were chosen as they had sufficient sample remaining for the number of punches required for this experiment.

Four different sample input amounts were tested: 2, 3 and 5 punches in 10 µL TE buffer and 10 punches in 15 µL TE buffer. All preparations were vortexed and incubated at room temperature for 10 minutes.

Pre-treatment with heated incubation - at 27 cycles: This experiment utilised samples 5, 7, 12, 18, and 19. The input amounts tested were 2 and 5 punches. Punches were incubated in 10 µL of TE buffer at 99°C for 10 minutes, using an Eppendorf Thermomixer™ (Eppendorf) without shaking.

4.2.2   Direct Amplification

GlobalFiler Express amplification reactions were carried out in DNA-free 0.2 ml strip tubes, with positive and negative control reactions and PCR cycling protocol as specified by the manufacturer (41). PCR cycle numbers and any additional modifications are described below.

For the direct input amount experiment, punches were first added to the PCR tubes followed by master mix with the volumes as specified by the manufacturer (41) (section 2.2.3), and the punches were added straight into the master mix. Amplifications were carried out at 25 cycles as per the manufacturer recommended GlobalFiler Express cycling protocol (41).

For the experiments with a pre-amplification incubation, the master mix was prepared as described in Table 4.2, with master mix added to 3 µL of the eluate. Amplifications were carried out at 25 and 27 cycles for experiments involving room temperature incubations, and at 27 cycles for the heated incubation experiment. Amplifications were conducted under the conditions recommended by the manufacturer for GlobalFiler Express (41).

Table 4.2. AM Buccal – Amplification reaction. Volume of individual master mix components and final master mix volume of a single PCR reaction for the GlobalFiler Express (GFE) and GlobalFiler (GF) kits.

Reaction components	Reagent volume (µL)
Master Mix	6
Primer Set	6
Total	12

4.2.3 Capillary electrophoresis

In all experiments, the PCR products were separated and detected by capillary electrophoresis on a 3500 Genetic Analyser under conditions specified by the manufacturer (Thermo Fisher Scientific, Australia), as described in section 2.2.4 for GlobalFiler Express reactions.

4.2.4 Data analysis

Following capillary electrophoresis, DNA profile data was analysed as described in Section 2.2.5 using GeneMapper™ ID-X (v1.6) (Thermo Fisher Scientific).

4.3   Results

Experiments were performed to develop a method for performing direct PCR with AM buccal FTA samples based on the cycle number determined for PM bloodstain samples (Chapter 3). With the experiments conducting direct PCR, varied input amounts (number of punches) of AM buccal FTA samples at 25 cycles were examined. While, with the experiments performing a pre-treatment, varied eluates were tested at 25 and 27 cycles.

4.3.1 <u>Input amount determination at 25 PCR cycles</u>

AM buccal FTA samples were tested at 25 PCR cycles, as this cycle number is acceptable for PM bloodstain samples (Chapter 3), and one of the aims is to have the same cycle number for both sample types.

Furthermore, a preliminary study conducted at the MBL (unpublished data) demonstrated that an input of 1.2 mm punch for AM buccal FTA samples at 26 cycles yielded profiles showing low allelic peak heights. Hence for this experiment, an attempt to increase the amount of DNA input into the PCR was tested at 25 cycles through firstly increasing the number of sample punches added directly and secondly eluting DNA into TE buffer with a high input amount and subsequently adding this eluate to the PCR reaction. All profiles obtained with the input amounts tested at 25 cycles produced concordant profiles.

Samples that had previously yielded DNA profiles with low peak heights were selected to determine the sample input for direct PCR at 25 cycles. Firstly, 2 punches were tested by following the standard direct amplification method. A complete profile was produced for two (samples 17 and 19) out of five samples (40%) tested (Table 4.3). Similarly, two out of five samples (40%) tested produced partial profiles, with sample 18 yielding 40 out of 42 alleles, and sample 21 yielding 19 out of 42 alleles (Table 4.3). In contrast, no allelic results were detected in one (sample 20) out of five samples (20%) analysed (Table 4.3). The target average peak height is between 1,200 to 14,000 RFU to minimise losing data below analysis thresholds or interpretation complication through overamplification effects. None of the input amounts analysed produced profiles in the aimed average peak height with all profiles producing an average peak height < 1,000 RFU (Table 4.3).

As results with 2 punches showed incomplete profiles obtained from most of the samples tested, as well as a low average peak height, it was decided to increase the sample input amount to 3 punches. A complete profile was produced in one (sample 19) out of five samples (20%) analysed, while one sample (sample 21) produced a partial profile, yielding 30 out of 42 alleles (Table 4.3). Furthermore, no alleles were produced in three (samples 17, 18 and 22) out of five samples (60%) tested (Table 4.3). The increase in number of sample punches appears to negatively impact the profiling outcomes, with poorer profiling outcomes obtained with the 3-punch method.

Therefore, it was decided to test the performance of a pre-treatment prior to amplification, to release the DNA from the FTA card into the TE buffer and then amplifying the eluate instead of the punch directly. This would hypothetically allow more sample to be used to increase the amount of DNA being added into the reaction.

Two pre-treatments were performed: 5 punches in 10 µL of TE buffer, and 10 punches in 15 µL of TE buffer, followed by a 10 minutes incubation at room temperature, with 3 µL of each eluate then amplified directly. For these pre-treatments, samples 19 and 21 were chosen because they had produced complete and incomplete profiles, respectively, when directly amplifying 2 and 3 punches input. Incomplete profiles were obtained for both samples tested with both pre-treatment methods. With the 5 punches eluate, 35 out of 42 alleles were produced in sample 19, and 39 out of 42 alleles were obtained for sample 21 (Table 4.3). While when using the 10 punches eluate, 41 and 32 out of 42 alleles were produced with sample 19 and 21 respectively. Poorer outcomes were obtained from the eluate tested compared to 2 and 3 punches direct based on profile completeness obtained and average peak height produced.

Table 4.3. AM Buccal– Sample input determination at 25 cycles. Profile completeness and average peak height (with standard deviation (±)) obtained from AM buccal FTA samples tested with the input amounts of 2 or 3 punches directly amplified and of 3 µL of a 5 punches eluate, or 10 punches eluate.

	Profile completeness (%)				Average peak height (RFU)			
Sample Preparation Method	**Direct**		**Pre-Treatment**		**Direct**		**Pre-Treatment**	
Number of Sample Punches	**2**	**3**	**5**	**10**	**2**	**3**	**5**	**10**
Sample 17	100	0			871 ± 366	0		
Sample 18	95	0			474 ± 326	0		
Sample 19	100	100	83	98	647 ± 206	679 ± 178	97 ± 39	109 ± 50
Sample 20	0				0			
Sample 21	45	71	93	76	121 ± 75	576 ± 565	138 ± 63	297 ± 213
Sample 22		0				0		

Input amount at 25 cycles – Overall findings

Results obtained from 2 and 3 punches with direct amplification, as well as from the pre-treatments performed, demonstrated that the input amount cannot be sufficiently increased to produce acceptable profiling outcomes at 25 cycles. The method that performed the best was the 2 punches added directly to the PCR reaction. However, this method produced profiling outcomes below the requirements for it to be effectively applied to identification casework. Given these results of low-profile completeness and low average peak height, it was evident that a higher cycle number would be required to obtain successful profiling outcomes. Therefore, a higher cycle number was trialled with PM bloodstain samples (Chapter 3) and was subsequently tested with AM buccal FTA samples.

4.3.1 Input amount determination at 27 PCR cycles

In Chapter 3, an alternative protocol producing acceptable profiles for PM bloodstains was established at 27 cycles. To match this protocol, 27 cycles was trialled with AM buccal FTA samples to determine if an appropriate protocol could be established.

A preliminary study at the MBL tested 1.2 mm punch input with direct PCR at 27 cycles (unpublished data). Results demonstrated 88% of samples producing complete profiles and 62% of the samples producing profile average peak heights within the ideal range of between 1,200 and 14,000 RFU, which did not meet the assessment criterion of >75% of samples producing profiles within this range. Also, 35% of the samples produced profiles with an average peak height of <1,200 RFU. Therefore, to improve the completeness of profiles and the average peak heights, it was decided to elute the DNA from different number of sample punches into TE buffer, and then amplifying the resulting eluate to theoretically increase the amount of DNA being available for PCR.

Six samples (samples 5, 7, 12, 18, 19, and 21) were used in this experiment. Pre-treatment involved 2, 3, and 5 punches from each sample suspended in 10 µL of TE buffer and incubated for 10 minutes at room temperature prior to the eluate being subjected to direct PCR. Also, 10 punches were tested with the same samples, but these were suspended in 15 µL of TE buffer.

Samples 19 and 21 were chosen in this experiment because in the preliminary study they produced profiles with an average peak height of <1,200 RFU. The objective of performing the pre-treatments was to determine if the average peak height of these samples could be increased by increasing the amount of sample used. Additionally, samples that produced the aimed average peak height (1,200 – 14,000 RFU) were tested to ensure that utilising this method did not negatively impact these samples. Performance of profiles obtained with the pre-treatment by testing the eluates from different number of punches, were compared to the corresponding results obtained with the manufacturer recommended protocol from the preliminary study at 27 cycles and these are presented in Table 4.4.

Table 4.4. AM Buccal– Sample input determination at 27 cycles. Percentage profile completeness, average peak height, average peak height balance, and average inhibition ratio calculated for the sample input amounts of 1 punch (preliminary study) and 2 punches, 3 punches, 5 punches and 10 punches eluates (this study).

Number of Sample Punches	Profile completeness (%)					Average peak height (RFU)				
	1	2	3	5	10	1	2	3	5	10
Sample 5	100	100	100	100	100	4,746 ± 1248	4,943 ± 1880	2,595 ± 2661	540 ± 279	1,046 ± 527
Sample 7	100	100	79	100	100	15,422 ± 3515	10,630 ± 8346	7,349 ± 8334	2,684 ± 1189	3,934 ± 1648
Sample 12	100	100	NR	100	100	8,867 ± 1759	11,298 ± 4354	NR	3,715 ± 1411	4,057 ± 1489
Sample 18	100	79	79	100	100	1,330 ± 790	1,468 ± 1406	1,465 ± 1431	1,561 ± 949	1,162 ± 645
Sample 19	100	100	100	83	100	588 ± 187	1,286 ± 460	584 ± 200	144 ± 72	238 ± 118
Sample 21	100	74	100	100	100	443 ± 318	749 ± 627	5,408 ± 2820	293 ± 185	781 ± 501

Number of Sample Punches	Average peak height balance					Average inhibition ratio				
	1	2	3	5	10x	1	2	3	5	10
Sample 5	0.91 ± 0.05	0.90 ± 0.09	0.76 ± 0.14	0.72 ± 0.24	0.82 ± 0.12	1.1 ±0.3	2.0 ±0.7	32.2 ±23.0	3.4 ±3.0	2.0 ±0.9
Sample 7	0.87 ± 0.10	0.78 ± 0.16	0.70 ± 0.15	0.86 ± 0.09	0.86 ± 0.10	1.1 ±0.5	11.8 ±13.6	243.7 ±231	2.2 ±1.2	2.4 ±1.0
Sample 12	0.91 ± 0.05	0.90 ± 0.04	NR	0.90 ± 0.08	0.90 ± 0.08	1.1 ±0.2	2.4 ±0.2	NR	1.7 ±0.5	1.6 ±0.4
Sample 18	0.82 ± 0.10	0.78 ± 0.12	0.79 ± 0.09	0.81 ± 0.12	0.85 ± 0.14	4.2 ±1.8	36.3 ±31.1	34.1 ±33.3	4.2 ±2.1	3.0 ±0.8
Sample 19	0.85 ± 0.10	0.84 ± 0.13	0.80 ± 0.13	0.55 ± 0.23	0.73 ± 0.17	1.2 ±0.5	1.1 ±0.5	1.5 ±0.5	2.8 ±2.4	2.1 ±1.2
Sample 21	0.78 ± 0.20	0.83 ± 0.13	0.88 ± 0.10	0.74 ± 0.17	0.77 ± 0.16	7.4 ±8.0	19.9 ±14.1	3.8 ±2.5	3.9 ±2.7	5.1 ±3.4

NR= No result

Input amount at 27 cycles- Profile concordance and completeness

All profiles obtained produced concordant profiles.

With the 2 punches eluate, four (samples 5, 7, 12, and 19) out of six (67%) samples tested produced complete profiles, while sample 18 and 21 produced partial profiles with 33 and 31 out of 42 alleles, respectively.

Using the 3 punches eluate, complete profiles were obtained in three (samples 5, 19, and 21) out of six (50%) samples tested. Two (samples 7 and 18) out of six (33%) samples tested produced partial profiles, with 33 out of 42 alleles present in both samples. No allelic results were obtained in sample 12. Comparing all inputs tested, the 3 punches eluate was the method where the least number of samples produced complete profiles, as well as being the only method where a sample failed to produce any allelic data.

With the 5 punches eluate, five out of six (83%) samples tested produced complete profiles. Sample 19 produced a partial profile where 39 out of 42 alleles were present. In contrast, when the TE buffer volume and number of punches was increased to 10 punches in 15 µL of TE, the eluate showed an improvement in results by obtaining complete profiles in all the six samples tested.

Input amount at 27 cycles - Average peak height

With a 2 punches eluate, five (samples 5, 7, 12, 18, and 19) out of six samples tested produced profiles with average peak heights within the desired range. Compared to the preliminary study, profiles from samples 19 and 21 produced an increase in average peak height with the 2 punches eluate, although sample 21 still produced an average peak height < 1,200 RFU.

With the 3 punches eluate, four out of six samples produced profiles with an average peak height within the target range. For Sample 21, this was the only input amount that produced profiles with average peak height > 1,200 RFU.

Using a 5 punches eluate, three (samples 7, 12, and 18) out of six samples tested produced profiles with average peak heights within the ideal range, while the other three samples produced profiles with average peak heights of < 1,200 RFU. This sample input amount was the only method which

produced a profile with average peak height < 1,200 RFU for sample 5. Additionally, samples 19 and 21 produced a lower average peak height than obtained in the preliminary study.

Despite the increase of sample input amount, the 10 punches eluate produced the highest number of samples of all the input amounts tested, with an average peak height lower than the desire range. With the 10 punches eluate, only two (samples 7 and 12) out of six samples produced profiles with average peak heights within the ideal range, while the other four samples produced profiles with average peak heights < 1,200 RFU, although sample 18 produced an average peak height of 1,162 RFU just outside the ideal range.

Input amount at 27 cycles - Average peak height balance

Compared to results from the preliminary study, the average peak height balance was lower with some samples when the pre-treatment was performed. Profiles showing a decreased average peak height balance compared to the preliminary study, had a concurrent increase of average inhibition ratio.

Input amount at 27 cycles – Inhibition ratio

Compared to the preliminary study, overall higher inhibition was observed in all of the sample eluates tested. Also, the 2 punches eluate showed particularly high inhibition ratios in samples 7, 18 and 21. With the 3 punches eluate, inhibition ratio was high in samples 5 and 18. While inhibition ratio obtained with the 5 punches eluate and 10 punches eluate were not as high as 2 and 3 punches eluates.

Input amount at 27 cycles – Overall findings

By performing the pre-treatment and increasing the number of punches used, it was expected it would increase the DNA input into the PCR resulting in improvement compared to profiles obtained from the direct PCR with 1 punch (preliminary study). Only the 10 punches eluate produced the same profile completeness performance as in the preliminary study, obtaining complete profiles in all six samples. However, this finding did not coincide with increases in other profile parameters, with insufficient increases in average peak height, increasing inhibition and decreasing peak height balance observed with all of the tested input amounts. No performance improvements compared to the preliminary study were obtained in all six samples with a

particular eluate. This could be the result of releasing more PCR inhibitors during the pre-treatment, as this was demonstrated by the profiles with a pre-treatment showing higher inhibition and lower peak height balance.

4.3.2 <u>Input amount at 27 cycles - Incubation at 99°C</u>

As the different eluates tested with room temperature incubation at 27 cycles did not improve the average peak height, a modification to the method by heating the punches in TE buffer at 99°C, was tested using 2 and 5 punches. Resulting percentage profile completeness and profile average peak heights were compared to those obtained for the same sample inputs incubated at room temperature. These comparisons are shown in Table 4.5 and Figure 4.1, respectively.

All profiles obtained were concordant to their known profile.

The 2 punches eluate with a heated incubation produced complete profiles in five out of six (83%) samples tested, with sample 21 producing a partial profile with 24 out of 42 alleles. Compared to the incubation at room temperature, sample 18 improved its performance when the incubation was heated by obtaining a complete profile (Table 4.5). However, sample 21 produced partial profiles with both incubation types, although the profile obtained from the room temperature eluate produced a greater number of alleles. The average peak height comparison between incubation temperatures for the 2 punches eluate is shown in Figure 4.1.A. This demonstrated that profiles for four samples (5, 7, 12, and 19) showed a higher average peak height when incubated at 99°C. Samples 18 and 21 produced a higher average peak height when the incubation was at room temperature. No improvement was seen compared to the profiles produced in the preliminary study.

With the 5 punches eluate, all six samples produced complete profiles when incubation was at 99°C. The incubation of five punches at 99°C demonstrated a marked increase in the average peak height in all samples compared to incubation at room temperature (Figure 4.1B). Also, five (samples 5, 12, 18, 19, and 21) out of six samples produced profiles with an average peak height between 1,200 and 14,000 RFU, but sample 7 produced an average peak height of 14,548 RFU just outside the ideal range. This is the only method tested at 27 cycles where no profiles

produced had an average peak height < 1,200 RFU. Also, the average peak height obtained in these profiles showed an improvement compared to the profiles obtained in the preliminary study (Table 4.4). The 5 punches eluate produced profiles from samples 19 and 21, with an average peak height greater than 1,200 RFU, while the corresponding sample profiles from the preliminary study produced average peak heights less than 1,200 RFU.

Table 4.5. AM Buccal– Pre-amplification treatment at 27 cycles. Comparison of percentage profile completeness between a pre-treatment incubation at room temperature (RT) compared to an incubation heated at 99°C for 2 and 5 punches eluate amplified at 27 cycles.

	Profile completeness (%)			
	2 punch eluate		5 punch eluate	
	RT	Heated	RT	Heated
Sample 5	100	100	100	100
Sample 7	100	100	100	100
Sample 12	100	100	100	100
Sample 18	79	100	100	100
Sample 19	100	100	83	100
Sample 21	74	57	100	100

Incubations – Overall findings

Based on completeness of profile and average peak height, improvements were seen for all samples tested in the 5 punches eluate incubated at 99 °C. This demonstrated an improvement over the profiles obtained with the preliminary study, as it was the only method that increased the average peak height of the six samples tested to higher than 1,200 RFU. However, additional testing on a larger sample set or different samples should be tested to determine if the method still performs as desired by producing 90% complete profiles with at least 75% of the samples with an average peak height between 1,200 and 14,000 RFU.
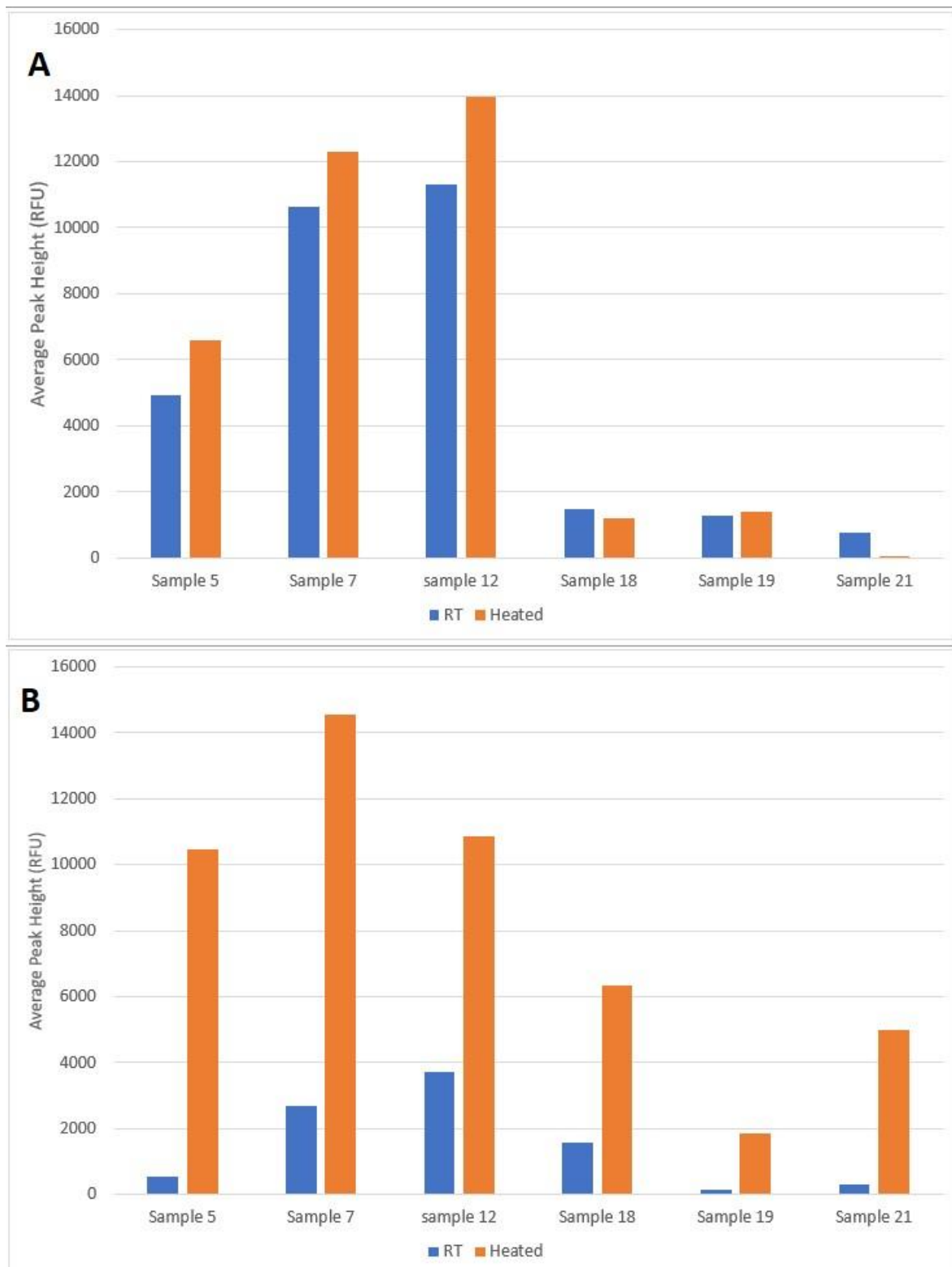
Figure 4.1. AM Buccal FTA – Pre-amplification treatment at 27 cycles. Comparison of the profile average peak heights obtained from pre-amplification incubation at room temperature (RT) and at 99°C (Heated) for A) 2 punches eluate; and B) 5 punches eluate amplified at 27 cycles.

4.4   Discussion

The GlobalFiler Express kit is designed to perform direct PCR with AM buccal samples (41). Nevertheless, a preliminary study at MBL (unpublished data) demonstrated that the use of GlobalFiler Express kit performing direct PCR with buccal samples at 26, 27, and 28 cycles, was producing lower than expected peak heights which would not be acceptable as a methodology to be validated at MBL for routine identification casework purposes (results from eight of the twenty-six samples tested can be seen in Chapter 2). However, one aim of the method development was to assess if the same PCR cycle number for PM bloodstain samples and AM buccal samples could be used in a direct PCR workflow, because buccal and bloodstain samples are often processed together as part of the same case. Consequently, by having the same cycle number, these samples could be amplified together with a single set of controls which would save time and costs.

Input amount determination at 25 cycles

Method development for direct amplification of AM buccal FTA samples started by increasing the sample input amount with 25 PCR cycles to match the cycle number determined for PM bloodstains. It was decided not to test the manufacturer recommended input amount of a single 1.2 mm punch as it was presumed that insufficient DNA input would result in sub-optimal profiling outcomes based on the preliminary study at 26 PCR cycles (unpublished data). As such, it was decided to first perform the method as recommended in the GlobalFiler Express protocol except for increasing the input amount directly into the PCR reaction. Therefore, the experiment at 25 cycles started by testing 2 punches.

Using this 2 punches method, partial profiles and low peak heights were observed. Consequently, the allele drop-out and the low peak heights could be the result of having low template DNA into the PCR (91). Hence, it was decided to test 3 punches, by hypothesising that the increase of sample amount into the PCR would increase the DNA input and allow for sufficient PCR product to be generated for detection.

It was expected that compared to the 2 punches input amount, the input of 3 punches would result in improved profiling outcomes, as the sample amount was increased. However, this did

not eventuate with the increase of sample amount negatively impacting profiling outcomes, with a greater number of samples either failing to produce results or to producing incomplete profiles. This might indicate that the issue was insufficient amplification cycle number for the amount of DNA input. Nonetheless, as the aim was to have buccal samples matching the PCR cycle number with PM bloodstains, it was decided to further assess amplification of buccal samples at 25 cycles. The adjusted sample preparation involved eluting the DNA from the FTA card into TE buffer, then using this eluate in the PCR. This might allow more sample to be used, potentially resulting in a higher concentration of DNA being added to the reaction. However, none of the eluates tested produced a complete profile. It is likely that AM buccal samples contain insufficient DNA to successfully generate high quality profiles when 25 PCR cycles are employed. Therefore, a higher PCR cycle number must be implemented to improve the performance obtained from direct PCR in buccal FTA samples.

It was concluded that for buccal FTA samples, 25 PCR cycles was insufficient.

Input amount determination at 27 cycles

Since 25 cycles were demonstrated to be insufficient for buccal FTA samples, a higher cycle number was tested. However, to achieve the aim of having the same amplification cycle number for bloodstains and buccal samples, a higher PCR cycle number was tested for bloodstains and resulted in 27 cycles producing acceptable profile quality (see Chapter 3). Therefore, for buccal samples the input amount was tested at 27 cycles to determine if the same PCR cycle number could be used for both sample types.

The preliminary study conducted at the MBL prior to this research, tested the direct PCR of 1.2 mm buccal sample punch at 27 cycles as recommended by the GlobalFiler Express kit (unpublished data). Results showed 35 % of the samples produced an average peak height < 1,200 RFU, which did not meet the acceptability criteria. Hence, different experiments were performed to explore the possibility of increasing the DNA input amount to produce profiles with average peak heights higher than 1,200 RFU at 27 PCR cycles.

To explore the possibility of increasing the average peak height obtained in the preliminary study, increasing number of punches (2, 3, 5, and 10) were incubated in TE buffer at room temperature, and these eluates were amplified. Because of the increase in amount of sample used, it was

expected these eluates would result in an improvement in profile quality, however this was not the case. The profiles obtained from the preliminary study with one punch directly amplified, demonstrated better quality compared to all eluates tested. The inhibition ratio was higher in all eluates amplified compared to the profiles obtained in the preliminary study indicating the increased number of punches used increased the input of inhibitors into the PCR. There is also the possibility that the elution of DNA from the FTA card is not efficient at room temperature. As such, a heated incubation was trialled.

Incubation heated

Incubation was changed from conducting it at room temperature to 99°C. It was hypothesised that the increased temperature would allow a greater amount of DNA to release from the card into the TE buffer, which might result in increased peak heights (90).

When heating the 2 punches, the average peak height increased but there was negligible difference compared to the same number of punches incubated at room temperature. In contrast, the heated eluate containing 5 punches was the only method where the profiles produced by all six samples were complete and also had average peak heights of > 1,200 RFU. This was also the only method where there was an improvement in the average peak height from the profiles obtained from the preliminary study. This improvement was marked in profiles from samples 19 and 21, which produced an average peak height < 1,200 RFU with the preliminary study, but this was increased to being higher than 1,200 RFU with the method of amplifying the 5 punches heated eluate.

This may be a suitable method, however testing with additional samples would be required to fully assess the suitability of this method. Additionally, this method was tested with 6 samples that had been extensively sampled throughout this study and as such, it cannot be confirmed that the sample punch came from the ideal location on the card. It is recommended that the 5 punches heated eluate is tested with an expanded sample set in order to confirm these results. If it shows the same trend as with the six samples tested in this study, that is producing all complete profiles with an average peak height > 1,200 RFU, it might be considered for validation as this would allow

buccal samples to be amplified at 27 cycles with bloodstains. Also, if it is decided that 5x 1.2 mm punches can be suitable, there is a Whatman^{TM} Harris micro punch of 6 mm size (GE Healthcare), which would make the collection easier and accurate by just having to punch once.

5. Chapter 5: TOENAILS

5.1   Introduction

Keratinous tissues are used as a source of DNA for identification (69). DNA is protected in these tissues reducing DNA degradation and the chance of cross-contamination (69). As a result of DNA being structured in keratin tissues, DNA is less impacted by the decomposition process than in non-keratinous tissues (69). DNA profiles have been successfully generated from nail samples collected from decomposed remains with standard DNA profiling processes (23, 92, 93). This has allowed a less invasive sample type to be collected from decomposed remains as historically, bone and teeth samples would be used as the DNA source in these cases and a surgical procedure may be required to collect the sample (23, 94-96). At the MBL, toenails are the preferred DNA source when bodies are decomposed (23). Toenails are preferred over fingernails, as toenails are less exposed to the environment and thus have less chance of obtaining mixed profiles (23).

This project aims to develop a methodology to perform direct PCR with toenail samples for identification purposes at the MBL. By performing direct PCR to produce DNA profiles from a toenail sample, the process could be less time consuming and more cost efficient than compared to the standard method that includes an extraction and a quantification step prior PCR. Direct PCR has been performed previously utilising AM fingernail samples, with 21 out of 40 samples producing partial profiles, which would not be an acceptable success rate for identification at the MBL (70). Additionally, Watherston et al., did not obtain allelic results from toenails when applying direct PCR with different incubations prior amplification (90).

The performance of direct PCR using toenails is limited in the literature, and the GlobalFiler Express kit (amplification kit used in the method development) does not have any recommended protocol for this sample type. As there is no recommended method to follow, trialling different modifications to sample input amount and assessing if sample pre-treatment is required will form part of this method development. Due to the availability of PM toenail samples for research being limited at the MBL, this method development study was conducted with AM toenail samples from volunteers. Also, a 29 cycle PCR protocol was used in all experiments, as by having a higher cycle is more likely to obtain profiles with the presence of allelic results.

The objective of this study was to develop a method for the direct amplification of toenail samples to determine if it is possible to generate complete DNA profiles by amplifying toenail samples directly without sample pre-treatment. However, with this method, no cell lysis has been performed prior to addition of the sample to the PCR and as such it is unclear if sufficient DNA would be available for the PCR with this type of protocol. If a direct PCR method is not suitable for toenails, a pre-amplification lysis protocol will be developed to improve profiling outcomes.

5.2   Materials and Method

5.2.1   Samples

Reference toenail samples were donated to this research project from four team members of the MBL with informed consent and ethics approval. Toenails were donated as clippings which were cut by each donor. Once received, toenail samples from individual donors were kept in sealed bags at room temperature.

Purpose of protocol to developed is to be applied in a forensic scenario with PM samples, therefore, toenails were collected from donors without enquiring any personal information such as time since last nail clipping, last shower, length of nail, etc.

5.2.2 Samples selection and preparation

Nails were first prepared by scraping all surfaces using a sterile scalpel and then were cut to the desired size (as described below in each experiment section) using sterile scissors and forceps. Conversely, the shaving size was obtained with the scalpel by scraping a layer of the surface from the underside of the nail.

Initial Testing: Samples 1, 2, and 4 were used in this experiment. Each sample was cut to approximately 1.2 mm (roughly the size of a 1.2 mm sample punch).

Sample Input Size Test: Sample 4 was used in this experiment – chosen by having the most quantity of sample left. The sizes tested were approx. 0.5 mm cutting; 1 shaving; and multiple shavings. Figure 5.1 shows a picture of the samples in the PCR tubes before the amplification mixture was added.
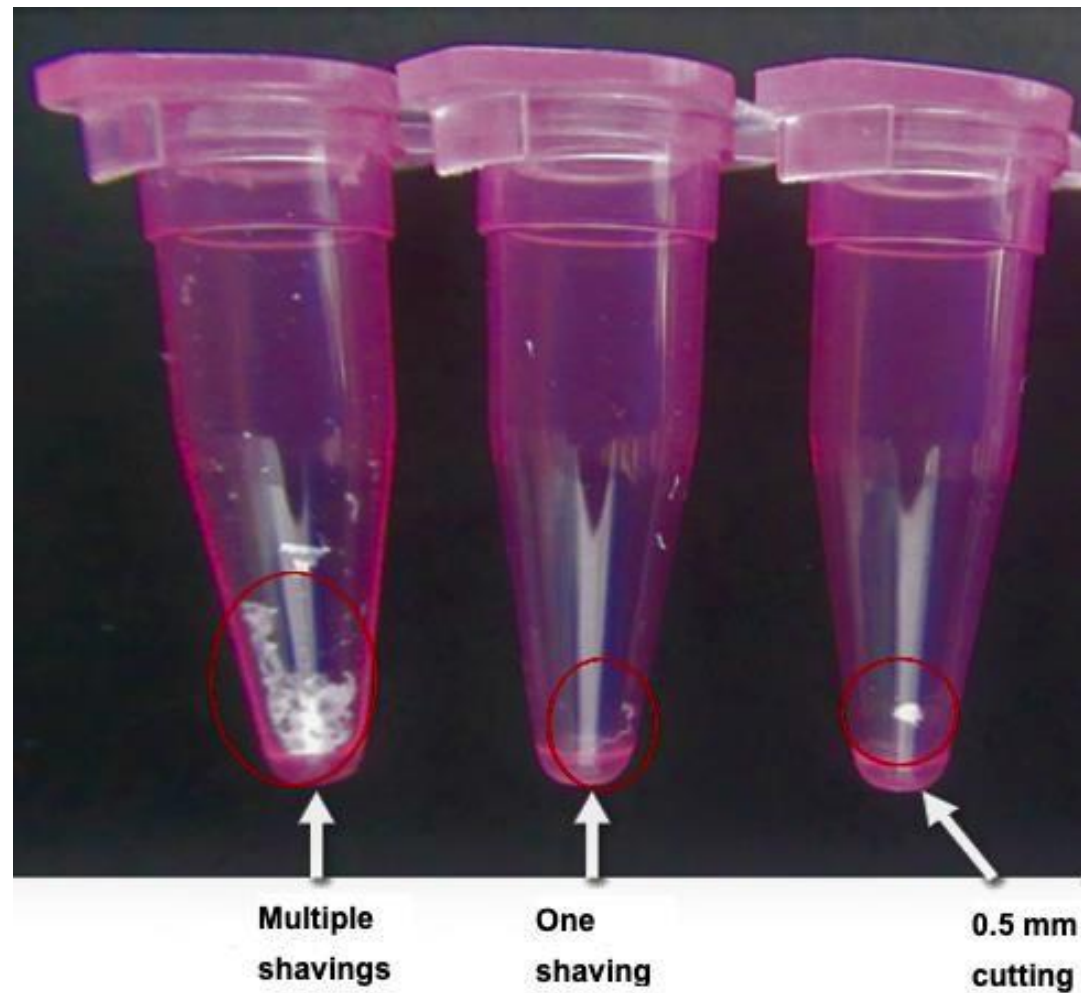
Figure 5.1. Toenail – Sample Input Size Test. Picture of the sample sizes tested inside the PCR tube prior to the master mix addition. All the sizes tested were obtained with sample 4. Sizes tested were multiple shavings; one shaving; and an approximately 0.5mm cutting.

5.2.3 Pre-treatment prior to amplification

An overview of the lysis protocols used is presented in Table 5.1.

General Lysis Protocol

Samples were added into individual 1.5 mL tubes with 100 µL of Qiagen 20 mg/mL Proteinase K (Qiagen, Australia) and 1.5 µL of sodium dodecyl sulfate (SDS) 20% (w/v). All tubes were vortexed for 10 seconds and briefly centrifuged for 5 seconds. Samples were incubated in the thermomixer for 52 minutes at 56^oC shaking at 900 rpm, following by 5 minutes at 95^oC to inactivate the Proteinase K. Following incubation, 3 µL of this 'Neat Lysate' was added into the PCR tube for amplification. Lysates were subsequently diluted as described in each section.

Initial Pre-Treatment Test

The pre-treatment performed was using sample 4 with the sizes; 0.5 mm cutting, one shaving, and 3 shavings. Post-performance of the lysis protocol, 300 µL of nuclease-free water was added to the lysate. The tubes were vortexed for 10 seconds and briefly centrifuged for 5 seconds. Following, 3 µL of the 'diluted lysate' was added into the PCR tube for amplification. The diluted lysate was then discarded, leaving the nail sample in the tube. This nail was then added into a PCR tube for amplification.

DTT Test with 1 piece of nail

Sample 4 was used in this experiment. The size used was one piece of the nail of about 3 – 5 mm. Two lysis reactions were performed; one as described with the general protocol and one with the addition of 10 µL DTT to the lysis mixture prior to incubation. The lysis mixture containing DTT was incubated for 30 minutes at 56^OC.

The neat lysate was serially diluted with 100 µL of nuclease-water added at each step and 3 µL of each diluted lysate removed for amplification. Eight and 10 dilution steps were carried out for lysates with and without DTT, respectively.

DTT Test with 2 pieces of nail

Sample 5 was utilised in this experiment. This experiment replicated the previous experiment with one piece of nail, except in this experiment the input was 2 pieces of nail approximately 3 – 5 mm in size. Ten dilution steps were carried out for lysates with and without DTT.

Modification of DTT concentration and sample amount

Sample 5 was used in this experiment with 2, 3, 4, 5, 6, nail pieces of approximately 3 – 5 mm tested.

All nail input amounts were subjected to lysis as previously described without the addition of DTT. Furthermore, inputs of 2, 3, 4 and 5 pieces were also lysed with the addition of 10 μL of DTT; and inputs of 2, 3 and 4 pieces were also lysed with the addition of 15 μL and 20 μL of DTT. All neat lysates were diluted with 800 μL nuclease-free water and 3 μL of this diluted lysate was used for amplification. Neat lysates were not amplified.

Table 5.1. Toenail – lysis solution preparation for all pre-treatment experiments. Sample input amount, volumes added to lysis reaction, and lysis incubation times for each pre-treatment experiment.

	Initial Pre-Treatment Test			DTT Test with 1 piece of nail		DTT Test with 2 pieces of nail		Modification of DTT concentration and sample amount			
Sample input	0.5 mm cutting	1 shaving	3 shavings	1 piece		2 pieces		2-6 pieces	2-5 pieces		2-4 pieces
20 mg/ml Proteinase K (µL)	100			100		100		100			
20 % SDS (µL)	1.5			1.5		1.5		1.5			
1 M DTT (µL)	-			0	10	0	10	0	10	15	20
Incubation time (mins) at 56°C	52			52	30	52	30	52	30		

5.2.4 Direct Amplification

GlobalFiler Express amplification reactions were carried out in DNA-free 0.2 ml strip tubes. For the experiments the nail was added directly to the PCR reaction, the nail was added to the PCR tube, followed by the master mix which was prepared with the volumes as specified in Table 2.2 (Chapter 2) described for the GlobalFiler Express kit (41). When a lysate was amplified, the master mix was added into the 3 μL of the neat or diluted lysate. Master mix was prepared as described in Table 4.2 (Chapter 4). Positive and negative control reactions were as specified by the manufacturer (41).

All amplifications were carried out at 29 cycles on an Applied Biosystems^{TM} Veriti^{TM} 96-well thermal cycler (Thermo Fisher Scientific, Australia) as per the GlobalFiler Express manufacturer's recommended cycling protocol (41) (section 2.2.3).

5.2.5 Capillary electrophoresis

In all experiments, the PCR products were separated and detected using capillary electrophoresis, on an Applied Biosystems^{TM} 3500 Genetic Analyser (Thermo Fisher Scientific, Australia) under conditions specified by the manufacturer for the GlobalFiler Express kit (Thermo Fisher Scientific, Australia).

5.2.6 Data analysis

Following separation and detection, DNA profile data was analysed as described in Section 2.2.5 using GeneMapper^{TM} ID-X (v1.6) (Thermo Fisher Scientific).

5.3   RESULTS

5.3.1 Direct amplification

Different toenail sizes were directly amplified to determine if DNA profiles can be obtained from toenail samples when the sample is added directly into the PCR reaction without the need for a prior lysis step. The initial test involved an amount of approximately 1.2 mm of toenail tested with three samples. Partial profiles were obtained with the three samples tested. The number of alleles obtained was low in all samples used; sample 1 produced 15 out of 42 alleles, sample 2 produced 6 out of 42 alleles, and sample 4 produced 19 out of 42 alleles (Table 5.2). Low peak heights were produced in all samples, with sample 4 having the highest average peak height of 137 ± 92 RFU, while sample 1 produced an average peak height of 93 ± 58 RFU and sample 2 an average peak height of 61 ± 28 RFU (Table 5.2).

Given the incomplete profiles obtained from approximately 1.2 mm input, a decrease of nail amount input into the PCR was tested to determine if results would improve. This by theorizing that the reason for not yielding complete profiles was the nail inhibiting the PCR, or because the DNA was not released from the cells and testing a sample input that provide a greater surface area may improve the results. Therefore, a sample input size test was performed using sample 4, by testing the sizes of; approx. 0.5 mm, a shaving, and multiple shavings. Partial profiles were obtained for all of the sizes tested, 17 out of 42 alleles were obtained with the approx. 0.5 mm input, while the shaving produced 9 out of 42 alleles and the multiple shavings 10 out of 42 alleles. Lower average peak heights were observed in the profiles for all sizes tested compared to approx. 1.2 mm size.

Extensive allele dropout prevented the calculation of inhibition ratio and peak height balance. However, signs of inhibition were shown with a ski-slope which is described as the alleles in the shorter locus being higher than in the longer locus. As seen in Figure 5.2, a ski slope is seen in sample 4 with the input amounts of approx. 1.2 mm, approx. 0.5 mm and the multiple shavings. This ski slope was seen in all dye channels with allelic results in the lower molecular weight loci and locus drop out in the higher molecular weight loci. Additionally, with these sizes, minus A peaks were produced in the purple dye channel which can be a manifestation of inhibition (Figure 5.3).

One shaving was the only size without showing a ski slope pattern or minus A peaks with all sizes tested. As the shaving is the smallest size tested, the minus A peaks and ski slope pattern shown in the other sizes could be an indicative of stochastic effects rather than inhibition.

Results obtained from performing direct PCR with different sizes of nail demonstrated that it was not possible to obtain a complete profile with the small input sizes tested. The allele dropout, low peak heights, and the ski slopes might be indicating inhibition, but also it might be insufficient DNA available for the PCR reaction without first lysing the cells. Therefore, further testing was done by performing a lysis pre-treatment before amplification.

Table 5.2. Toenails – profile attributes for direct amplification. Percentage profile completeness and average peak heights obtained from toenail samples tested with different input amounts amplified directly.

	Complete profile (%)				Average Peak height (RFU)			
	1.2 mm	*0.5 mm*	*Shaving*	*Multiple shavings*	*1.2 mm*	*0.5 mm*	*Shaving*	*Multiple shavings*
Sample 1	36				93 ±58			
Sample 2	14				61 ±28			
Sample 4	45	40	21	24	137 ±92	81 ±27	88 ±21	87 ±27

±: Standard Deviation

Figure 5.2. Toenails – Green dye channel. Screenshot of the green dye channel obtained from sample 4 when performing direct amplification of the input amount size of (from top to bottom): (A) the 1.2 mm, (B) the 0.5 mm, (C) the shaving, and (D) the multiple shavings.
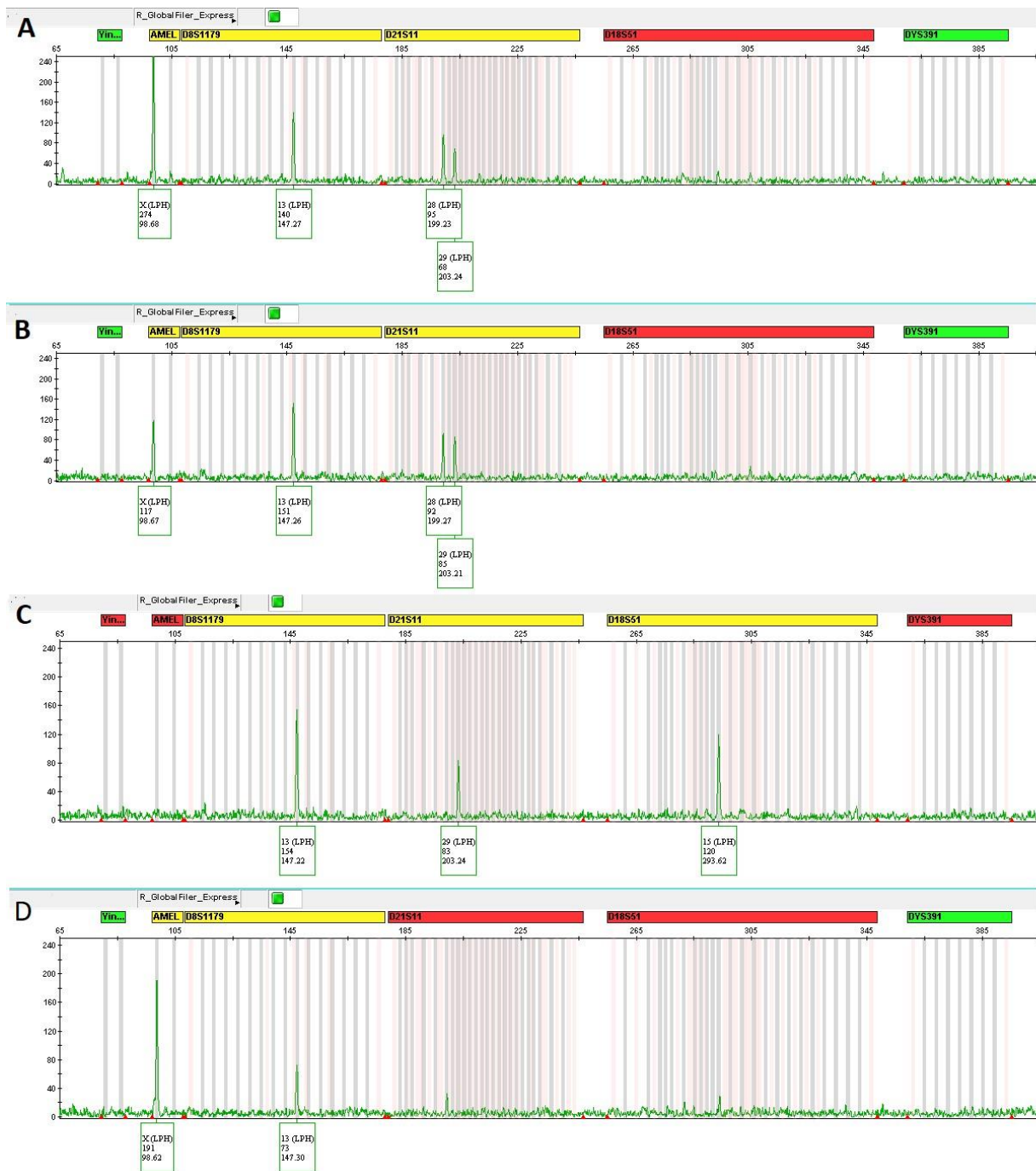
Figure 5.3. Toenails – Purple dye channel. Screenshot of the purple dye channel obtained from sample 4 when performing direct amplification of the input amount size of (from top to bottom): (A) the 1.2 mm, (B) the 0.5 mm, (C) the shaving, and (D) the multiple shavings.
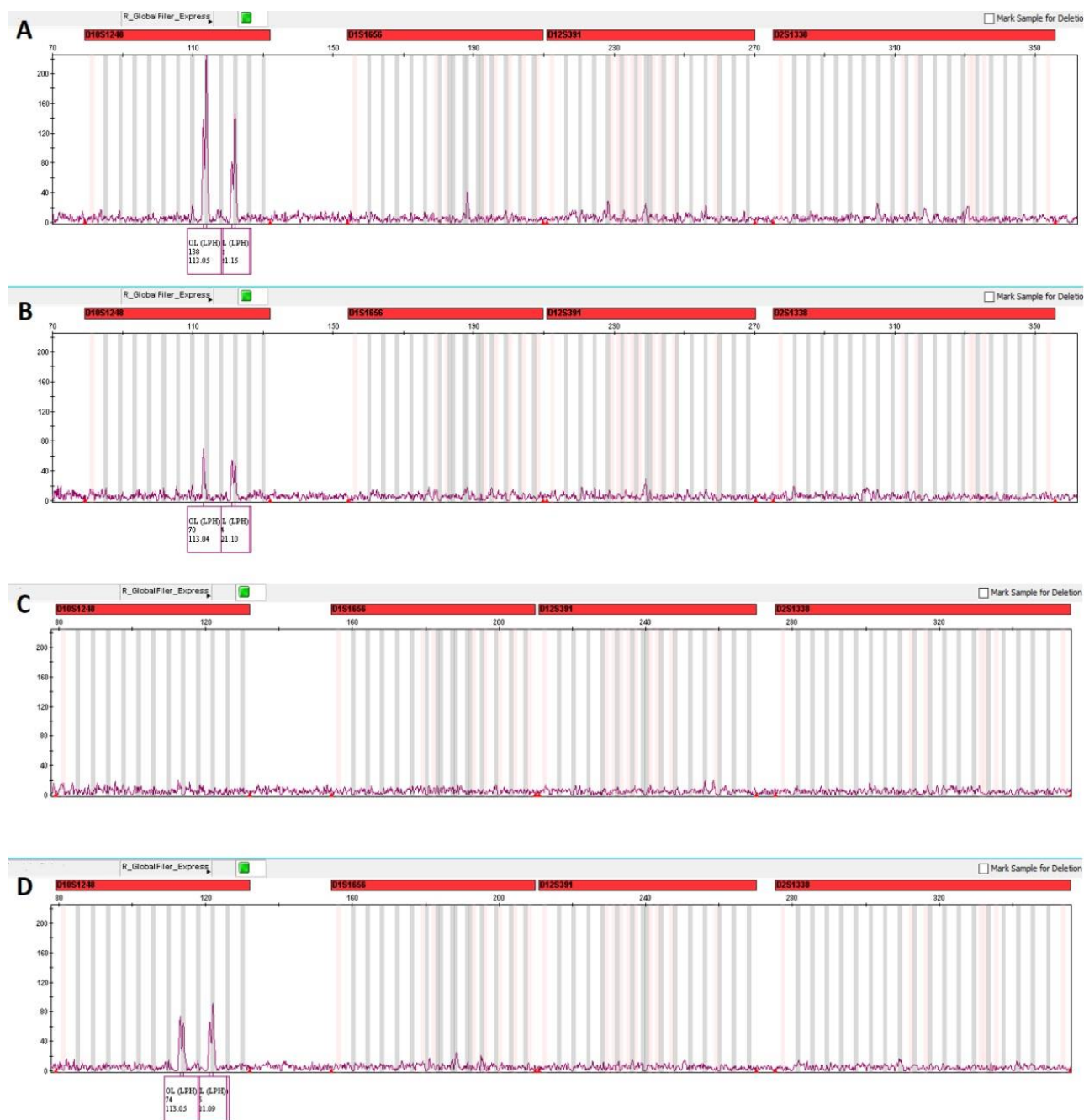
Initial Pre-treatment test

Initial testing of a lysis pre-treatment involved incubation of three different sizes of nail with a lysis solution of proteinase K and SDS. Both a neat and diluted lysate were subsequently amplified, as well as the direct amplification of the post-lysis nail.

Operator contamination was observed in the profile obtained from the 3 shavings diluted lysate and as a result the shared alleles were removed in calculations.

No allelic results were obtained with any of the neat lysates tested. Poor results were also obtained with direct amplification of the treated nail, with allelic data only seen in the 0.5mm sample by producing 7 out of 42 alleles (Table 5.3). Improvement in profiling outcomes was seen in profiles obtained from the diluted lysate, with improving completeness and average peak height. Using 3 shaving lysates produced full profile, using 1 shaving lysate produced 39 out of 42 alleles, and with 0.5 mm lysate produced 17 out of 42 alleles. Average peak height balance did also increase when increasing sample input with the diluted lysate, resulting in the 3 shavings being the highest profile with an average peak height of 755 ± 359. Additionally, the inhibition ratio also increased with the increases in sample input. Peak height balance was low in all sample inputs tested which, coupled with the low average peak height, was indicative of insufficient DNA input resulting in stochastic effects.

This experiment demonstrated that for all input sizes tested, the best profiling outcomes were obtained from amplification of the diluted lysates, which could be due to dilution of proteinase K and SDS as potential PCR inhibitors. Also, it demonstrated that the increase of toenail input into the lysis increased the completeness of profile and the average peak height, although peak heights obtained were still low with evidence of stochastic effects present. As such, it was decided to test an increase in amount of nail used and different lysate dilutions. Furthermore, the addition of DTT to reduce proteins and extract enough DNA from the toenail to increase the average peak height.

Table 5.3. Toenails – Profile attributes with a pre-treatment. Percentage of profile completeness, average peak height (RFU), peak height balance, and inhibition obtained from profiles when amplifying the treated toenail direct, the neat lysate, and the lysis mix from using the toenail size of; 0.5 mm, a shaving, and 3 shavings.

	% Profile completeness			Average peak height (RFU)			Average peak height balance			Average Inhibition ratio		
	0.5 mm	*1 Shaving*	*3 Shavings*	*0.5 mm*	*1 Shaving*	*3 Shavings*	*0.5 mm*	*1 Shaving*	*3 Shavings*	*0.5 mm*	*1 Shaving*	*3 Shavings*
Treated nail direct	17	0	0	117 ±66	0	0	NC	0	0	NC	0	0
Neat Lysate	0	0	0	0	0	0	0	0	0	0	0	0
Diluted Lysate	40	93	100	123 ±83	328 ±192	755 ±359	0.59 ±0.2	0.59 ±0.2	0.65 ±0.2	3.1 ±1.5	4.2 ±5.2	2.1 ±1.5

NC= Not Calculated

Modifications in the pre-treatment

Due to the insufficient peak heights seen in the initial test, it was decided to increase the amount of sample input into the lysis by testing 1 and 2 larger pieces of toenail, as well as the addition of DTT to the lysis solution in an attempt to release more DNA from the toenail. Also, a serial dilution was performed on the lysates to dilute out potential inhibitors to the point where inhibition had little to no impact on profiling outcomes.

DTT test with 1 piece of toenail

Consistent with the initial experiment, the neat lysate (dilution 0) did not produce any allelic results with DTT and without DTT (Figure 5.4 A). In contrast, all the dilutions produced allelic data with and without DTT, which were concordant with their known profile. As the dilution of the lysate increased, higher completeness of profile and lower inhibition was seen. Additionally, higher profile completeness and average peak height was seen in the lysates that contained DTT. Compared to the pre-treatment without DTT, an improvement was obtained with DTT, however, all average peak heights were < 500 RFU (Figure 5.4 B). Dilutions did not have an effect on the average peak height. For both with and without DTT no trend was seen between the average peak height balance and the dilutions, although peak height balance was consistently low. In general, higher inhibition levels were observed with the presence of DTT (Figure 5.4 D).

DTT test with 2 toenail pieces

The results from using 1 toenail demonstrated low levels of average peak height; therefore, the same experiment was repeated but with adding 2 pieces of toenail into the pre-treatment instead of 1. It was hypothesised that by adding the double of sample amount into the lysis mix, this may increase the average peak height. Results for this experiment are also graphed in Figure 5.4.

All profiles obtained from the lysates were concordant to their known profile. The neat lysate with DTT produced a partial profile (8 out of 42 alleles), being the only neat lysate from all experiments that produces any allelic results. For the dilution series, profile completeness improved as dilution increased for lysates both with and without DTT, with full profiles obtained from dilution 4 and dilution 5 onwards respectively. Without DTT, decrease in profile completeness began from dilution 7 onwards. Although,

in dilutions from 1 to 4 the profile completeness was higher with the lysis without DTT compared to with DTT.

Similar profile average peak height was observed between the lysate with DTT and the lysate without DTT (Figure 5.4 B). The lysate without DTT with dilution 1, produced the highest average peak height (1,573 RFU) of all lysates tested, although this did not coincide with a full profile being obtained. In both lysates, the profile average peak heights did not decrease linearly with increasing dilution.

The average peak height balance was calculated and graphed in Figure 5.4 C. Both with and without DTT produced low average peak height balance, but it was higher without DTT. However, when the 2 pieces of toenail were used without DTT, the peak height balance was markedly higher compared to when using 1 piece without DTT. However, as this used different samples, it cannot be presumed that the increase of sample amount increases the peak height balance.

Average inhibition ratios were calculated for all partial and complete profiles and graphed in Figure 5.4 D. Overall, the highest inhibition levels were observed in lower dilutions, particularly dilutions 1 and 2, regardless of the presence or absence of DTT in the lysis solution and decreased at higher dilutions. For the lysis solution without DTT, dilution 1 (that also produced the highest average peak height) produced the highest average inhibition ratio of 24.1. Observed inhibition decreased with increasing dilution, to ratios ranging between 1.3 and 5.5 for dilutions 3 to 10 (except dilution 7). A very similar trend was observed for the lysis solution containing DTT, with dilution 2 producing the highest average inhibition ratio of 31. Again, inhibition decreased with increasing dilution, with the lowest inhibition ratios of 2.1, 2.4 and 3.4 observed for dilutions 5, 6 and 8, respectively.
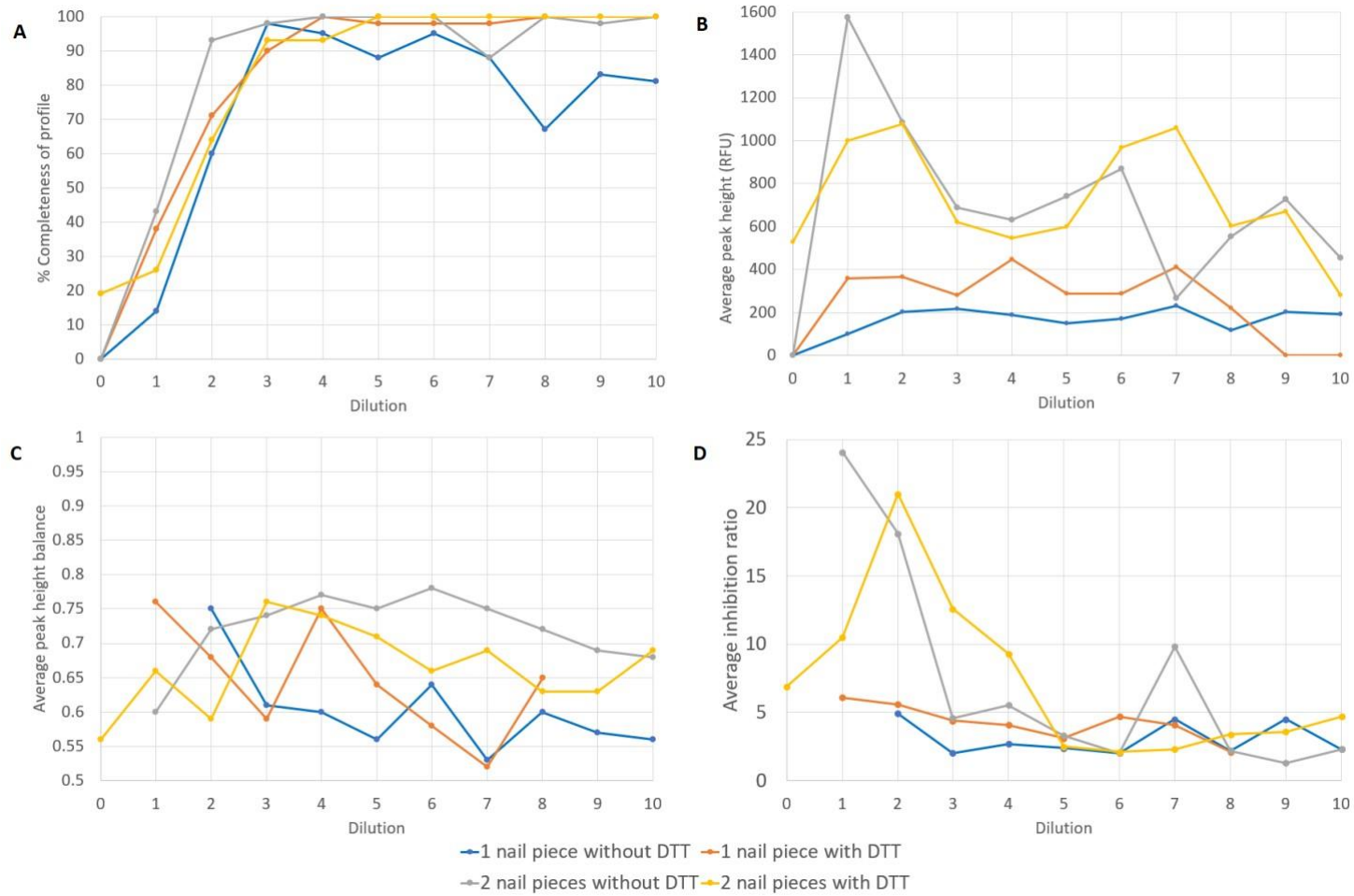
Figure 5.4. Toenails – Profile attributes for the DTT test with 1 and 2 pieces of nail. A) Percentage profile completeness; B) Average peak height; C) Average peak height balance; and D) Average inhibition ratio, obtained from amplification of neat and diluted lysates, with or without the addition of 10 µL of DTT using 1 or 2 pieces of toenail sample input.

Results showed that the completeness of profile improved when the dilution increased as there was less inhibition. The lysate with 2 pieces of toenail produced a higher percentage of profile completeness than the lysate with 1 toenail. Also, the average peak height and the average peak height balance were higher when using the 2 pieces of toenail than 1 piece of toenail. The addition of DTT did not show a big difference on the improvement of the profile quality.

Overall, it was demonstrated that amplification of diluted lysates containing DTT resulted in greater profile completeness than diluted lysates without DTT for both sample inputs of 1 or 2 toenail pieces, and that increasing dilution of the lysate coincided with a reduction in average inhibition ratios and an increase in profile completeness. When the amount of toenail in the lysate was increased from 1 to 2 toenail pieces, further improvement in profiling outcomes and, in particular, profile average peak heights, was seen regardless of the presence or absence of DTT in the lysis solution, indicating greater toenail input had a more significant effect on positive profiling outcomes than did DTT. However, as different samples were used with 1 and 2 toenail pieces, further assessment on the increase of sample amount into the lysis solution should be evaluated using the same sample. Across the dilution series, however, the highest average peak heights were still too low for the purposes of casework. Therefore, it was decided to test larger sample input amounts, using more pieces of toenail, as well as higher concentrations of DTT in order to determine if a higher amount can be added to increase average peak heights even further. As these results demonstrated overall that dilution 8 showed a combination of higher average peak heights, the greatest proportion of complete profiles and lowest average inhibition ratios with and without DTT, further testing was performed just with dilution 8.

Modification of DTT concentration and sample amount

This experiment consisted of using 2-6 pieces of toenail in the lysate and testing various concentrations of DTT (Figure 5.5). All combinations were performed for 2, 3 and 4 pieces. All conditions were not tested with 5 and 6 pieces due to insufficient sample availability. After the incubation, all lysates were diluted by adding 800 µL of nuclease-free water. Results are shown in Figure 5.5.

All profiles obtained were concordant to their known profile. The lysate containing 15 µL of DTT, showed complete profile with all pieces of toenail tested (2, 3, and 4 pieces). The lysates with 0 and

with 10 μL of DTT produced complete profiles with 2 and 4 pieces of toenail, while for 3 pieces of toenail a partial profile was produced. The lysate with 20 μL of DTT, produced partial profiles with all the pieces of toenail tested (2, 3, and 4 pieces). This demonstrated that the increase of sample into the lysis solution, has a positive impact by increasing profile completeness, average peak height, and average peak height balance. Also, the addition of 10 μL of DTT produced the same results as without DTT, while the increase of DTT to 20 μL inhibited the PCR resulting in a decrease of profile completeness, average peak height, and average peak height balance.

Generally, the profile average peak heights increased as increasing amount of toenail input used. In the presence of 10 and 15 μL of DTT, inputs of 2 and 3 toenail pieces generated profiles with low average peak heights, while the greater input of 4 pieces showed marked improvement in profile average peak heights. However, no further improvement was seen with additional input of 5 toenail pieces. In the absence of DTT, greater toenail inputs generated increasingly higher profile average peak heights, and indeed of all the lysates tested, the highest average peak height was obtained from the 6 pieces without DTT (3,677 RFU).

The average peak height balance was higher when more pieces of toenail were used, regardless of DTT inputs, except for 20 μL DTT, for which the average peak height balances were slightly lower. With inputs of 4, 5, and 6 pieces of toenail, with a successful profile average peak height balance ranged between 0.8 to 0.87, which has been the highest obtained in all toenail experiments from this project.

Average inhibition ratios were generally lower when more pieces of toenail were used. Not all of the nail inputs were tested with all volumes of DTT, so cannot be reliably compared; however, inhibition levels were higher when using 20 μL of DTT.

In conclusion, the increase of amount of toenail added into the lysis solution, as opposed to the addition of DTT, provided the greatest overall improvement in all measures of profile quality that were assessed. The lysate without DTT with an input of 6 pieces of toenail produced a complete profile with a suitably high average peak height of 3,677 RFU, a high average peak height balance of 0.87, and a low inhibition ratio being the lowest from all the pieces tested. This was the only method tested that produced results profiles of acceptable quality that would be suitable in identification casework analysis. However, this method would need to be further tested with other subset of samples to determine if results are reproducible.

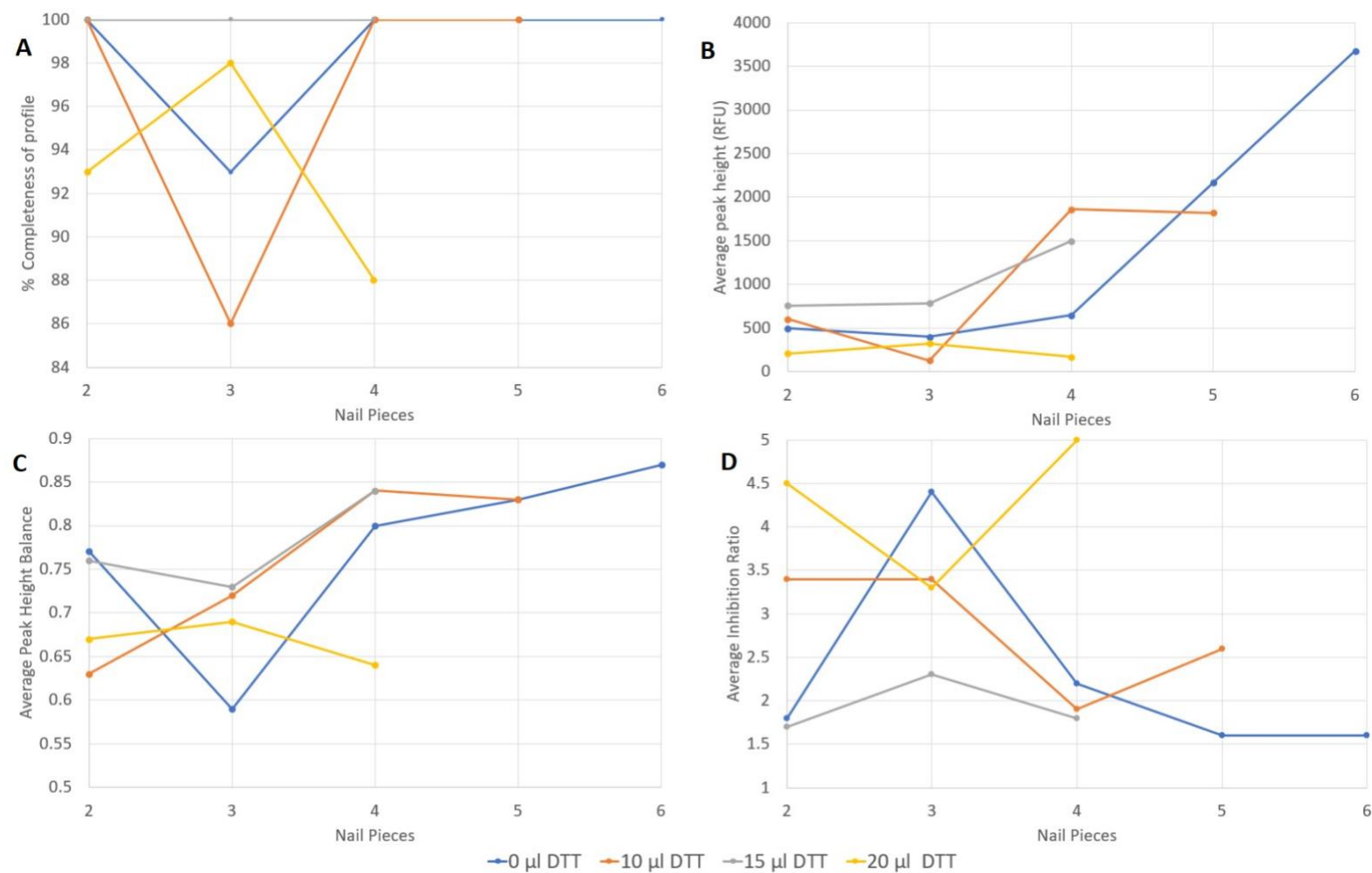**Figure 5.5. Toenails – Profile attributes for the modification of DTT concentration and sample amount**. A) Percentage profile completeness; B) Average peak height; C) Average peak height balance; and D) Average inhibition ratio, obtained from amplification of lysates with or without the addition of 10, 15 or 20 μL of DTT, containing 2, 3, 4, 5 or 6 pieces of toenail, and diluted with 800 μL of nuclease-free water.

5.4   Discussion

At the MBL, toenails are the second most common PM sample type received for DNA identification of deceased individuals and this is the preferred PM sample type for identification in cases when the body is decomposed (93). Hence, development of direct PCR method using toenails was initiated, with the intention for its eventual use during casework at the MBL, as an effective direct PCR method would save on time and costs during routine identification casework. For the method development, it would be ideal to use PM toenails as these are used in casework; however, such samples were in limited supply.

Therefore, the strategy for this research project was to first develop a method with live donor samples. Subsequent studies would then test the method with PM samples once a promising method was identified. Using AM toenail samples, the method development strategy for this research project started with direct sample input without any pre-amplification treatment(s), and then proceeded with developing a pre-amplification protocol treatment as needed.

All the experiments were performed using the GlobalFiler Express kit and amplifying the sample at 29 PCR cycles. As there are no parameters or recommendations from the manufacturer for this sample type, a high cycle number (29 cycles) was chosen to have a higher chance of observing allelic data and then to modify the cycle number if necessary. However, no modifications were required to the PCR cycle number.

This discussion will be divided into two sections; (i) direct amplification – to discuss the experiments where no pre-treatment was included, and (ii) pre-treatment – to discuss the development of this treatment.

5.4.1   Direct amplification

Evidence of direct PCR with toenails is limited in the literature. However, Ottens *et al.* produced DNA profiles from fingernails by performing direct PCR (70). Although a different amplification kit to the one used in this project was utilised, this demonstrated that it is possible to obtain profiles from toenail by performing direct PCR (70). Hence, it was decided to explore the performance of direct PCR without any lysis pre-amplification treatment.

As there is no GlobalFiler Express kit manufacturer's recommended protocol for the direct amplification of toenail samples, and no evidence in the literature of this method using this amplification kit with this sample type, it was unsure the amount of nail that should be input

into the PCR. Firstly, it was decided to use a similar amount to the 1.2 mm punch as this is the size recommended to use for buccal and bloodstains by the GlobalFiler Express kit manufacturer (41). However, a punching tool was not used since it was unclear if the Harris™ Micro punch would be able to cut the toenail as it is designed for paper. Instead, the punch size was cut with scissors and it is unclear if the intended size was achieved.

Poor profiling outcomes were obtained with all of the sample input amounts tested with direct amplification. The smallest amount of sample used showed evidence of stochastic effects indicating insufficient DNA input into the reaction. However, higher sample amounts demonstrated ski slopes and incomplete adenylation consistent with inhibition. Ottens *et al.* reported minus A peaks with direct PCR of fingernail clippings at 29 cycles, they further recommended to dilute the PCR product (70), which would be unsuitable for these profiles as sufficient peak heights were not observed. It is possible that the inhibition in higher sample amounts is due to elements from within the nail being released into the PCR reaction. A likely candidate is calcium, which is known to be contained within the toenail structure (97). Calcium ion can inhibit the PCR by binding into the Taq polymerase by interfering with the magnesium (79, 80).

Nevertheless, the ski slopes and incomplete adenylation observed in the toenail profiles, could also be explained by the DNA not releasing from the cell or toenail structure due to lacking the extraction step. This would not be surprising as DNA in the nail is structured in keratin tissues (69) which might need the lysis of the cell membrane to release the DNA from the cell and allow a proper amplification. This was not an issue in the other samples tested in this project: PM bloodstains and AM buccal FTA samples (see chapters 3 and 4), because these are in FTA cards which lyses the cell before amplification.

Results from this project did not reflect Ottens *et al.* outcomes, which produced complete profiles from 17 out of 40 samples tested (70). The method used in this project involved removal of cells adhering to the nail prior to use. For PM samples, this serves to remove tissue adhering to the nail which is likely highly decomposed. Ottens *et al.* did perform this type of cleaning step prior to sample processing (70). By leaving out the cleaning step, it is possible that profiles obtained were from DNA on the surface or beneath the nail (98), rather than from inside the nail structure. However, this method adds the disadvantage of obtaining contamination which was the case in 22.5% of the profiles (70). This study performed a

cleaning step which consisted of scraping the toenail with a scalpel to remove the surface of the nail, which was aimed at reducing contamination risks, but incomplete profiles were obtained. Additionally, a method that has the risk of obtaining a mixed profile is not acceptable for the casework purposes at the MBL, as just single source DNA profiles are optimal for analysis in this laboratory.

In conclusion, this study was not successful to produce complete profiles from performing direct PCR with toenails. Given that is likely that the reason for these results is PCR inhibitors or the DNA not lysing from the cell, a lysis pre-treatment protocol was further developed.

5.4.2 Pre-treatment protocol

The addition of proteinase K and SDS was explored aiming to lyse the cell membrane in order to release the DNA. No results were seen with any of the neat lysates tested, it is likely that extensive inhibition was responsible for this failure. Diluted lysates produced allelic data. Inhibition causing amplification failure is further supported by improvement in profiling outcomes with the diluted lysates.

To determine an optimal dilution for this lysis protocol, lysates were amplified with varying levels of dilutions. At higher dilutions, the DNA profile improved by showing allelic results, however, all dilutions profiles exhibited signs of insufficient DNA input into the PCR. Increasing the amount of sample added into the PCR to 6 pieces of toenail, demonstrated an improvement by resulting in acceptable quality profiles. The addition of DTT added did not appear to have any net benefit to profiling outcomes.

5.4.3 Conclusion and Further study recommendations

In conclusion, it was demonstrated that toenails do not produce a nDNA profile without a lysis step and a dilution. The 6 pieces lysate showed optimal results, which was achieved by performing an incubation with Proteinase K and SDS, as well as diluting the lysate before amplification. This illustrates that the toenail needs a lysis step to release the DNA from the cell. The dilution step allowed to obtain results by diluting the PCR inhibitors. Inhibitors might be from the toenail, as well as from the reagents used. The method of 6 pieces lysates demonstrated that complete profiles with an average peak height > 1,200 RFU, can be

obtained. However, this was only tested with one sample which makes it not possible to confirm that the method is success. It is recommended to test this method with more samples to confirm that results can be reproducible. Also, a limitation of this method was that the 6 pieces were not weighted so this amount would not be replicable. Additionally, if the 6 pieces demonstrates to be suitable when tested with more samples, this might allow to do further testing in order to reduce the incubation time to speed the process.

6. Chapter 6: General discussion

The following discussion centres on the research findings described in Chapters 2 to 5, and how these may impact the workflow at the Molecular Biology Laboratory (MBL), Victorian Institute of Forensic Medicine.

The MBL currently performs DNA analysis with a standard method consisting of a workflow with the following steps: DNA extraction, quantification, amplification, capillary electrophoresis and analysis of profiles. The MBL aims to replace the standard method with a direct PCR method to reduce working time by having a shorter process and decreasing the annual costs by not needing the reagents used in the extraction and quantification steps. Hence, a method development described in this thesis was conducted to perform direct PCR using PM bloodstains, AM buccal FTA samples, and toenails, for identification casework at the MBL.

The main objective of this method development project was to develop a method for direct PCR of the most common sample types received for routine identification of deceased individuals, by producing nDNA profiles that can be analysed and reported during such casework at the MBL. The assessment criteria to determine an analysable profile consisted of a complete profile with minimal evidence of stochastic or overamplification effects. The method was developed for each sample type separately; however, specific aims were to determine if it was possible to utilize the same amplification kit for all sample types and to determine whether the same PCR cycle number could be used for PM bloodstains and AM buccal FTA samples. Minimising the number of amplification methods would be beneficial as this would allow for more streamlined processing of samples received for identification.

Prior to the commencement of this research project, a preliminary study conducted at the MBL using 1.2 mm punch of AM buccal FTA and PM bloodstains with the GlobalFiler Express kit as per recommended by the manufacturer (41), demonstrated high inhibition in profiles generated from the PM bloodstains samples ((72), unpublished data). Based on the inhibition levels observed for these PM bloodstain samples, it was decided to test the GlobalFiler amplification kit as it has a larger reaction volume (73, 75) which may dilute out the inhibitors (99, 100). The first step of the method development was to compare the performance of direct amplification protocols for AM buccal and PM bloodstain samples with the GlobalFiler Express kit and the GlobalFiler Amplification kit (Chapter 2). For both sample types, the GlobalFiler

Express kit produced higher quality profiles exhibiting less impacts of inhibition and reduces instances of incomplete adenylation which results in minus A peaks. As such, it was more likely that the GlobalFiler Express kit would produce more consistent high-quality profiles than the GlobalFiler kit. In identification casework, the potential lack of consistency of the GlobalFiler kit may result in these profiles not being able to be analysed which would probably result in reamplifying the sample or reprocessing with a standard profiling workflow. This would increase the cost and the time of reporting the case. Therefore, it was decided to use the GlobalFiler Express kit for the rest of the project.

From the preliminary study with PM bloodstains, it was unclear if direct PCR would consistently produce profiles of sufficient quality to be an effective method due to the high levels of inhibition observed with the manufacturer recommended protocol ((41, 72), unpublished data). Therefore, the input amount of sample was reduced to a 0.5 mm punch with the theory that the smaller sample size may reduce the number of inhibitors added into the PCR reaction (see Chapter 3). The 0.5 mm punch showed a decrease in inhibition levels compared to the 1.2 mm punch (72) and also improved the profile peak heights. Some inhibition was still seen in profiles when using the 0.5 mm punch, however, this was accepted as it was lower than with the 1.2 mm punch. Also, it was expected to have inhibition present specially with a direct PCR method as the sample is not purified before the amplification. Nonetheless, part of this method development was to minimise the inhibition level present to reduce the likelihood of allele drop out, peak height imbalance, or minus A peaks within the profiles obtained from this method. Hence, the 0.5 mm punch was the input amount chosen for subsequent method development experiments.

The determination of the most appropriate PCR cycle number for PM bloodstains, resulted in 25 cycles being suitable (see Chapter 3). Given one of the aims of this project was to attempt to establish the same cycling protocol for AM buccal and PM bloodstains, a 25-cycle protocol was tested with AM buccal samples. The increase of AM buccal sample input into the PCR did not result in sufficient peak heights in the profiles, indicating that this cycle number was insufficient for this sample type. Therefore, the focus went back to PM bloodstains to determine if a higher cycle numbers (27, 28, and 29) could be utilised, with the purpose of then testing the determined cycle in AM buccal samples. As it was expected that PM bloodstains would produce profiles exhibiting overamplification effects at these higher cycle numbers, the PCR product was diluted before undertaking capillary electrophoresis. The

method of 27 cycles with a 1 in 8 dilution of the PCR product prior to capillary electrophoresis, resulted in acceptable completeness of profile and average peak heights, however the level of incomplete adenylation in the profiles requires further exploration to assess the impact on profile interpretation.

Nonetheless, if the method determined at 27 cycles diluted with PCR product dilution results in being acceptable based on the minus A peaks, this would probably be positive for the method overall if the method for AM buccal samples could also be acceptable at this cycle number. The dilution step in the PM bloodstain method would not be time demanding and it could allow the PM bloodstains and AM buccal samples to be amplified together which, according to internal analyses, would save annual reagent costs by more than half compared to a standard profiling protocol (Zoe Bowman, personal communication). The use of a dilution step prior capillary electrophoresis would also have the advantage that if a profile is showing incomplete profiles or over amplification effects, instead of reamplifying the samples, the scientist would be able to use the PCR product and then alter the dilution if necessary, depending on the results. By avoiding reamplification this could save time and costs.

For AM buccal samples, 27 cycles were tested to match the PM bloodstain PCR cycle number. Different numbers of AM buccal sample punches were tested with a pre-incubation step at room temperature by amplifying the eluate at 27 cycles, but this resulted in partial profiles and low peak heights. The five punches eluate with incubation at 99°C, was the only method that showed marked improvement by producing complete profiles with an average peak height > 1,200 RFU for all samples tested. These results show promise; however, further testing is recommended with additional samples to determine if results are reproducible.

It was aimed to determine the same cycle number for AM buccal and PM bloodstain samples and therefore both sample types were tested at 27 cycles. Amplification of these samples with different cycling conditions would require the use of multiple positive and negative control reactions for processing a single case. Results from this study indicated that in order to have the same cycle number for both sample types, a pre-PCR incubation step for AM buccal samples and a post-PCR dilution step for PM bloodstains would need to be performed. However, these steps would not be time consuming and would still allow cost savings. At present, it is unclear of the full impact these method changes may have on first-pass profiling success rates and further testing and analysis is required to establish this. Consequently, it is

also uncertain if the cost benefits of processing these samples together may be negated by a higher amount of rework to produce profiles. Such rework would not be saving costs and would be increasing the time of process. If that is the case, it is recommended the most suitable cycle number for each sample type be implemented, which was determined to be 25 cycles for PM bloodstains in this study.

Performing direct PCR using PM bloodstains and AM buccal samples at the MBL for casework purposes would be an improvement over the standard method even if a different cycle number between sample types is used. This improvement would be on saving costs and time by removing the extraction and the quantification steps from the process.

For toenails samples, the main aim was to determine if DNA profiles can be produced from a direct PCR method using toenails, and if not, to determine if pre-treatment protocol prior to amplification could produce acceptable profiles. No complete profiles were obtained by directly amplifying the toenails, which was likely due to insufficient cell lysis and the nail inhibiting the PCR. As these partial profiles would not produce sufficient information to facilitate kinship matching, a pre-treatment protocol was then explored. A pre-amplification lysis step using proteinase K and SDS demonstrated that better profiling outcomes could be obtained than the direct protocol, though dilution of the lysate prior to amplification was required due to high inhibition levels. However, it was demonstrated that the increase of sample input into the lysis solution, greatly improved the results. When amplifying the diluted lysate from 6 pieces of AM nail a complete profile with an average peak height between 1,200 and 14,000 RFU was produced, showed promise as a method to use on PM toenails. Further testing on this method is required as it was only tested with one sample. Although, if this method is reproducible, it would mean that a direct PCR method including a pre-treatment could be performed for identification at the MBL.

Even though this method includes a pre-treatment, this would still be an improvement over the standard method, as in saving cost and time by removing the extraction and the quantification steps. Additionally, this could also benefit the forensic field by producing, in a much faster way, DNA profiles for identification from decomposed bodies. Human remains can be identified by utilising nail, as it has been demonstrated that environmental conditions would not affect the quality of the DNA for at least one month of exposure (96). For example, in a DVI situation, using nail clippings might facilitate the process and the sample collection

compared to other samples collected in mass disasters such as bones that are collected by medical practitioners, because toenail clippings can be collected by an individual with simple training being an easy and timeless collection process. Bones and teeth are commonly used in DVI situations (5) but these requires a long and complicated method in order to extract the DNA (101), which could be improved by using toenails as a sample type in DVI. By performing direct PCR in a DVI situation, which would usually be a large number of samples, would allow to speed the process of reporting profiles, resulting in the families getting a quicker answer in such a stressful time.

Also, as mentioned before, it would be ideal that the same PCR cycle number is used between sample types, however, this project just explored toenails at 29 cycles and further testing would be required to determine if a suitable method could be developed using 27 PCR cycles. In DVI situations, as well as any identification casework, the process would be shorter, and costs would be reduced if all sample types are amplified together. Nonetheless, performing a direct PCR over a standard method is an improvement which reduce the time and cost even if the cycles are different between sample types.

Conclusion

Firstly, several planned experiments aimed to further refine the direct PCR of sample types tested – in particular toenails – were unable to be conducted due to COVID-19 restrictions. The restrictions severely limited the access to the MBL (as casework was prioritised over research activities and limits on staff/student attending were implemented) since March 2020.

Secondly, as an overall project it can be concluded that in order to consider a method for validation, further analysis and testing is required in all sample types. However, it was demonstrated that when performing direct PCR with PM bloodstains and AM buccal FTA samples, the GlobalFiler Express kit produced DNA profiles with less inhibition and incomplete adenylation than the GlobalFiler kit. Additionally, it was established that a reduction in the manufacturer recommended sample input amount in amplification of PM bloodstains with the GlobalFiler Express kit reduced the inhibition levels. Furthermore, it was demonstrated that performing direct PCR with toenails obtained partial profiles which is not a method suitable for the MBL. Also, by applying a pre-treatment protocol to toenails, successful results

were obtained meeting the requirements to be used for casework at the MBL, but just one sample was used, hence further testing is needed to consider this method for casework.

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