DUAL DETECTION OF LATENT FINGERMARKS AND DNA

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Degree of Doctor of Philosophy (Science)

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CERTIFICATE OF AUTHORSHIP AND ORIGINALITY

I certify that the work in this thesis has not previously been submitted for a degree nor has it been submitted as part of requirements for a degree except as fully acknowledged within the text.

I also certify that the thesis has been written by me. Any help that I have received in my research work and the preparation of the thesis itself has been acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

Alicia Khuu-Maitre

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Dedicated to

My Grandparents and My Parents

~ I owe my every success to you ~

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RESEARCH COMMUNICATION

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ABBREVIATIONS

% ..... Percent
[ ] ..... Concentration of
×g ..... Times gravity
≤ ..... Less than or equal to
≥ ..... Greater than or equal to
°C ..... Degrees Celsius
µ ..... Micro
µM ..... Micromole
φ29 ..... phi29
$\bar{x}$ ..... Mean
ACE-V ..... Analysis, Comparison, Evaluation-Verification
ACS ..... American Chemical Society
AG ..... Analysis grade
Ag^{+} ..... Silver ion
ANOVA ..... Analysis of Variance
AR ..... Analytical Reagent
AUD ..... Australian Dollar
bp ..... Base pair
BSC ..... Biological Stain Commission
BY40 ..... Basic Yellow 40
CA ..... Cyanoacrylate
CAST ..... Centre for Applied Science and Technology
CB ..... Cyanobloom
cm ..... Centimetre
COOH ..... Carboxylic acid
DAB ..... Diaminobenzidine
DFO 1,8-Diazafluoren-9-one
DMAB ..... para-Dimethylaminobenzaldehyde
DMAB-IND ..... para-Dimethylaminobenzaldehyde-Indanedione

DNA ..... Deoxyribonucleic acid
dATP ..... Deoxyadenosine triphosphate
dCTP ..... Deoxycytidine triphosphate
dGTP ..... Deoxyguanosine triphosphate
DMSO ..... Dimethyl sulfoxide
dNTP ..... Deoxynucleoside triphosphate
dTTP ..... Deoxythymidine triphosphate
dsDNA ..... Double stranded deoxyribonucleic acid
EDTA ..... Ethylenediaminetetraacetic acid
Em ..... Emission
Ex ..... Excitation
g ..... Grams
HFC ..... Hydrofluorocarbon
IED ..... Improvised explosive device
IFRG ..... International Fingerprint Research Group
ILS ..... Internal lane standard
IND ..... Indanedione
IND-Zn ..... Indanedione-Zinc
kb ..... kilobase
L ..... Litre
LC ..... Lumikit^{TM}
LSD ..... Least significant difference
MDA ..... Multiple displacement amplification
mm ..... Millimetre
mM ..... Millimolar
mtDNA ..... Mitochondrial deoxyribonucleic acid
NCFS ..... National Centre for Forensic Science
NIN ..... Ninhydrin
n ..... Number of samples
ng ..... Nanogram
nm ..... Nanometre
PBS ..... Phosphate buffered saline
PC ..... PolyCyano UV
PCR ..... Polymerase chain reaction
PD ..... Physical developer

PEG ..... Polyethylene glycol
pg ..... Picogram
pL ..... Picolitre
q.s. ..... *quantum satis* (to a final measurement of)
R6G ..... Rhodamine 6G
RCA ..... Rolling circle amplification
RFLP ..... Restriction fragment length polymorphism
RFU ..... Relative fluorescent units
RH ..... Relative humidity
RUVIS ..... Reflective ultra violet imaging system
SD ..... Standard deviation
SDS ..... Sodium dodecyl sulfate
SE ..... Standard error
SPR ..... Small particle reagent
SS ..... Stock Solution
ssDNA ..... Single stranded deoxyribonucleic acid
STR ..... Short tandem repeat
TBE ..... Tris-borate-EDTA
TE ..... Tris-EDTA
TEG ..... Triethylene glycol
Tris ..... Trisaminomethane
TX-100 ..... Triton X-100
U ..... Units
UNIL ..... University of Lausanne
UTS ..... University of Technology Sydney
UV ..... Ultraviolet
v/v ..... Volume per volume
WGA ..... Whole-genome amplification
WS ..... Working solution
w/v ..... Weight per volume
$ZnCl_2$ ..... Zinc chloride

ABSTRACT

In forensic investigations, fingermarks and deoxyribonucleic acid (DNA) are often considered as valuable traces as they could allow for the identification of the source of the trace or act as a vector of information, providing a link between a touched object and an individual. These traces are often latent, which means they need to be detected before recovery. While there are many well established methods for the detection of fingermarks across a variety surfaces, no routine method is in place for the detection of DNA (that is not clearly visible from the biological material). The general consensus regarding the profiling of touch DNA is that detection and localisation of the specimen needs to occur prior to analysis to improve DNA profiling outcomes. However, emerging work in the area of DNA detection in casework focuses on touch DNA on non-porous surfaces and often disregards the impact of the detection agent on the fingermark component of the trace. Currently, no technique for DNA detection has been reported that is suitable for operational use let alone a dual detection method for latent fingermarks and DNA.

The aim of this project was to address this gap in knowledge and investigate possible modes of detecting both latent fingermarks and DNA for non-porous and porous surface types. The use of biological and fingermark detection methods was explored to determine the effect of the respective method on the alternate trace to find a suitable technique to allow for detection of both traces. As with the treatment of fingermarks alone, it was found that a separate application technique was required for non-porous and porous surfaces types. In order to achieve successful dual detection, it was also determined that the fingermark needed to be apparent first following the application of the technique as

the size of this trace was more easily visualised. This would therefore allow for a more targeted area to localise touch DNA.

For non-porous surfaces, 14-day old fingermarks could be enhanced following the use of one-step luminescent cyanoacrylates (CAs; PolyCyano UV and Lumikit™). Additionally, the application of these treatments also allowed for cells originating from a spike solution to be easily visualised using an alternative light source (ALS) and, particularly with PolyCyano UV, cells originating from the fingermark residue could be detected microscopically and differentiated from the fingermark. The impact of the application of one-step luminescent CAs on DNA was also assessed and it was found that DNA was recoverable and quantifiable following treatments as were controls (conventional CA treatments and no treatment). In terms of profiling, it was found that the quality of PolyCyano UV-treated DNA tended to be poorer than that of Lumikit™-treated and control DNA. However, this study also found that DNA was more significantly impacted by time than the respective CA treatment, with larger fragments of the 14 day old treated samples showing significant degradation than compared to fresh DNA, regardless of the treatment.

For porous surfaces, a combination of the indanedione-zinc (IND-Zn) reagent with *para*-dimethylaminobenzaldehyde (DMAB) was found to allow for the visualisation of fingermarks both faintly, under ambient laboratory lighting, and strongly, using an ALS. In this combined reagent (DMAB-IND), the concentrations that presented the best compromise between the visualisation of touch DNA and fingermarks was found to be 0.4% DMAB : 0.06% IND. The DMAB-IND reagent was compared to IND-Zn and it was found that while the intensity of DMAB-IND-treated fingermarks was less than that of IND-Zn-treated, cells were able to be visualised using fluorescent microscopy following treatment with the former, while not following treatment with the latter. When

DNA profiling of the treated marks was compared, it was found that DMAB-IND treatment achieved significantly better quality profiles with more alleles being called. This is attributed to the targeted recovery of cells, while cells in IND-treated samples could not be visualised, and therefore achieved profiles similar to that of no-treatment controls. The stability of the DNA following treatment was also assessed and it was found that the quality of DNA decreased with time following treatment.

Overall, this project investigated two potential methods that enhance latent fingermarks and enable cells to be localised thus improving the quality of DNA profiling. This allows for either trace to be exploited should it be required. While dual detection is possible, one of the parameters that has significant impacts on the analysis of touch DNA, in particular, is time. Increased time between deposition and detection, and detection and analysis was found to be detrimental to the trace. Future work could be conducted to decrease the sensitivity of the technique to time and thus assist in preserving the DNA and allow for more time between detection of the trace and analysis of DNA.

Chapter 1. INTRODUCTION

1.1 The Traces

Fingerprints have long represented the leading approach for the identification of individuals [1]. Following contact of the fingerprint skin with a surface, a fingermark is typically left on the surface as a representation of the fingerprint. As the papillary ridge pattern that makes up the fingerprint is extremely polymorphic in nature, the analysis of fingermarks can allow for the subsequent identification of the source. During the contact event, deoxyribose nucleic acid (DNA) can also be transferred from the fingerprint skin to the surface. The transferred trace is known as touch DNA [2]. Due to allelic variations, DNA in itself exhibits considerable polymorphism between individuals, which can also be exploited in order to identify the source except among identical multiple birth siblings whose DNA is inherently identical [3].

While the detection and recognition of traces is fundamental to forensic science, it often one of the most challenging steps. Both latent fingermarks and touch DNA may exist on a substrate following contact, but finding and determining the significance of these traces is integral to their exploitation. However, there are no reported techniques used to simultaneously detect fingermarks and DNA. Currently, fingermarks are detected using the appropriate technique and DNA analysis is conducted on the mark to obtain further information when required. This presents a potential loss of information. Alternatively, DNA processing may occur first, with an area that is likely to have been touched swabbed

for the trace. However, in this case, there is no knowledge of whether the touch DNA specimen existed in the first instance, and further, when an uninformative profile is obtained, any potential fingermarks will have been destroyed during the DNA collection process. The collection and subsequent impact of the collection of these traces will be discussed further in this chapter.

1.2 Fingerprints and Fingermarks

Fingermarks are depositions formed from the papillary ridge pattern on the epidermal skin of an individual's fingertips, also known as their fingerprint skin. The distinction here is that a fingermark is an uncontrolled reproduction of the friction ridge skin on one's fingertips; a fingerprint can be construed as a controlled reproduction of the friction ridge pattern on the fingertip, or the friction ridge pattern itself [4]. Ridge pattern formations are also present on the palms of the hands as well as the toes and soles of the feet. While these complex ridge formations are believed to assist in the improvement of grip and aid in the perception of touch [1, 5], they have also become a reliable means of identification having been used in a forensic context for well over a century. Due to the polymorphic nature of these patterns, among fingers of each hand and between individuals, they have high discriminatory power that results in high evidentiary value of the trace.

The custom of using fingerprints for identification purposes began centuries before the specifics of friction ridges were intensely studied. The earliest instance of this is believed to have occurred in China with the Qin Dynasty (221-206 B.C.), where authors of documents would impress their fingerprint into clay seals as a means of authenticating the document [6]. This practice continued through to the Tang Dynasty (A.D. 617-907)

in China, while other Asian countries also used friction ridge skin to adopt works before their significance was recognised in the Western world [6-8].

In the late 19th century, Alphonse Bertillon introduced the use of body measurements for forensic differentiation of individuals. Bertillon's system was adopted in most developed countries. However, due to inadequacies in the anthropometric method, where inconsistencies in the measurement of an individual frequently occurred, and the numerous misidentifications of individuals with similar body measurements, a more reliable identifying means was required. The significance of fingerprints was identified through a series of independent works by Sir William Herschel and Dr Henry Faulds and expanded on by Sir Francis Galton, who demonstrated uniqueness and persistence [8, 9]. Based on this work, Juan Vucetich, an Argentine police official, integrated fingerprinting alongside the Bertillon system. Following the successful use of fingerprint evidence in the case against Francisca Rojas, Argentina ceased the use of the Bertillon system in favour of fingerprint classification in the early 1890's [8, 9]. Fingerprint classification was soon adopted by police forces worldwide, becoming the predominant method of identification [9].

Friction ridge patterns that form the fingerprint skin are persistent and remain unchanged from the time of birth until death, growing with an individual as they age [10]. The pattern originates from the basal layer and hence the fingerprint surface is restored following an injury to the epidermis. In the case of injuries or skin conditions that occur at the basal layer, permanent disfigurement to the pattern results [1, 11, 12]. The extreme variability of a pattern comes from the development of minutiae rather than the general pattern and ridge count. Minutiae have long been believed to form as a result of various biochemical and physical processes *in utero* that affect the cellular environment and impact cell development and proliferation. However there is evidence to suggest there is some

hereditability in the total minutia count [13] and that Merkel cells stimulate the formation of the ridge pattern [14], suggesting some biological influence to the establishment of minutiae. While modelling of ridge formation has been performed to support theories about ridge development [14, 15], it is extremely difficult to replicate the ubiquitous factors that govern ridge development from individual to individual or digit to digit, and hence influence the development of minutiae, making friction ridges patterns highly polymorphic among individuals [1, 11, 16]. Typically, minutiae present as bifurcations, where a ridges splits into two; ridge endings, where a ridge terminates; and enclosures, where a ridge separates and recombines [1, 12, 17].

The friction ridge patterns may be transferred to other surfaces to give stamp-like impressions of the region of contact called fingermarks. These marks are either visible without enhancement, or latent and requiring of enhancement to differentiate them from the surface of deposition. Fingermarks are composed of bodily secretions, cellular components and external contaminants [1]. The constituents of the fingermark residue play a significant role in its capacity to be detected and enhanced [18]. Friction ridges have sweat ducts which run from the eccrine sweat gland, located in the dermal layer to the surface of the ridge. These sweat ducts create pores on the epidermal surface of the friction ridges which allow perspiration to be expelled onto friction ridges [1]. This secretion results in the eccrine fraction of the fingermark residue and is largely made up of water soluble components such as salts, metal ions, amino acids, sugars and proteins [1]. Friction ridges can also accumulate sebaceous and apocrine secretions through contact with other regions of the body, as well as extraneous contaminants through contact with the external environment. Sebaceous secretions come from glands located all over the body except on the friction ridge skin, and result in the lipid soluble portion of the fingermark [1]. Apocrine secretions originate from the armpit and groin areas, and

primarily contribute water soluble components to the fingermark residue [1]. The bodily secretions and contaminants on the friction ridges allow the ridge pattern to be deposited onto surfaces of contact in a stamp-like manner, leaving behind an impression of the friction ridge pattern called the fingermark.

1.3 DNA and Touch DNA

Within the fingermark deposit, cells and cell-free DNA can also be found. This allows for another form of identification to be performed. The human body consists of trillions of cells each containing the genetic information of an individual in the form of DNA. DNA is found in two forms within the cell: nuclear DNA (nDNA), which is found within the nucleus; and mitochondrial DNA (mtDNA) which exists in the mitochondria. Typically nDNA is used in DNA typing, as it holds greater discriminating power than mtDNA which cannot differentiate between persons from the same maternal line, although mtDNA can be exploited where genomic DNA is not available or severely degraded [19].

nDNA is inherited from the biological parents of an individual. This DNA is tightly packed in chromosomes, and through a series of recombination events during cell division, the DNA of the parents is combined and mixed resulting in a sequence of chemical bases or nucleotides (adenine, guanine, cytosine and thymine) from which each zygote (fertilized egg) is formed. The result is a genotypically and phenotypically unique individual. While the bulk of the 3 billion bases making up the genetic code is identical among individuals, the polymorphisms are found in small regions of vast variation throughout the genome. The majority of these varying sequences occur in the non-coding

region of the DNA, that is, outside of areas that code for proteins. Hence, each individual's genetic code is different, with the exception those resulting from the same zygote, that is, identical twins or other identical multiplets [20, 21].

Each cell in the body contains an exact copy of an individual's DNA [22]. These cells and DNA can be transferred either directly, through shedding, for example, flaking skin and contact with objects, as well as the act of expelling bodily fluids, for example the transfer of saliva through sneezing; or indirectly, through transfer by a secondary article, for example the transfer of DNA from one person to another onto an object [23]. Similar to fingerprints, biological material presents a means of individualising most people and providing links to a crime. Initially, ABO blood typing, a system discovered in 1900 by Karl Landsteiner, was used to discriminate between human blood groups (type A, B, AB or O) and exclude individuals as possible donors of biological specimen; however a large pool of possible donors could still result [24]. In 1984, Alec Jeffreys described a number of locations in DNA, which showed banding patterns found to be unique among individuals resulting from different zygotes [20, 21, 24]. This technique is known DNA profiling and since then has evolved to provide an absolute means of exclusion and probabilistic identification [20]. DNA profiling techniques will described in further detail in Section 1.4.2.

Shed cells come from the epidermis or outermost layer of the skin. This layer of the skin provides the body with protection against the external environment while retaining essential body fluids. Desquamatation, or the shedding of cells from the epidermis, is a major contributor of touch DNA. While this process occurs throughout the body on the surface layers of all membranes and tissues, when DNA containing cells are shed from the fingertip skin with a fingermark secretions, the DNA is referred to as touch DNA [2]. Terms such as low copy number DNA and low template DNA have also been used to

describe touch DNA owing to the minute amount of DNA that can be recovered from this sample type. These terms however can also be used to describe DNA from any biological source. To distinguish one source of DNA from another, the term "touch DNA" is used in this thesis to define DNA that originates from fingerprint skin.

Similar to the formation for fingermarks, the process of cell shedding begins in the basal layer of the skin with keratinocytes [25]. These cells are the most prevalent type on the epidermis. In their life cycle, keratinocytes migrate from the basal layer, where these cells form and proliferate, through the spinous, granular layers, where they differentiate into corneocytes at the cornified layer, the layer where cell shedding occurs [25, 26] (Figure 1).

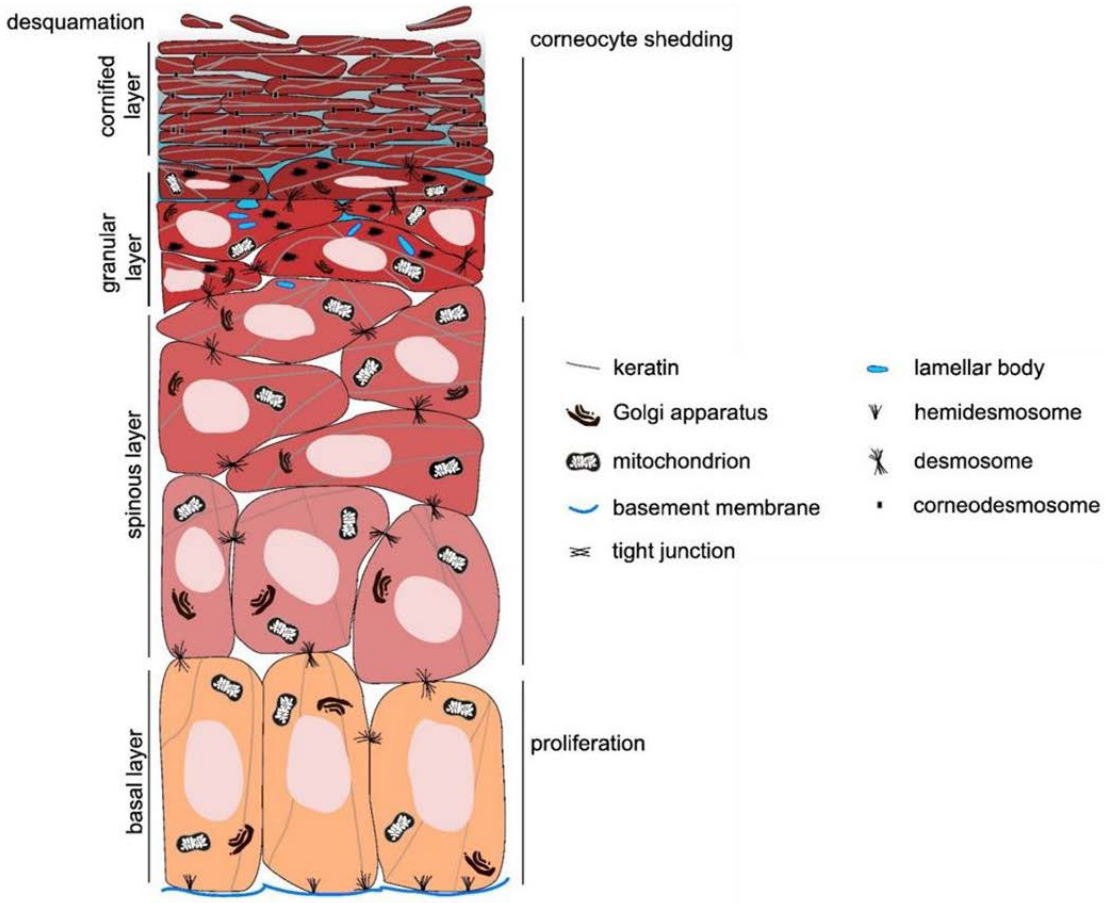


Figure 1: The cornification process leading to cell shedding

Adapted from Eckhart *et al.* 2013 [26].

During this cornification process, the organelles in the cell are degraded. This includes the DNA containing nucleus, which is broken down as it moves to the surface of the skin (Figure 1), leaving the cell anucleated and possibly devoid of DNA. However, studies have shown that touch DNA results from both nucleated cells and free nuclei present in the fingermark deposit [27, 28] or fragmented DNA in cells from the cornified layer where the low quality DNA can result in a usable profile where enough cells are obtained [29]. This is supported by a study indicating that fuller profiles are obtained from those with drier hands where the lack of moisture in the hands caused cells to flake off the surface of the skin [30].

Successful profiling of touch DNA was first reported by van Oorschot and Jones [31] in 1997 using short tandem repeat (STR) analysis. Since this breakthrough, the transfer and recovery of touch DNA has been investigated [32-41]. It has generally been found that due to the low quantity of touch DNA found in a specimen and the transfer and contamination events that can occur, care must be taken with its interpretation [32-34, 38, 42, 43]. Additionally, targeted approaches to the collection of DNA and techniques to maximise its recovery are required in order to increase the level of success in profiling [35, 36, 39-41, 44]. While touch DNA is genetic material that is recovered from fingermarks [2], the originator of the DNA may not necessarily be the source of the fingermarks [32]; this adds another obstacle to the interpretation of touch DNA profiles.

1.4 Analysis of Fingermarks and DNA

1.4.1 Friction Ridge Patterns

1.4.1.1 *Types of patterns and identification*

Fingerprints, or friction ridge patterns on the fingertips to the first joint, are widely studied and are the main friction ridge surface utilised in the identification of individuals [11]. Fingerprints are typically classified by their general pattern. The Henry Classification categorises fingerprint patterns into four groups: arch, loop, whorl (**Figure 1**) and composites. Two important fixed points also exist that assist in the differentiation of fingerprint types: the delta and core. The delta is located on point of the ridge nearest to the central area of digression, while the core is located at the uppermost point of the most central recurving ridge [45].

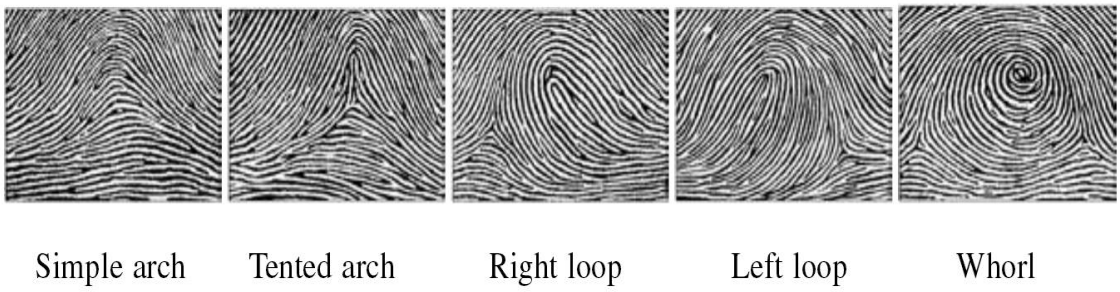


Figure 2: General fingerprint patterns

Sourced from Champod *et al.* [1].

There are three levels of ridge detail that may be accounted for when conducting identifications [35, 46]. The first level applies to the general pattern of the friction ridges (Figure 2), the flow of the friction ridges and their relative dimensions and focal points. While first level characteristics are not necessarily unique, they allow the initial classification to be conducted. Second level characteristics account for minutiae, as well

as other obvious distinctive features such as wrinkles and scars [1, 46]. The most common forms of minutia are ridge endings, bifurcations and dots (Figure 3). Finally, third level features are the natural characteristics of the ridges, including spatial arrangement, appearance of edges, and the size and position of pores. While the detection of features at each of these levels is significant to identification process, the inherent value of the feature must also be assessed for its discriminating power in determining the source of the fingermark [1, 47].

Figure 3: Level 2 detail, the three main types of minutiae

Sourced from Champod *et al.* [1].

Fingerprint examiners employ the analysis, comparison, evaluation and verification process, also known as ACE-V, when conducting mark examinations. This process allows for a systematic approach to the assessment friction ridge patterns and an objective interpretation of the detail contained in the mark [46, 48].

1.4.1.2 *Deposition of fingermarks*

Fingermark deposition is impacted by both pre-transfer conditions and transfer conditions. Pre-transfer conditions refer to the state of the fingerprint skin prior to the contact event, such as secretions levels and residues present [18, 49]. Fingermark residue contains proteins, peptides and amino acids. These are the principle components of eccrine secretions [18]. Sebum may also be a component of fingermark deposits as sebaceous glands are present throughout the body except on the palms of the hands and

the soles of the feet. The sebaceous secretions primarily contribute squalene, fatty acids and phospholipids to the deposit. These are transferred to the fingers following contact with other areas of the body, particularly the face and hair [18]. Apocrine secretions are less common in fingermark deposits as these glands are located in the pubic, auxiliary and breast areas, and contribute carbohydrates and cholesterol to the deposit [18, 50]. The eccrine and apocrine secretions form the water soluble component of the fingermark, while the sebaceous secretions form the lipid soluble portion of the fingermark [1, 18].

Compounds from the epidermis are also found in fingermark deposits. These are present due to the desquamation process, where dead cells are excreted from the basal layer. Proteins such as keratin and cathepsin D have also been found to be released in this process [18]. DNA is another endogenous component of the fingermark residue which is typically found in epidermal cells which have been sloughed from the skin onto the surface of contact [51].

Contact with external surroundings introduces extrinsic components to the fingermark residue. This includes food and cosmetics residues, and bacterial and dust contaminations [18]. Nicotine [52] and other drugs and their metabolites [53] have also been detected in fingermark residue as a result of both physical transfer and secretion following metabolism. These external contaminants change the original composition of the fingermark and can affect the rate of degradation of the mark [18].

Substrate type also influences how fingermarks are deposited and retained, and play an important role in the detection and enhancement of the mark. Substrates are generally classified into three categories: porous, non-porous and semi-porous [1, 54]. Porous substrates, such as paper, cardboard and untreated wood, are absorbent and readily retain the eccrine fraction of the fingermark residue with the water within the substrate, while

the water insoluble fraction remains on the surface of the substrate [1, 54] (Figure 4). Non-porous substrates, such as glass, smooth plastics and metals do not absorb any constituent of the fingermark, with the residues remaining on the surface of the substrate and degrading over time hence marks on this surface type are more prone to loss [1, 54] (Figure 5).

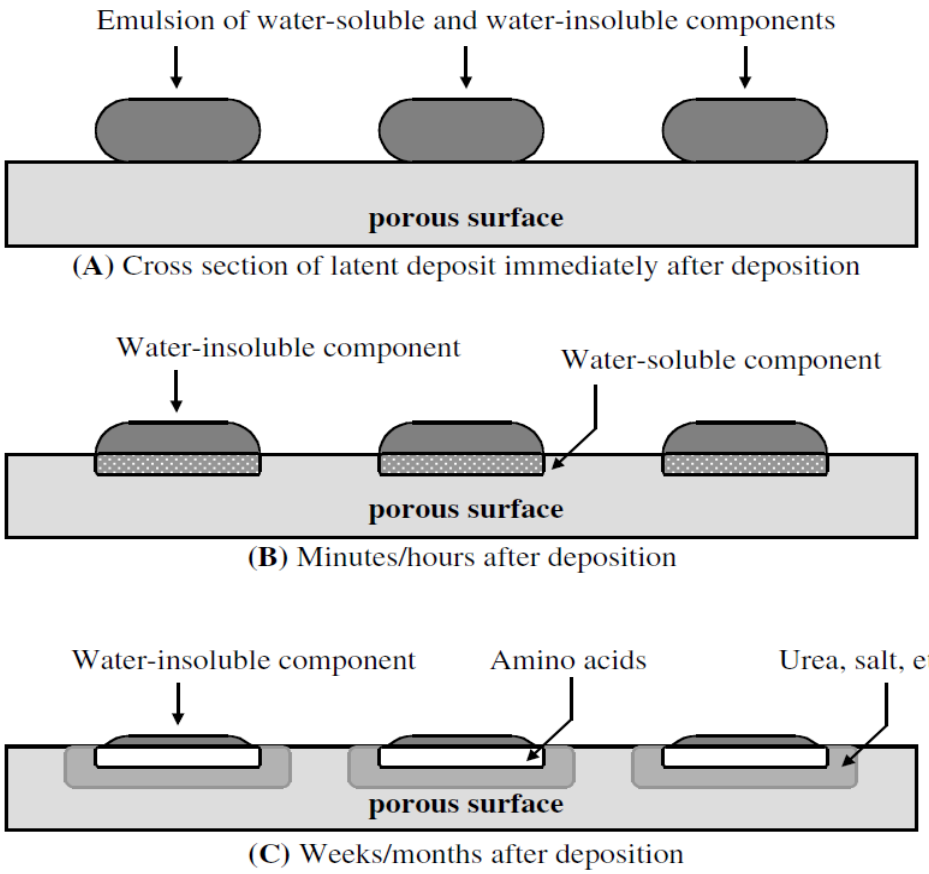


Figure 4: Ageing of a latent fingermark on a porous substrate

Sourced from Champod *et al.* [1].

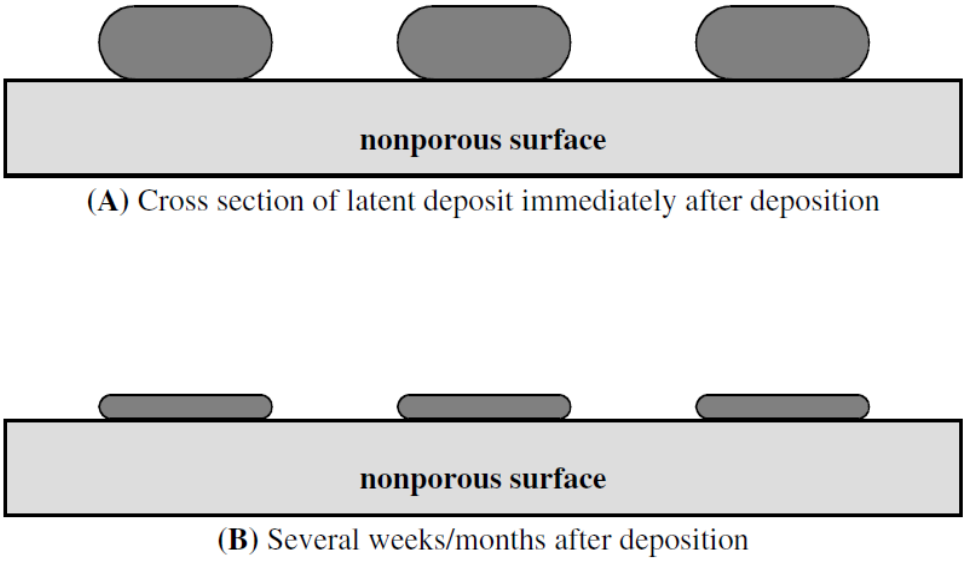


Figure 5: Ageing of a latent fingermark on a non-porous substrate

Sourced from Champod *et al.* [1].

Semi-porous substrates, such as polymer banknotes and glossy cardboard, however, are an intermediary of porous and non-porous surfaces. Semi-porous substrates absorb the water-soluble component of the fingermark deposit; however, at a slower rate than that of porous surfaces. They also retain the lipid soluble portion of the fingermark deposit for a shorter period than that of a non-porous substrate [1, 54]. The detection method used to visualise latent fingermarks is determined initially by the nature of the substrate and the environment and context in which it is found [42]. This will be further discussed in Section 2.2.

1.4.2 Touch DNA

1.4.2.1 *RFLPs and STRs*

As with fingerprints, specific regions of the genome show polymorphism among individuals. The initial method of discriminating DNA described by Jeffreys isolated minisatellites using restriction fragment length polymorphisms (RFLPs) [55].

Minisatellite sequences are typically thousands of bases long, showing a number of tandem repeats in the sequence, generally 10-15 base pairs (bp) in length, which varies significantly from person to person [55]. Using the RFLP technique, lengths of DNA are cleaved with restriction enzymes at specific sequence of bases, resulting in different sized fragments of DNA, which are then separated though gel electrophoresis [55]. The separation pattern of an unknown DNA specimen can then be compared to that of reference DNA to determine that they are from the same source. DNA from the same source will exhibit the same separation pattern, similarly the more closely related the two specimens are, the more closely the separation patterns will resemble one another [3]. A limitation of this method is that it relatively large quantities (more than 25 ng) of high quality DNA are required for the analysis facilitate the binding of a considerable amount of probe for visualisation purposes [56]. Such amounts and quality of DNA is often difficult to locate in forensic investigations as DNA may have degraded over time and traces such as touch DNA naturally do not contain large quantities of DNA and are difficult to detect.

The analysis of RFLP is assisted by the development of polymerase chain reaction (PCR). In the mid-1980s, Kary Mullis developed a protocol to amplify DNA [57]. The PCR protocol draws on DNA replication *in vivo* exploiting complimentary base pairing. PCR amplifies DNA in three temperature dependent stages as summarised in Figure 6. Firstly, denaturation of DNA occurs at 94^{o}C, where the two strands of the double helix are separated. Secondly, the system is cooled to 50-60^{o}C to allow for primers to anneal to the DNA. Primers are short sequences of single-stranded DNA (ssDNA) that are complimentary to each of the template DNA strands. Thirdly, the mixture is incubated at 72^{o}C to allow DNA polymerase to synthesise the complimentary strand of the DNA from the primers. Currently, *Taq* polymerase is the enzyme used in the extension stage. It is

derived from the thermophilic bacterium, *Thermus aquaticus,* which renders it heat stable up to 95ºC, where typical DNA polymerase is sensitive to such high temperatures. The denaturation, annealing and extension steps are repeated 20-30 times to achieve millions of copies of the template DNA [20-22, 58]. Thus, PCR has enabled minute samples of DNA to be profiled, which may be construed as a double-edged sword as contaminating DNA may also be amplified resulting in difficulties in interpretation.

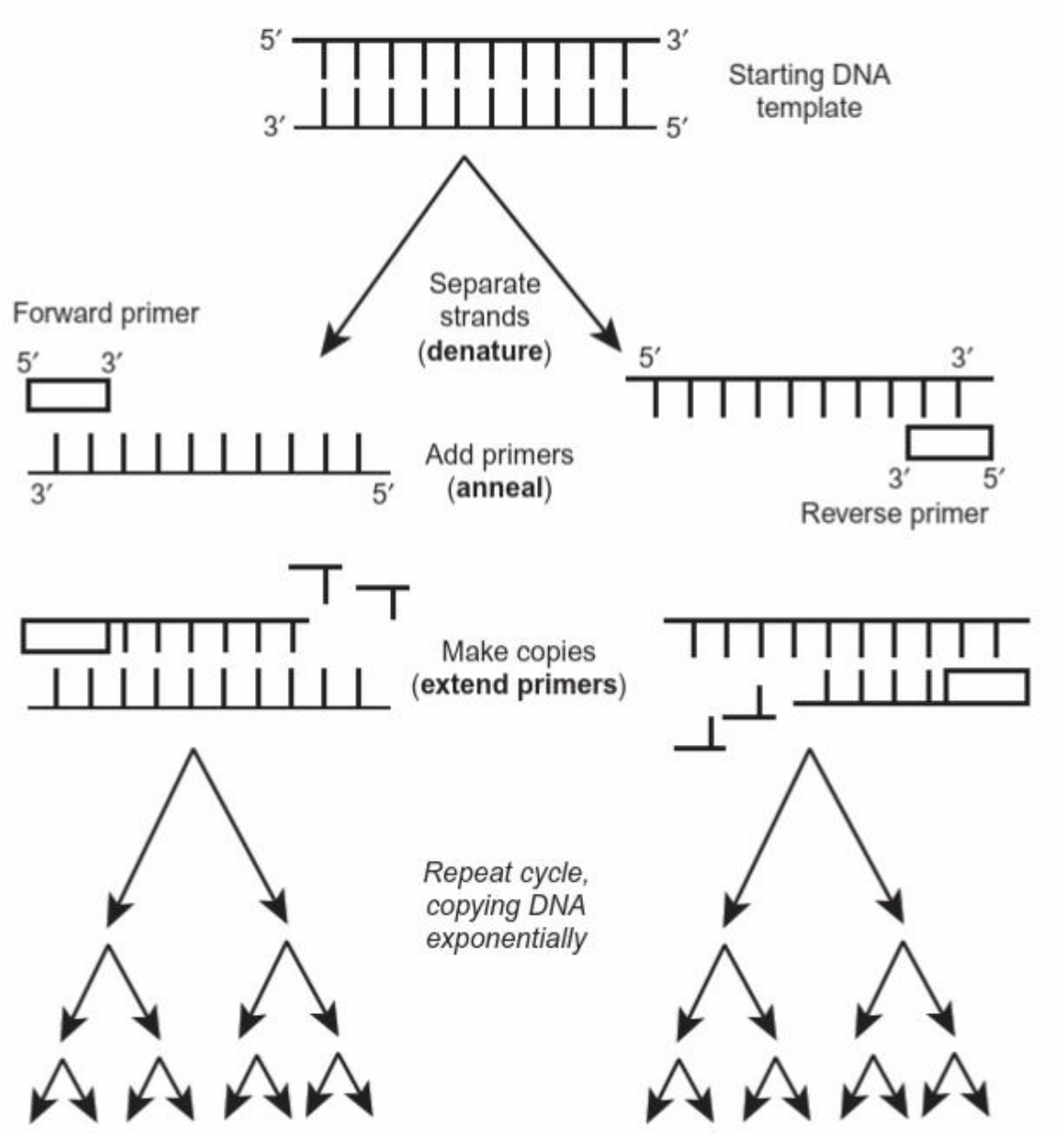


Figure 6: DNA amplification process with polymerase chain reaction

Sourced from Bulter [56].

The analysis of DNA has also evolved from RFLPs to the analysis of STRs, which describes sequences of DNA which are 2 to 5 base pairs in length, successively repeated throughout the genome at particular loci. Similar to RFLPs, the number of repeating units is measured. However, in STR analysis, the areas of interest are amplified only, rather than cleaved prior to amplification. Hence, the advantage of this technology over RFLP is that STR analysis can be successful on degraded or fragmented DNA specimens and requires a substantially smaller quantity of DNA (approximately 0.5 ng). STRs are can be found in all humans, however, the number of repeating units at a particular loci are polymorphic among individuals in a population. In this technique, a multiplex of sequence specific primers are used to locate STRs and the sequences are amplified using PCR with a multiplex of fluorescently tagged primers. The templates are subsequently sequenced with a fluorescent scanner or sequencer and a electropherogram is generated under the same principles as that described for RFLPs [20, 21, 58].

1.4.2.2 *DNA profiles*

The different sized DNA amplicons can be distinguished from one another through separation using capillary electrophoresis. This process takes advantage of the negatively charged backbone of DNA. With the application of a negative current, the DNA molecules move through a DNA-inert capillary towards the anode. The capillary that the DNA moves through is filled with a polymer that hinders the flow of the molecules based on size [59]. Hence, smaller DNA fragments are able to move through the polymer at a faster rate than larger fragments, allowing the fluorescent signal of smaller fragments to be detected prior to that of larger fragments. In the analysis a number of STR loci are evaluated in order to increase the discriminating power of the technique.

Multiplex STR analysis analyses multiple autosomal STR markers, which allows for high power of discrimination between profiles. The UK and the USA were among the first regions to establish National DNA databases in 1995 [60] and 1998 [61], respectively. In doing so, core STR loci were specified which allowed commercial kits to be developed. Most kits have between 15 and 22 core loci [43] which enables DNA profiles to be compared to databases both nationally and internationally. The results of a STR analysis are generally presented as the different alleles (variant of a gene) present at a particular locus. From this, the minimum number of donors that contributed to the specimen can be determined. The results of the generated profile can also be compared to that of a known sample or DNA from another exhibit to determine the likelihood that the two share a common source. Where a partial profile is obtained, it may be used to determine if a person should be included or excluded from further investigation.

While DNA analysis has high discriminating power, it is not without its limitations. Interpretation of results is prone to error especially in regards to samples that have more than three contributors and specimens that have low levels of DNA such as touch DNA. Many methods have been developed in order to increase the sensitivity of DNA analysis. While these methods allow for low-template DNA to be analysed, there is the risk of drawing erroneous conclusions.

1.4.2.3 *Profiles from touch DNA*

Profiling of touch DNA was first reported in 1997 [31] by van Oorschot and Jones. This study determined that significant amounts of DNA could be recovered from the palms of hands (2 – 150 ng) and a range of touched non-porous surfaces after regular handling (1 – 75 ng). While this initial study indicated that it was possible to generate profiles from touched objects, it also demonstrated the difficulties associated with interpreting results

obtained from objects touched by multiple people [31]. Since then, the factors influencing the quality of DNA profiles from touch DNA has been investigated such as an individuals shedding status [32, 62-64], the surface of deposition and transfer events [33, 35, 38, 65, 66], time between deposition and recovery of DNA [34, 67-69], and DNA recovery methods [30, 36, 40, 70].

Shedder status distinguishes an individual's inherent capacity to deposit DNA onto surfaces and thus the likelihood of obtaining a full DNA profile, for example, a full DNA profile is more likely to be obtained from a "good shedder" than a "poor shedder". Studies by Lowe *et al.* [32], Allen *et al.* [63], Goray *et al.* [64] support the idea that individuals can be categorised as either good shedders, where full DNA profiles are generally always obtained following contact with a surface, and poor shedders, where DNA profiles are generally uninformative. Phipp and Petricevic [62] highlighted the difficulty in classifying individuals into these categories due to the wide range of factors that can intrinsically affects an individual's ability to deposit DNA at a given time. In the study conducted by Phipp and Petricevic [62] good shedders as defined by Lowe *et al.* [32] were not able to be achieved. However, a phenol/chloroform extraction method was used in the former [62] as opposed to the use of the more specific QIAGEN QIAamp DNA Mini Kit used in the latter study [32] that could account for the lack of good shedders.

DNA transfer will also influence the quality and type of profile that is achieved following recovery. Transfer of touch DNA is affected by the surface type with it being more readily transferred to porous substrates than non-porous. Daly *et al*. [38] achieving higher DNA yields from primary transfer to wooden and fabric surfaces than from a glass surface, with DNA profiles also reflecting this trend. Similarly, Goray *et al*. [66] found higher DNA recovery when DNA was deposited on cotton by touch, than on plastic, also noting that secondary transfer of touch DNA occurs more readily from non-porous to porous surfaces

and increased significantly when friction was applied. This suggests that the roughness provided by the pores of porous surfaces more readily retain DNA and this renders it capable of "scrapping" DNA when friction is applied, thus providing superior amounts of DNA than compared to non-porous surfaces. However, secondary transfer can present some challenges for interpretation. Not only can secondary transfer of touch DNA occur from one inert object to another but also from contact between two individuals and onto an inert object. Lowe *et al*. [32] investigated the phenomenon and found that shedder status of the individuals determined the profile that is recovered. It was found that when good shedder made contact with a poor shedder prior to the latter making contact with a plastic tube, the profile of the good shedder presented as the primary component of the profile [32]. Additionally, when a time delay of up to 1 hour was introduced between the two transfer events, the good shedder's profile constituted approximately half of the recovered profile [32]. This suggests that transfer can occur readily from good shedders and reiterates the need for care when interpreting profiles. In situations where mixed profiles may be recovered or questions relating to the transfer events in the lead up to the deposition, additional information needs to be taken into account. Thus in these cases, the dual detection of the touch DNA trace along with the fingermark, as incomplete as it may be, could provide enough complementary information to identify the source.

Due to the extreme polymorphism of papillary ridge patterns, the analysis of fingermarks is one of the most predominant methods used for the identification of individuals [1]. In addition, fingermarks left behind following contact with an object are also a source of genetic material, known as touch DNA [2]. Touch DNA has been successfully typed since 1997 [31] and, as DNA in itself is so highly polymorphic, this also presents an additional avenue of analysis that that can aid in the identification of individuals [3] when the fingermark fails to provide meaningful information. However, techniques used to recover

DNA are detrimental to fingermarks as they involve the swabbing the surface [71]. The impact of prioritising the detection of one trace over another will be explored in Chapter 2.

Chapter 2. DETECTION OF THE TRACES

2.1 The Dilemma

The detection and recognition of traces is fundamental to forensic science and yet is one of the most difficult steps of the investigative process [72]. In terms of fingermarks and touch DNA, these traces are recorded in different scales: fingermarks at the macro level; DNA at the molecular level. Thus, these scales play a role in the order of their detection and exploitation. The enhancement and recording of fingermarks typically occurs prior to the collection and analysis of DNA to minimise the destruction or loss of traces [71]. This is because DNA detection methods, in a forensic context, are far less developed than that of fingermarks and current routine work generally calls for the swabbing of suspected DNA containing surfaces, which is destructive to latent fingermarks [71]. Further, swabbing without knowledge of whether the area contains the relevant trace leads to challenges in terms of both effectiveness and interpretation. Over the years many studies have reported success in DNA analysis following the application of certain latent fingermark development techniques [73-84], while DNA visualisation studies are slowly gaining momentum.

2.2 Fingermark Detection and Impact on DNA Processing

2.2.1 Optical Techniques

While the nature of the substrate determines the type of technique that will be used to detect the fingermark, an initial examination typically starts with optical examination before physical and chemical techniques are employed for the specific substrate. Optical techniques are non-destructive to the fingermark [42] and rely on the use of different optical devices or light sources to detect fingermarks with the aim of increasing the contrast between the fingermark and the background. This can generally be achieved with the use of diffused reflection and ultraviolet (UV) illumination, which exploit the diffusion and absorption properties of the fingermark to increase their visibility as well as the use of fluorescence [1, 42].

The use of diffused reflection is particularly effective for detecting fingermarks on non-porous, glossy surfaces. Episcopic coaxial illumination is a lighting configuration that involves a light source perpendicular to the suspected fingermark. The light is reflected onto the surface using a semitransparent mirror that is then diffused by the fingermark deposit and reflected by the surface. This results in dark ridges on a light background [42, 85]. While this technique cannot be used on every surface, negative impacts on DNA resulting from the use of this technique have not been reported. However, as with the use of any fingermark detection technique, DNA contamination and loss as result of investigations is always a possibility.

UV illumination may utilise either short (200 – 300 nm) or long (300 – 400 nm) UV wavelengths or both. As fingermarks, along with a number of other biological fluids, fluoresce under exposure to UV-wavelengths, the use of UV illumination is particularly

useful for their detection [86]. In UV illumination techniques, the surface is subjected to short wavelength UV and a long wavelength UV barrier filter is used to enable the recording of detected fingermarks [87]. A limitation of this technique is that the fluorescence of the fingermarks tend to fades as the exposure time increases due to photochemical degradation [86]. Additionally, short UV wavelengths, which most commercial systems, such as the Reflective Ultra Violet Imaging System (RUVIS), utilise, cause degradation to DNA [88, 89], hence increased exposure can result in the loss of potential information. This is particularly significant in delicate specimens such as that of touch DNA where the quantities of DNA are initially low.

Episcopic coaxial illumination is not a widely used technique for visualising latent fingermarks [42] and the use of UV illumination requires the use of precise angles of incidence as well as high intensity [90]. Hence, optical enhancement may fail to detect latent fingermarks in the first instance. These techniques can, however, be performed again following treatment with another detection technique to further increase the contrast of the fingermark to the background.

2.2.2 Techniques for Non-Porous Surfaces

2.2.2.1 *Powders*

Commonly used techniques for non-porous substrates include powders, small particle reagents (SPR) and cyanoacrylate (CA) fuming. Fingerprint powdering is a simple technique of detecting latent fingermarks and can be used at crime scenes. These powders enhance visualisation of fingermarks by adhering to the moisture component of the residue and are most successful on fresh marks on smooth, dry surfaces [91]. Fingerprint powders tend to lose effectiveness on older fingermarks. While there are a range of

fingerprint powders commercially available, the colour of the powder is generally chosen to increase the contrast of the fingermark to the background. Luminescent and magnetic powders are also available for use when the mark is difficult to differentiate from the surface, and for delicate surfaces, respectively. Powders are gently applied to surfaces to detect latent fingermarks, before photographs of the enhanced fingermark are taken under the appropriate conditions. The enhanced fingermark is generally also lifted using gel-lifters or non-gel lifters such as hinge or tape lifts [54].

When incorporated with surfactant solutions, a fingerprint powder suspension is formed and the technique is capable of developing fingermarks on non-porous surfaces that are wet or have been exposed to moisture. This technique is referred to as SPR, or small particle reagent. There are a number of SPR formulations, however, generally, they include a powder (typically a black or white powder) in an aqueous surfactant base, and enhance fingermarks by reacting with the lipid component of the residue [92]. Application of the technique is via spraying with the SPR or submersion into the SPR before rinsing off excess reagent. Enhanced fingermarks are subsequently treated per powdered fingermarks with photography and lifting of the fingermark.

Both fingerprint powdering and SPR techniques present challenges for the recovery of DNA profiles. Due to the use of brushes to apply the fingerprint powders, any DNA that brushes come into contact with can be retained on the brush and subsequently, if the brush is reused on other surfaces or objects or in other cases, secondary transfer of DNA can occur. Sutherland *et al*. [93] found that it was possible to obtained a full profile from a fingerprint brush used in case work. Similarly, studies by van Oorschot *et al*. [94], Proff *et al*. [78], Bolivar *et al*. [95], and Szkuta *et al*. [96], indicate that DNA can be transferred from a detected fingermark to another via the brushes used during powdering. The amount of DNA transfer depends on the specimen. Hence, when specimens resulting from

biological fluids are brushed, the risk of secondary transfer of DNA is higher than when fingermarks are brushed [78, 96]. Similarly, when fingermarks of good shedders are powdered, secondary transfer is more likely than from the specimens of bad shedders [78, 96]. This essentially correlates with the initial amounts of DNA present in the specimen, where low amounts are less likely to be picked up by brushes and, further, less likely to be passed onto the next specimen.

Proff *et al*. [78] and Raymond *et al*. [79] recommend the use of new brushes for significant cases to avoid DNA transfer from powdering methods and preserve the integrity of the specimens. Ballantyne *et al*. [97] suggest a 1% solution of hypochlorite followed by 70% ethanol is effective at removing DNA from laboratory surfaces. Studies by Szkuta *et al*. [96] confirmed that the use of a 1% solution of hypochlorite solution successfully removed DNA from fingerprint brushes and a 5% solution Virkon^{TM} was able to remove low level DNA contamination such as that resulting from touch DNA. However, it was recommended that the use of these cleaning solutions be limited to squirrel hair brushes as the integrity of fibreglass brushes was strongly affected following decontamination procedures [96].

Magnetic powders may also negatively impact on PCR of treated fingermarks. This technique uses a magnetic wand to attract iron filings covered in coloured particles. When the filings are brushed over a surface, the coloured particles are transferred onto moisture residue, such as that found in fingermarks. While magnetic wands may be an advantage to conventional powdering on delicate surfaces, Norlin *et al*. [98] observed lower DNA recovery than when treated with black powder, while Lin *et al*. [99] also reported on interference with DNA recovery caused by magnetic powders. Similarly, Laurin *et al* [100] found the use of MAGNA^{TM} Jet Black magnetic powder inhibited extraction when using a magnetic bead extraction kit. These results [98-100] suggest that the magnetic

particles interfere with the extraction process. Roux *et al*. [101] has also reported inhibitory effects on PCR when magnetic aluminium powder was used to treat blood stain specimens while work by van Hoofstat *et al*. [83] was not in agreement, finding that the use of BVDA magnetic grey and magnetic black for fingermark enhancement did not negatively impact DNA analysis. It was found, however, that fingermarks treated with metallic powders inhibited PCR. The magnetic powders used in this study [83] were BVDA Special Silver and Faurot Aluminium Bronze. Both of these powders contain aluminium, and along with copper, which is likely found in the latter powder, and iron, that is found in magnetic powders, are inhibitors of PCR [102]. These metals form complexes with DNA, obstructing the molecule from undergoing PCR. This would also account for the results observed by Norlin *et al*. [96], Laurin *et al* [100] and Roux *et al.* [101].

Conversely, the results from a study conducted by Lin *et al*.[99] would suggest that the choice of extraction method influences the successfulness of DNA analysis following the use of aluminium powders. When compared to untreated controls extracted with the same kits, Lin et al [99] reported powdering with aluminium powders resulted in a significant decrease in DNA recovery when extracted with QIAsymphony (QIAGEN), while no significant impact was observed when extracted with DNA IQ (Promega) and QIAcube (QIAGEN) kits. This suggests that the latter two kits may be more efficient at removing aluminium inhibitors than QIAsymphony.

A study by Zamir *et al.* [103] found the use of SPR does not impede on the physical processes of DNA analysis reactions, with complete profiles obtained on each tested substrate. This method, however, analysed dried blood droplets following the application of an unnamed SPR [103]. Hence, the DNA from the untreated inner portion of the droplet was likely preserved following the application and wash steps allowing for full profiles

to be generated. Studies by Au *et al.* [104] showed that the use of SPRs primarily developed the fingermark portion of bloodied marks however found on average 91% of DNA in bloodied fingermark specimens can be lost following application. This is not only a significant loss of DNA for bloodied marks, but on touch DNA specimens that have no other biological fluid contamination, the use of this technique can result in the complete loss of exploitable DNA.

2.2.2.2 *Cyanoacrylate fuming*

The most commonly used laboratory technique for enhancing fingermarks on non-porous substrates is CA, or superglue, fuming. With the emergence of CA fuming wands, this technique can also be used at crime scenes [1, 105], however there are safety concerns associated with CA vapours. In this technique, fingermarks are enhanced in a three-phase reaction: initiation, propagation, and termination. During initiation, CA is vaporised and the resulting CA monomer react with initiators found in the fingermark residue, such as amine, carboxylic groups and other nucleophilic groups, resulting in a nucleophilic CA molecule. This sets-off the propagation phase, where the other CA monomers react with the initial nucleophilic CA molecule to form a polymer. In the termination phase, the polymerisation reaction finishes due to the exhaustion of CA monomer or nucleophiles [54].

The result of CA fuming is a hard white polymer corresponding to the ridges of the fingermark. If the reaction is allowed to continue for prolonged periods, overdevelopment of fingermarks can result under humid conditions where the CA monomers continue to build upon each other and the ridge detail is no longer discrete. Hence fuming should be closely monitored to ensure fingermark enhancement is not compromised. Alternatively, CA fuming can be developed under vacuum conditions to limit overdevelopment [54].

The resulting white ridges of enhanced fingermarks is easily visualised on dark backgrounds but can be difficult to visualise on light backgrounds. Hence, post-treatment with fingerprint powders, or coloured or luminescent staining solutions can be employed to enhance the contrast with the background [1]. Powders are applied via brushing, while staining solutions may be applied to CA treated fingermarks or fumed items may be submerged into staining solutions, with excess solution rinsed off.

A study by Wickenheiser and Challoner [84] suggests that this technique is not detrimental to DNA and, in fact, recommends its use as the CA forms a protective barrier over the DNA until collection and extraction can occur. The effect of CA fuming on DNA processing has also been investigated. Stein *et al*. [106] and Bhoelai *et al.* [74] reported no significant impact of CA fuming on RFLP and STR analysis, respectively, where successful profiling was achieved following CA treatment in both cases. Von Wurmb *et al.* [82] had similar findings for STR analysis, however, also reported an adverse impact on PCR depending on the extraction method used. CA treated specimens were less compatible with chelex extraction than with Invisorb Forensic Kit [82]. Similar to Phipps' and Petricevic's [62] findings, this suggests that DNA extraction methods play a significant role in the successful PCR of samples. "Quick and dirty" methods such as phenol/chloroform extraction and chelex extraction do not appear to remove PCR inhibitors as successfully as DNA isolation kits.

The use of staining agents to assist in visualisation of the CA-developed fingermarks showed negligible impacts on the resulting profiles [77, 79, 99], which suggests that some exploitable DNA is trapped and protected within the CA polymer. Results by Leeman *et al*. [77], however, must be interpreted with care, as donor pool and sample size was small for experiments investigating DNA profiling following CA treatment and compared quantities of DNA prior to and following treatments. Due to the high variability in the

deposition of touch DNA [62], it would be more useful to compare the quality of DNA profiles. Similarly, Raymond *et al*. [79] used a small donor pool consisting of a single donor for CA treatment and post-treatment experiments. While this donor is suspected to be a poor shedder, the DNA recovery result may have been a result of processing method, where the substrate was cut and placed into extraction tubes [79]. Hence, secondary transfer of available DNA onto the cutting instruments may have occurred, which may have resulted in a low overall recovery from the donor. Additionally, the amount of DNA recovered has been found to vary depending on whether the fingermark donor is a good shedder or a poor shedder [77]. Similar to fingerprint powdering, the more starting DNA prior to the fuming process, the more likely a profile will be obtained. The use of stains also presents the risk of contamination if the same solution is used in the submersion of treated specimens.

In recent decades, one-step luminescent CAs have been developed as a time saving, safer alternative to the conventional CA method, which eliminates the need for post-treatment of fumed fingermarks [107-116]. This CA is also marketed for use on semi-porous surfaces that present difficulties when using non-porous or porous detection techniques [117, 118]. While these one-step luminescent CAs seem to show comparable development to conventional CA depending on the surface of the substrate, their luminescent abilities is inferior to that of conventional stains such as basic yellow 40 (BY40) [110, 112] and rhodamine G6 (R6G) [111, 115]. There has been suggestion that these one-step luminescent CAs are compatible with DNA analysis [118], complementary to DNA recovery [119], and assist in the preservation of DNA by limiting any loss due to staining and wash steps [117]. Hence, the impact these one-step luminescent CA has on recoverability of DNA needs to be assessed.

2.2.3 Techniques for Porous Surfaces

2.2.3.1 *Physical developer*

Typically, the techniques used to enhance fingermarks on porous substrates are conducted in the laboratory. There are techniques that can be utilised for surfaces that have been wetted or those that have remained dry. For porous surfaces that have been in contact with high levels of moisture, physical developer (PD) is typically the technique that is used. The use of this technique generally results in the loss of any DNA present as a number of washing and rinsing steps are required to prepare the substrate for the deposition of silver onto the ridges of the fingermark [98]. The treatment consists of an initial water bath of distilled water to wash the item. This is followed by soaking the item in a solution of maleic acid then rinsing in distilled water to remove excess acid before the item is ready to be submerged in PD solution to develop any fingermarks. The PD solution consists of silver nitrate in a redox solution of ferrous nitrate, ferrous ammonium sulfate and citric acid buffer, and detergent. The PD solution is then rinsed in multiple water baths until the water is clear before being allowed to dry [1]. Thus, due to the number of submersion steps in this technique, it is considered detrimental to DNA recovery [98].

The mechanism of action of PD is still largely unknown; however, a study by de la Hunty *et al.* [120] suggests that this technique targets the eccrine component of the fingermark that is potentially emulsified in a hydrophobic layer that allows it to be retained on the substrate when in contact with aqueous solutions. This finding was supported by Frick *et al* [121] and suggests the retained eccrine components is then available for interaction with the silver deposit [120]. While this technique is routinely used in forensic laboratories for wetted surfaces, it is also destructive to the integrity of

the paper and hinders further forensic examination, hence it is typically employed at the end of a detection sequence [122]. In addition to being destructive on the substrate, due to the number of submersion steps in this method, and considering this method is employed for surfaces that have previously been wetted, treatment with PD limits the recovery of DNA from detected fingermarks following this treatment [98] and presents the risk of contamination if solutions are used to treat multiple items [74].

Further, a study conducted by Lin *et al*. [99] found that where spiked DNA was retrieved from PD treated samples, the quality of the profile was adversely impacted when compared to the untreated controls. In addition to the decreased starting quantity of DNA contributing to the loss of integrity in the profile, the negative effects could be contributed to the components of the PD solution. The PD solution contains detergent, which could lyse the cells, thus leaving the DNA valuable to further degradation prior to collection. The exposed DNA is also sensitive to chelation by silver ions, a primary constituent of PD, where Ag^+ interacts specifically with the bases of DNA rather than with the sugar moity or phosphate backbone [123]. With guanine, Ag^+ forms a complex around the N_7 and C_6O groups, while chelation also occurs at the N_7 and C_6NH_2 groups in adenine [123]. Ag^+ preferentially binds G-C base pairs over A-T base pairs [124]. The DNA/Ag^+ complexes may account for decreased amplification of the template DNA seen when PD is applied to the specimen and thus a quality profile results as seen in Lin *et al*'s work [99]. Further, PD solution also contains ferrous components. As seen with magnetic fingerprint powders, the presence of iron may complex DNA and also hinder PCR [102], resulting in poor quality DNA profiles. As DNA recovery and processing are both negatively impacted with the use of PD, the ability of treated specimens to provide useful DNA information is significantly reduced.

2.2.3.2 *1,8-Diazafluoren-9-one*

Methods of fingermark detection that are less destructive include 1,8-Diazafluoren-9-one (DFO), ninhydrin (NIN), 1,2-indanedione (IND) and IND-zinc chloride (IND-Zn). These techniques are used on porous surfaces that have not previously been in contact with high levels of moisture. The DFO solution consists of DFO in dichloromethane, methanol and acetic acid, diluted with petroleum ether or HFC4310. The porous item is briefly submerged in the working DFO solution, allowed to dry then heated at 180°C for 10 seconds in a heat press between absorbent paper. The amino acids present in the fingermark residue react with DFO and form red ridges that are faintly visible under white light and highly luminescent under the 450-570 nm excitation range with 550-650 nm barrier filter [1]. DNA recovery rates have been reported to decrease following treatment with DFO depending on the substrate with up to 60 % loss observed when compared to untreated controls [75, 125]. However, with an adequate amount of starting material, negligible effects have been observed on STR analysis following DFO treatment [74, 75, 103, 125]. Due to the use of a submersion technique, the use of DFO also presents the risk of contaminating sample if the same working solution is used for treatment [74].

2.2.3.3 *Ninhydrin*

Where latent fingermarks cannot be visualised with DFO, post-treatments with NIN can also increase the instances and quality of developments [126]. This reagent generally consists of NIN dissolved in a simple alcohol with ethyl acetate and acetic acid, diluted in a carrier solvent [1]. NIN is used on porous surfaces and targets amino acids present on the item. The treatment requires 24-48 hours for development to occur, with dark purple ridges, known as Ruhemann's Purple, resulting [1]. The reaction can be

accelerated with the use of ovens up to 80°C [54]. The use of NIN presents similar limitations to that of DFO in regards to DNA recovery. The recovery of DNA has been found to be lower than that of untreated controls and comparable to the loss from DFO treated samples [75]. A study by Schulz *et al*. [127] found that NIN treated fingermarks were a source of genetic material from a crime scene successfully conducting STR analysis on a number of treated specimens at the scene. This is in agreement with Bhoelai *et al*. [74], Sewell *et al*. [75] and Lee *et al*. [125], who also reported that the quality of resulting DNA profiles were not found to be significantly impacted.

2.2.3.4 *Indanedione and indanedione-zinc*

Another compound that is now frequently used to detect latent fingermarks on porous surfaces is IND, a NIN analogue, that produces pink ridges following development, known as Joullié's pink, and are also highly luminescent. This reagent is prepared in a similar manner to NIN, however, with the addition of a zinc-chloride solution. The incorporation of the zinc salt has been found to be more effective than DFO and resulting in more intensified luminescence than DFO and the conventional IND solution [128, 129]. As with DFO and NIN, when an item is submerged in the IND solution, the molecule will react amino acids present. The item is then allowed to dry at ambient temperature before heat treatment at 160°C for 10 seconds. Again, with the submersion of the item into the loss and contamination of DNA specimens is always possible.

Tsai *et al*. [73] observed similar, if not better, DNA recovery from paper that was rubbed with all five digits following IND treatment than compared to untreated controls when the formulation was prepared without acetic acid. This positive result may be due to being able to localise a complete area of touch. A study by Azoury *et al*. [81] on saliva specimens treated with one of two IND formulations, showed no difference in the

resulting DNA profiles of the two formulations or when compared to untreated controls. An observation from this research was that time following IND treatment had a crucial impact on the quality of DNA profiles, where no alleles were detected from samples extracted 6 days following IND treatment [81]. In research by Yu and Wallace [76], only 1% of fingermark samples on thermal and carbonless papers returned full profiles following IND treatment. However, using their test parameters, alleles were still called for up to 21 days between treatment and DNA processing, where 99% of profiles called in this study were partial [76]. It should be noted that there are some differences that need to be observed when working with thermal paper that do not need to be observed for other porous surfaces. The differences in IND treatment between the two studies [76, 81] is outlined in Table 1. The use of a submersion method in Azoury *et al*.'s [81] study may have washed away a significant amount of DNA, with the remainder degrading quickly following treatment. The use a spray application may have assisted Yu and Wallace [76] in preserving the quantity of starting DNA and similarly, as heat treatment cannot be used on thermal paper. The lack of full profiles from post treated samples maybe due to the nature of touch DNA, where the starting quantities are generally low.

Table 1: Differences in IND treatment methods between Azoury et al. [79], and Yu and Wallace [74]

Component	Azoury *et al.* [81]		Yu and Wallace [76]
	Formulation 1	Formulation 2	
Indanedione	0.025% w/v	0.2% w/v	0.2% w/v
Ethyl acetate	90 mL	90 mL	70 mL
Acetic Acid	10 mL	-	-
HFE-7100	q.s 1000 mL	q.s. 1000 mL	q.s. 1000 mL
Application method	Submersion	Submersion	Spray
Heat treatment	100°C; 10 minutes	100°C / 60% RH; 20 minutes	Ambient

2.3 DNA Collection Before Detection of Fingermarks

The collection of DNA prior to fingermark detection is generally not recommended as DNA collection methods are considered destructive to fingermark traces. At the time of writing, only one study was found to have determined the effect of conducting DNA collection prior to fingermark enhancement in an attempt at dual recovery [130]. In their study, Parsons *et al*. [130] investigated how tape-lifting, gel-lifting and swabbing with nylon swabs used to recover DNA impacts the fingermarks and subsequent enhancement with fingerprint powdering for hard and smooth non-porous surfaces, CA for textured and soft non-porous surfaces, and NIN for porous surfaces. While no full profiles were obtained from their investigations, a number of partial profiles resulted following use of the respective collection methods.

Their results indicate that swabbing of fingermarks completely destroys any informative information that can be garnered from the specimen [130]. This was believed to be due

to the use of a wet swab and the researchers suggest the use of a dry swab only. However, Pang *et al*. [36] recommend the use of a wet and dry swab sequence to recover DNA as higher amounts can be recouped using this technique. Additionally, if a swab, be it wet or dry, makes contact with a region of the fingermark, it is likely to remove that ridge detail as the fingermark residue will become smeared on contact with the swab on non-porous surfaces, while the fibres of porous surfaces are likely to lose their integrity.

It was also found that the use of gel- and tape-lifts to recover DNA resulted in the distortion and transfer of ridge details [130]. The distortion from gel-lifts was attributed to the act of powdering the remaining residue [130]. It could also be possible that the gel-lifter itself may have distorted the fingermarks during the lifting process by warping the fingermark during lifting. Minitaping primarily showed displacement of ridge detail due to the fingermark residue adhering to the contact surface minitape and redepositing on the next contact [130]. These DNA collection methods show varying degrees of destructiveness to fingermarks due to the interactions of the solid DNA collection mechanism against the surface of deposition. This sequence is also less constructive than the detection of fingermarks prior to collecting DNA as touch DNA does not always result in useful profiles and friction ridge detail is destroyed in its collection, hence the result is incomplete or unusable traces. However, in case work, where best practice has not been followed and surfaces have been swabbed for DNA prior to fingermark detection, this study does indicate there is a possibility of retrospectively retrieving some fingermark traces depending on the DNA collection method used [130].

Table 2: Summary of the impacts of prioritising one trace over another

NB: ✓ indicates no negative impact; × indicates increasing negative impact; - indicates impacts not stated.

Priority	Technique		Impact on Secondary Trace		
			Integrity	**Quantity**	**STR Profiling**
Fingermarks	Non-porous	Optical	✓	✓	✓
		Powdering [78, 79, 93-100]	✓ / ×	×	×
		CA [62, 74, 82, 84, 106]	✓	×	×
	Porous	PD [74, 98, 99]	-	×××	×××
		DFO [74, 75, 103, 125]	×	××	×
		NIN [74, 75, 80, 125]	×	××	×
		IND [73, 76, 81]	×	××	×
			Distortion	**Displacement**	**Usability**
DNA	Lifts	Minitape [130]	×	×	××
		Gelatine lifts [130]	×	×	××
	Swabs	Nylon Flocked [130]	×××	×××	×××

2.4 Techniques to Visualise DNA

2.4.1 Detection of Biological Specimens

2.4.1.1 *Blood techniques*

Techniques to visually detect DNA are not as common or well developed as for fingermarks and this remains a major drawback of touch DNA. The targeted recovery of touch DNA has been recommended to improve the quality of profiles [35, 36, 39-41, 44], however there is no such technique that specifically targets touch DNA in routine use. Methods to detect other types of biological specimens, such as those described to detect fingermarks, are often relied upon instead. Such indicators allow for the assumption that DNA is associated with the biological specimen.

While methods for presumptive testing of respective biological specimens are well established, their use in the detection of DNA holds some limitations. The use of blood detection agents often relies on the presence of heme in blood to catalyse oxidation-reduction reactions to present a colour change [131]. Thus, the use of this method does not indicate the presence of genetic material. The heme group of haemoglobins is found only in red blood cells. These cells are devoid of the nucleus in order to maximise oxygen transport around the body. In addition, the use of some blood detection techniques has been shown to negatively impact DNA analysis. The use of the Kastle-Meyer and leucomalachite green assays each decrease the recoverable quantity of DNA, while leucomalachite green also hinders DNA amplification [132] and the extent of its impact on DNA amplification may increase with time between treatment and processing [133]. Similarly, the DNA from stains tested with Hemastix® appear unable to proceed with some DNA extraction kits and cannot be recovered as the dye in

Hemastix® appears to interact with the magnetic beads required to bind DNA [134, 135]. This technique seems to be compatible with chelex extraction [132]. The use of luminol to detect blood has been found to be compatible with DNA analysis procedures [132, 136], however, this may be dependent on blood concentrations [137] and time between treatment and processing [138].

2.4.1.2 *Saliva and semen techniques*

Presumptive tests for saliva typically rely on a reaction with α-amylase, the enzyme found in saliva that is responsible for breaking down polysaccharides such as starch [131]. As with blood, these detection methods indicate the presence of the bodily fluid rather than the genetic material. The use of Phadebas® Forensic Press to indicate the presence of saliva appears to present no inhibitory effects on PCR following contact, and the transfer of DNA from the from the surface of deposition to the Phadebas® Forensic Press is minimal [139]. The impacts of other saliva detection reagents on DNA processing have not been reported.

A target for seminal fluid is often seminal acid phosphatase, however much like the detection of blood and saliva, the detection of seminal fluid does not necessitate that sperm cells are present in a specimen. The use of Fast Black to detect seminal acid phosphatase has been found to be compatible with DNA analysis [140]. No other compatibility studies were found regarding semen detection and DNA analysis, however these detection methods are often used in case work, to both provide context on events as well as to identify the source. As with saliva and blood, if a stain is visible, a portion maybe sampled and tested without impacting the remainder of the stain. While the presence of a biological fluid may assist in establishing what occurred in the case, there is no guarantee that DNA is present. This is often unknown until DNA analysis occurs,

which is a costly and time-consuming test. Thus, the ability to detect and target DNA following identification of a biological stain would allow for DNA analysis to be streamlined. Additionally, this could potentially allow for transfer events to be studied.

2.4.2 Detection of DNA

In recent years, research has been conducted on detecting DNA to assist in forensic casework. Haines *et al.* [141] investigated the use of intercalating DNA dyes: GelGreen and SYBR Green I, to visualise fingermarks through the detection of touch DNA within the specimen. These DNA dyes are typically used to detect DNA in separation gels following processing. While it is unrealistic to expect this technique to be successful in all donors due to shedder status and the vast number of other variables which impact touch DNA deposition, fluorescence from DNA was reported in this study along with signals from fingermarks [141]. It is unclear whether DNA or fingermark residue was the target for these dyes. Interesting, the manufacturers of GelGreen state that the molecule has been designed so that it is unable to cross cell membranes [142]. It can be assumed that the fluorescent signals were derived from DNA, as these dyes are intercalating agents, and hence, have high affinity for DNA, hence it is possible that free DNA is detected. A limitation of the use of these dyes is that they are not specific for double stranded DNA and thus will also detect the presence of bacterial DNA, as illustrated by the fluorescent signals in response the presence of *Escherichia coli* in phosphate buffered saline (PBS) [141]. This study was also limited by working volumes of 5 µL being used to detect fingermark specimen [141], which is not a feasible volume for use in case work due to the unlocated nature of latent fingermarks, the large unknown surface areas and the large number of fingermarks that may be encountered. The use of these low volumes is likely due to the cost of these dyes, with SYBR Green I retailing for more than AUD700 for

1 mL of 10000x concentrate, while GelGreen is more cost effective at approximately AUD100 for 0.5 mL of 10000x concentrate. As a reagent for the singular detection of fingermarks, this does not compare to the cost of conventional fingermark detection reagents.

GelGreen and SYBR Green I are both intercalating agents. While they are among the safer of such reagents, they are still low level mutagens [143-145]. With over exposure they are likely to present future health problems. Additionally, due to the intercalating nature of SYBR Green I, it has an inhibitory effect on PCR as reported previous molecular studies where DNA stained with these dyes showed decrease PCR efficiency when the dye was included in the reaction [146, 147]. PCR efficiency appears to be lacking in the presence SYBR Green I in a study conducted by Haines *et al*. [148] that reported faint bands corresponding to this treatment. Conversely, no PCR inhibition was observed when SYBR Green I was added to extracted DNA samples, or used to stain fingermarks in a study by Tonkrongjun *et al*. [149]. The latter result suggests that extracted DNA concentrations may have been high enough so as to allow for some non-bound DNA to proceed in PCR, and in touch DNA samples SYBR Green I merely binds to cell components rather than DNA.

Again, conflicting STR analysis results have been reported. The study by Tonkrongjun *et al*. [149] found that DNA profiles were able to be obtained after SYBR Green I applied to an improvised explosive device (IED). However Haines *et al*. [150] observed allele dropout and obtained a 70% profile from hair follicles treated with SYBR Green I, while in another study, Haines *et al*. [148] found a significant impact on STR analysis when SYBR Green I was added to a DNA containing solution with only two alleles, of the shorter fragment sizes, detected on average. This result is likely due to PCR inhibition that was also reported in the presence of SYBR Green I [148] and suggests that this dye

is not ideal for the processing of the cellular material it detects. An investigation of GelGreen showed no such issues with amplification or profiling of DNA [150]. It is unclear if this is due to GelGreen's inability to permeate the cell membrane [142]. This would indicate that GelGreen also has an affinity for components of the outer cell membrane, thus is removed during the extraction process.

Touch DNA detection studies have also been conducted with Diamond™ Nucleic Acid Dye. This dye is a groove binding agent and binds not only double stranded DNA, but also single stranded DNA and RNA as well [151]. Kanokwongnuwut *et al.* [152] showed Diamond™ Nucleic Acid Dye is effective in detecting cellular material in fingermarks deposited on glass. This study utilised the direct PCR technique potentially to minimise the loss of DNA caused by the extraction process. From the detected material, DNA profiles were able to be generated and categorised according to shedder status. The relative fluorescent units (RFU) from these generated profiles correlated to the number of cells counted in the respective fingermark source. Two full profiles were able to be generated in this study [152] while the study conducted by Haines *et al*. [150] could not recover full profiles in DNA solutions (1 ng/μL) treated with Diamond™ Nucleic Acid Dye. This suggests that some loss of DNA was incurred due to the extraction process used in the latter study, however it is unclear how long the specimens were exposed to the dye and if this had an impact on the results. Alternatively, the lack of full profiles may be attributed to the Diamond™ Nucleic Acid Dye being bound to the DNA, where the Promega Corporation [151] has indicated that this hinders electrophoretic mobility of the DNA when run in gels. Thus, the direct amplification method may not be sufficient to remove the dye from the DNA groves, causing similar interference on STR profiling.

While touch DNA was able to be visualised with Diamond™ Nucleic Acid Dye, in a research setting, the reported concentration (20-fold dilution) [152] is much higher than

the suggested concentration (1:10,000 dilution) [151]. This may render the use of Diamond™ Nucleic Acid Dye a costly technique for the detection of touch DNA, at approximately AUD140 for 0.5 mL, especially when the location of the fingermark is unknown. This dye has also been assessed at the recommended dilution (1:10,000) and found to be non-toxic, however, the authors stress that the concentrated dye is still cytotoxic when undiluted and even a dilution of 1:1,000 was showed genotoxicity [153]. The results of the safety study [153] would indicate that a 20-fold dilution [152] is likely to have toxic effects. The combination of the exorbitant cost and the related health and safety issues related to the use of Diamond™ Nucleic Acid Dye as a DNA detection agent make its operational use unlikely.

The use of Diamond™ Nucleic Acid Dye stained tape-lifts has also been evaluated for the detection of latent DNA [154]. While this method shows good visualisation and recovery of DNA using Diamond™ Nucleic Acid Dye and EvaGreen stained tape-lifts, DNA contamination appear to be an issue, likely attributed to the sticky nature of the tape-lift. In addition, this study only assessed with dried saliva samples on a glass surface [154]. The success rate for use on latent stains or touch DNA would therefore be low as the target area is unknown. To apply this technique in the search for latent DNA would appear costly and could damage fingermark traces if best practice for fingermark detection is not concurrently observed.

2.5 Current Challenges

Studies have shown touch DNA to be a valuable source of information and the detection of fingermarks plays a significant role in the recovery of this trace. Fingermark detection

techniques reveal the location that contact of the skin has been made with a surface. When the fingermark is of an insufficient quality to provide valuable information or if there is no correspondence with reference fingerprints, then it may be processed for DNA. The exploitation of DNA is an expensive and time-consuming process; knowledge that genetic material exists within the specimen would increase the chances of success. However, DNA detection outside the field of molecular biology has emerged the recent years with a focus on nucleic acid stains.

Studies on use of nucleic acid stains focus primarily on touch DNA detection on non-porous surfaces. The impact of each of these dye techniques on the remaining trace, the fingermark, was not expressed. Depending on the solvent used in each case, it may dissolve or wash away the fingermark residue, while cellular material remains fixed. Small volumes of the detection solutions have been used in each of these cases, suggesting that while latent DNA within a visible stain can be easily detected, these techniques are less effective on truly latent specimens. Another limitation that these studies fail to highlight is the use of specialised equipment, such as fluorescent microscopes, required with the use of these staining techniques. For larger exhibits found at, or fixed to, crime scenes, portable equipment would have to be used in order to visualise DNA specimens, especially if the specimen is minute. In addition to the costs in purchasing these nucleic acid stains, and the potential loss of the fingermark trace, forensic resources may be better utilised without this added screening step. Ideally, a technique to detect latent fingermarks and then analyse the fingermark for touch DNA would allow for the preservation of both traces.

2.6 Project Aims

At current, there is no routine technique in use to both detect latent DNA and fingermarks. Techniques used to detect latent fingermarks are well established in casework, and their impact on DNA processing has been well described. The detection of DNA is a relatively new area for forensic research and studies focus primarily on locating latent DNA, specifically touch DNA, however these techniques are generally expensive and the research has neglected the two-fold nature of this trace – it exists within a fingermark. The overall aim of this project was to establish techniques for the dual detection of latent DNA and fingermarks that limits the negative impact on each of the traces while allowing both to be located and exploited.

The initial objective of this project was to determine suitable reagents and techniques that would be compatible with both fingermarks and DNA detection. The difficulty in dually detecting both of these different traces exists in the compatibility of the detection method with each trace. Many constituents make up the fingermark residue, and what is deposited and retained on the substrate is not always complementary to cellular material or DNA and vice versa. Hence, the reagent or technique applied to the traces may negatively impact on one, while positively impacting the other. Additionally as fingermark detection is primarily dictated by substrate type, this project investigated dual detection methods according to the type of substrate (i.e. non-porous and porous). Thus, the first questions that had to be answered in this research were:

What method of detection can best be applied to simultaneously detect fingermarks and DNA?

and

Can this method be applied to different surface types?

Thus, many techniques used in fingermarks detection and cell biology were investigated to determine their compatibility with the alternate trace, and how they can best be applied different surface types. However, while the successful detection of fingermarks and DNA provides investigators with the knowledge of where they should analyse or sample from, there is no guarantee that these traces are intact following application. This raises the next question:

How does the detection technique impact on the quality of the trace after application to the surface?

Hence, after techniques were determined to allow for visualisation of fingermarks and DNA, the effect of the use of these techniques were assessed and reported.

Thus, in this thesis, Chapter 3 and Chapter 4 look to address the issue of what methods can be employed for dual detection. Once techniques for the dual detection were identified, their impact on DNA and DNA processing was then evaluated. Chapter 5 investigates the effect of a dual detection technique for a non-porous surface on DNA analysis, while Chapter 6 explores the detection capabilities of a novel reagent for dual detection on a porous surface and its impact on the analysis of DNA.

The International Fingerprint Research Group (IFRG) provides guidelines for the evaluation of new fingermark detection techniques [155].There are four key phases to the assessment of new techniques. These are, Phase 1: Pilot studies; Phase 2: Optimisation and comparison; Phase 3: Validation; and Phase 4: Operational evaluation and casework trials [155]. In this project, as the overarching aim was to develop a dual detection

technique for fingermarks and DNA, hence Phase 1 of evaluation process was used a guide for the development of experiments in this project.

Chapter 3. INVESTIGATION INTO POTENTIAL TECHNIQUES FOR DUAL DETECTION OF FINGERMARKS AND DNA

3.1 Detection with Biological Stains

3.1.1 Introduction

A relatively simple method of detecting either DNA or fingermarks is the use of stains. There are a number of staining techniques used in biological fields to detect the presence of and study the cell nucleus and DNA. These stains typically colour the nucleus of cells a distinct colour to that of cytoplasm and do not impart colour to the background environment [156]. Similarly, there are many fingermark stains that are in operational use; however, these are generally used on porous surfaces or as post-treatments to enhance the visualisation of CA-developed fingermarks on non-porous surfaces. Fingermark stains have been discussed in Section 2.2.3. For porous surfaces, the stains typically react with the fingermark residue, imparting colour or luminescence on the ridges to contrast with the background. Stains applied to CA-treated fingermarks become trapped within the CA polymer and increase the contrast with the background [1, 157].

Generally, nuclear stains do not specifically target the nucleus, but have high affinity to the acidic constituents of the cell thus are attracted to the presence of DNA, where dye

molecules bind to the phosphate backbone. Common nuclear stains include acridine orange, methylene blue, nile blue, rhodamine B and toluidine blue. Acridine orange is a aminoacridine fluorescent dye that intercalates with DNA giving a green fluorescence when excited with blue light, showing the presence of DNA at 530 nm [158]. Nile blue is a fluorescent intercalating phenoxazine dye, excited at between 625 – 635 nm and viewed at 665 – 675 nm, depending on whether the solvent is water, methanol or ethanol [159], that also imparts a blue colour to the nuclei under ambient light. In solutions of 1% nile blue or greater, hydrolysis of the molecule can occur resulting in the presence of nile red [160]. Nile red is used primarily to stain lipids in cells, showing fluorescence in organic solvents over aqueous [161]. Rhodamine B is a fluorescent xanthene dye that preferentially binds to the minor groves of DNA [162], excited at 553 nm and can be viewed at 627 nm. Toluidine blue is an acidophilic thiazine dye. This dye therefore binds to DNA and RNA, giving them a blue colour, while carbohydrates, to which they also have affinity for, are stained red [156]. Similarly, methylene blue is a phenothiazine dye with strong affinity for DNA, forming a complex by intercalating with DNA with preference for alternating G-C base pairs in the sequence to give dark blue nuclei [163].

While these dyes have been used extensively in a histological settings to examine nucleic acids, their use in forensic casework for DNA detection is yet to be reported on. The use of methylene blue, nile blue, nile red and rhodamine B has been investigated in fingermark detection. Methylene blue was intercalated with bentonite clay particles to form a complex that was brushed over fingermarks on a glass surface, with blue ridges visible under while light after application [164]. Additionally, methylene blue has been combined with carbonised banana peel powder and found to give well visualised fingermarks when brushed glass at a concentration 0.2% [165]. While its impact on touch DNA was outside the scope of these studies, it is unlikely that the dye had any interaction

with the DNA as it was complexed prior to application in the former study [164], and in both studies [164, 165] devoid of a liquid carrier, thus unable to diffuse through the cell and nucleus.

Nile blue does not emit luminescence but when acidified, spontaneous hydrolysis of the molecule to nile red occurs. Nile red presents with luminescence at an excitation wavelength of 505 nm and can be visualised at 550 nm. A 0.5% w/v solution of nile blue in water was found to develop fingermarks on copy paper, giving a blue colour that also showed luminescence, suggesting presence of nile red in the solution [160, 166]. Interestingly with nile blue, neutral components of the cell are coloured red while a blue colour is visible in acidic compounds [160] thus could allow for DNA to be differentiated from fingermark residue in lipid rich fingermarks while fingermark residue with high concentrations of trapped acidic components may cause issues with contrast.

Rhodamine B has been used in conjunction with nanoparticles to detect fingermarks. When fingermarks deposited on non-porous surfaces were treated with SiO_2 nanoparticles doped with rhodamine B, fingermarks were able to be observed at 590 nm when excited at 515 nm [167]. The impact of this technique on touch DNA was, again, outside the scope of this study. It is likely that, as the dye is incorporated into the nanoparticle lattice, it cannot migrate through cells to the nucleus. Additionally, the use of long submersion times (30 – 60 minutes) and a rinse step [167] may result in a loss of touch DNA.

Thus, the aim of this experiment was to determine a staining technique that would be compatible with both touch DNA and fingermarks to allow for the detection of both traces following a single application. This was investigated through the preparation of a number of solutions individually containing these dyes and applying it to fingermarks deposited on paper and glass surfaces to determine the compatibility of these solutions with the

traces. The solvents chosen were glycerol, chloroform, triethylene glycol and tris-HCl buffer. These solutions are compatible with cellular material; however, consideration was given to their ability to preserve the fingermark secretions and, hence, limit their destructiveness on the fingermark.

3.1.2 Methods

3.1.2.1 *General methodology*

Solutions of 2% w/v dye (acridine orange (Biological, ProSciTech), methylene blue (Certified Biological Stain Commission (BSC), Sigma-Aldrich), nile blue A (Microscopy, Sigma-Aldrich), nile red (Microscopy, Sigma-Aldrich), rhodamine B (Fluorescence, Sigma-Aldrich) and toluidine blue O (BSC, Sigma-Aldrich)) were prepared in each of chloroform (Analytical Reagent (AR), Chem-Supply), glycerol (99.5%, Sigma-Aldrich), triethylene glycol (TEG; 99%, Sigma-Aldrich) and tris-HCl (3.5 mM, pH 8, Sigma-Aldrich) solvents. Charged fingermarks, created by rubbing the fingertips across sebum-rich skin surfaces of the face, were deposited onto grid microscope slides (glass, uncoated, ProSciTech) and copy paper (Carbon Neutral 20 % Recycled A4, STAPLES®). The dye solutions were applied to the fingermarks for 5 minutes on glass and 20 minutes on paper before being rinsed with Milli-Q water. Treated fingermarks were allowed to dry at ambient temperature. Fingermarks deposited on paper were mounted onto microscope slides (Premium Glass, Livingstone International). All fingermarks were then examined under microscopy (Leica DM4 M microscope with Leica LQ-HXP 120-L-Multipole external light source and Leica H3 filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm), Leica Microsystems^{TM} Pty Ltd) and using the Poliview® IV System (Polilight PL500, Peltier Cooled 12 bit CCD camera and lens,

with Poli-V®++ software, Rofin Australia), under both white light and luminescence mode.

3.1.2.2 *Procedure*

Acridine orange, methylene blue, nile blue, nile red, rhodamine B and toluidine blue were each prepared to a final concentration of 2% w/v in each of the chloroform, TEG and tris-HCl solvents. Due to the viscosity of glycerol, the dyes were prepared to a final concentration of 2% w/w.

Two donors each deposited 24 charged fingermarks onto each of the two surfaces (glass microscope slides with grids and copy paper) to allow for each of the six dyes in the four solvents to be tested in duplicate. Fingermarks were charged by rubbing the donor fingerprint surface on the back of their ears to ensure the presence of cellular material. Fingermark deposits were left overnight, uncovered, under ambient laboratory conditions before they were treated with 25 µL of the respective dye solution. For fingermarks on glass, the dye treatment was left for 5 minutes before being briefly rinsed with water. Care was taken to avoid rinsing of untreated portions of the fingermark. For fingermarks on paper, the treatment was left for 20 minutes before being rinsed to remove excess stain, and then hung to dry in the dark at ambient temperature overnight, before being mounted onto glass microscope slides. Treated fingermarks were then viewed under a microscope at 50 times magnification, under both bright field and H3 fluorescence mode and under the Poliview® system under white light and luminescence mode.

3.1.3 Results

3.1.3.1 *Dyes in glycerol*

In preparing these dyes in glycerol, it was observed that nile red was not soluble at 2% w/w and had the appearance of suspended particles. Following the treatment of fingermarks on glass with each dye solution, DNA or cellular components were visible following treatment with acridine orange, nile blue, nile red and rhodamine B in glycerol, while fingermark secretions are weakly luminescence following treatment with acridine orange and nile red in glycerol (Figure 7). The use of glycerol on glass generally appears to cause slight degradation to the secretion residue, while rinsing with water appeared to remove some of the deposited fingermark secretion. In the case of methylene blue in glycerol (Figure 7; B), the treatment appears to have a strong negative impact on the fingermark secretions. No luminescent fingermarks or cells were observed on glass with the Poliview® system under any of the excitation and barrier filter combinations available in the system, up to an exposure time of 5 seconds.

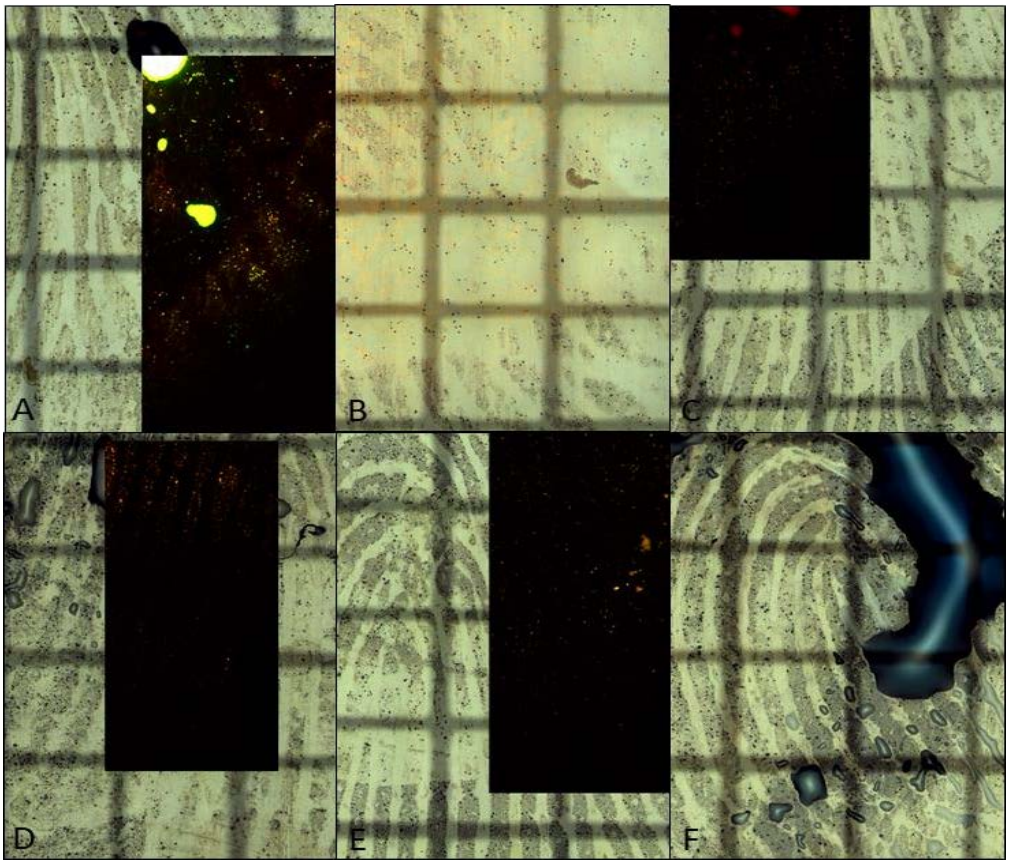


Figure 7: Fingermarks on glass microscope slides treated with 2% w/w (A) acridine orange, (B) methylene blue, (C) nile blue, (D) nile red, (E) rhodamine B and (F) toluidine blue in glycerol

Fingermarks viewed with Leica DM4 M microscope at 50 times magnification; light areas indicate viewing under bright field, dark area indicate the presence of luminescence in in the treated area; where no dark area is present, no luminescence was observed.

On paper no fingermark ridges or cells were evident following treatment of glycerol based dyes when viewed with either the microscope or the Poliview® system (Figure 8 and Figure 9). The stain appears to have penetrated through the paper; however, there is no differentiation of fingermark secretions or DNA from the treated areas of the paper that was observed using the Poliview® system under luminescence mode (Figure 9). The luminescent conditions that provided some luminescence of the dye application spot are listed in Table 3.

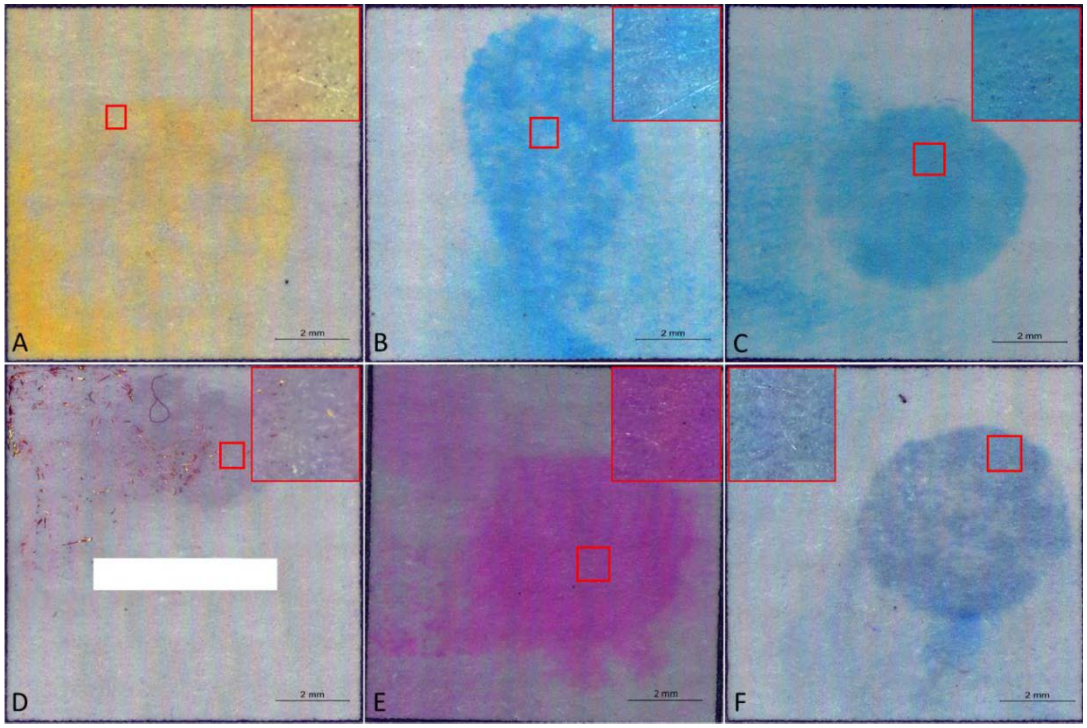


Figure 8: Fingermarks on paper treated with 2% w/w (A) acridine orange, (B) methylene blue, (C) nile blue, (D) nile red, (E) rhodamine B and (F) toluidine blue in glycerol

Fingermarks viewed with Leica DM4 M microscope at 50 times magnification under bright field; blank areas indicate areas that could not be stitched due to lack of difference from surrounding areas; area encompassed by red box has been enlarged in the corner of the image.

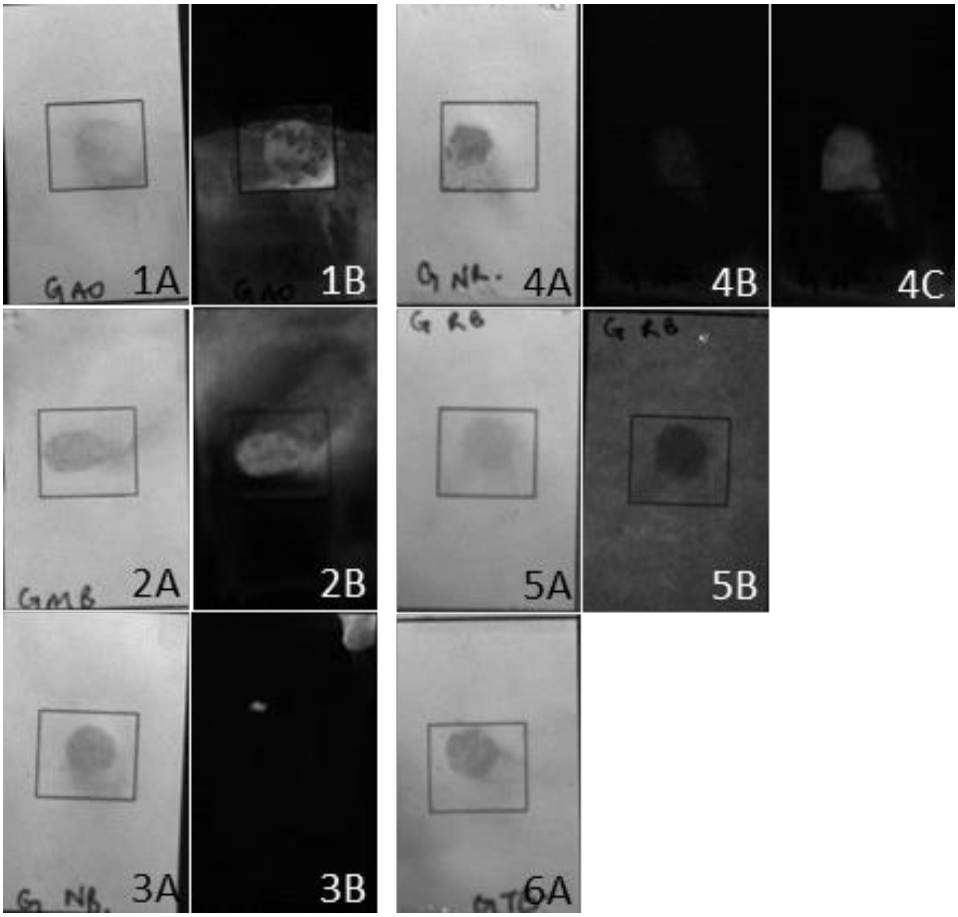


Figure 9: Fingermarks treated with 2% w/w (1) acridine orange, (2) methylene blue, (3) nile blue, (4) nile red, (5) rhodamine B and (6) toluidine blue in glycerol

Fingermarks viewed with the Poliview® IV System under (A) white light; and (B and C) under luminescence mode.

Table 3: Luminescent conditions used on the Poliview® IV System according to dye

Dye	Excitation (nm)	Barrier Filter (nm)
Acridine Orange	460	650
Methylene Blue	650	700
Nile Blue	650	700
	505	610
Nile Red	555	650
	505	530
Rhodamine B	450	530
Toluidine Blue	-	-

3.1.3.2 *Dyes in chloroform*

Similar to that seen following treatment with dyes in glycerol, it was apparent that DNA or cellular material were visible following treatment with acridine orange, nile blue, nile red and rhodamine B in chloroform, while the fingermark secretion residue themselves do not appear to luminescence (Figure 10). The use of chloroform on glass has caused some of the fingermark residue to be washed away, however the stained portion appears to be lightly fixed to the glass surface as unstained portions appear to have been washed away during water rinse. While luminescence was observed under fluorescence mode using the microscope, no luminescence fingermark or cells were visible under the Poliview® system under any of the excitation and barrier filter combinations available in the Poliview® IV System, up to an exposure of 5 seconds.

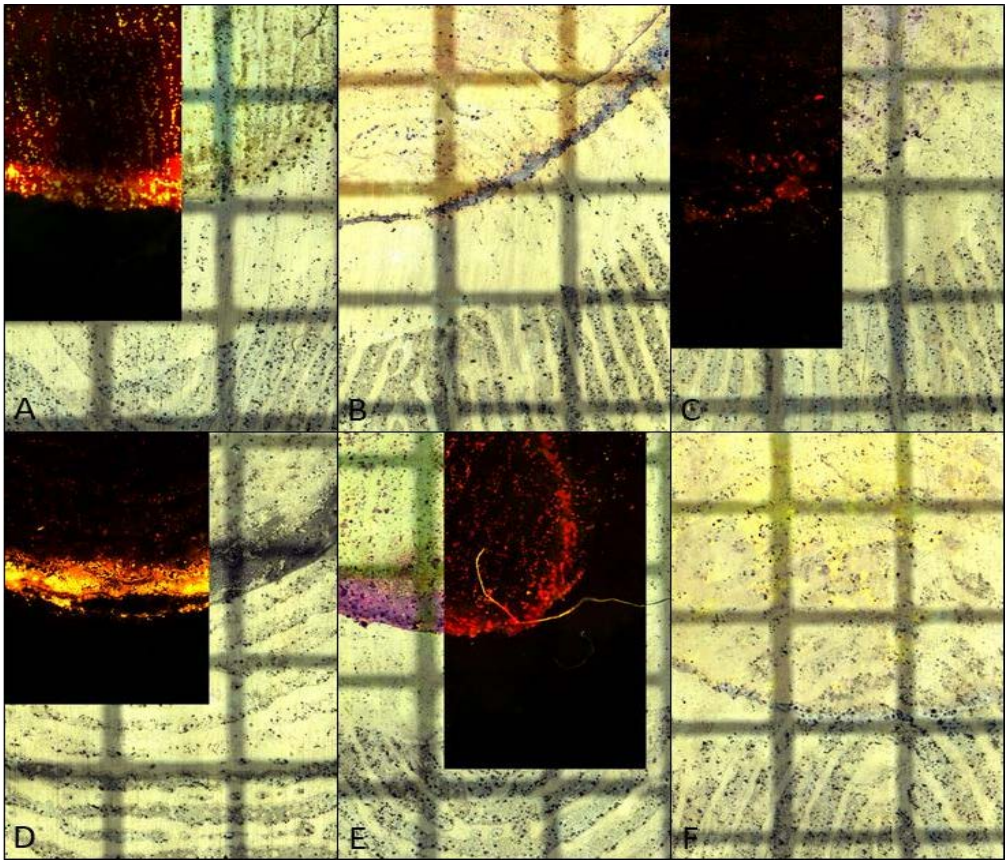


Figure 10: Fingermarks on glass microscope slides treated with 2% w/v (A) acridine orange, (B) methylene blue, (C) nile blue, (D) nile red, (E) rhodamine B and (F) toluidine blue in chloroform Fingermarks viewed with Leica DM4 M microscope at 50 times magnification; light areas indicate viewing under bright field, dark area indicate the presence of luminescence in in the treated area; where no dark area is present, no luminescence was observed.

It was thought that the use of chloroform would accommodate the dye solutions into the paper and assist the dye in reacting with the fingermark secretion residues or with DNA. However, no discernible fingermarks nor cells were evident following treatment of chloroform-based dyes on paper. The stain appears to have penetrated through the paper; however, ridge detail and cells were not detected under luminescence mode following treatment (Figure 11). Similarly, no differentiation of the fingermark nor cells from the background could be observed under the fluorescence mode using the microscope, similar to that seen in Figure 8. The luminescent conditions listed in Table 3 were used.

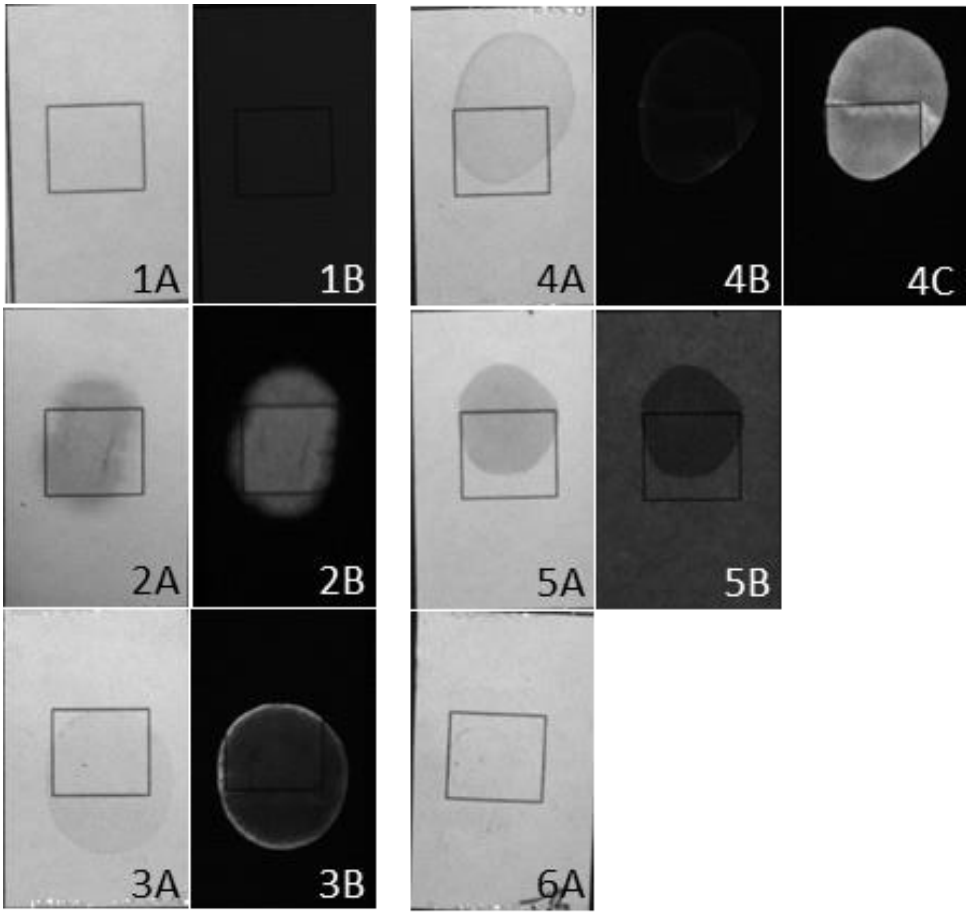


Figure 11: Fingermarks on copy paper treated with 2% w/v (1) acridine orange, (2) methylene blue, (3) nile blue, (4) nile red, (5) rhodamine B and (6) toluidine blue in chloroform

Fingermarks viewed with the Poliview® IV System under (A) white light; and (B and C) under luminescence mode.

3.1.3.3 *Dyes in TEG*

Again with TEG, the only dyes to show luminescence were acridine orange, nile blue, nile red and rhodamine B (Figure 12). These were only apparent on glass and the luminescence was observed in cellular material being quite faint following treatment with nile blue and rhodamine B. The fingermark secretions appear to have been washed away following treatment with the dyes in TEG.

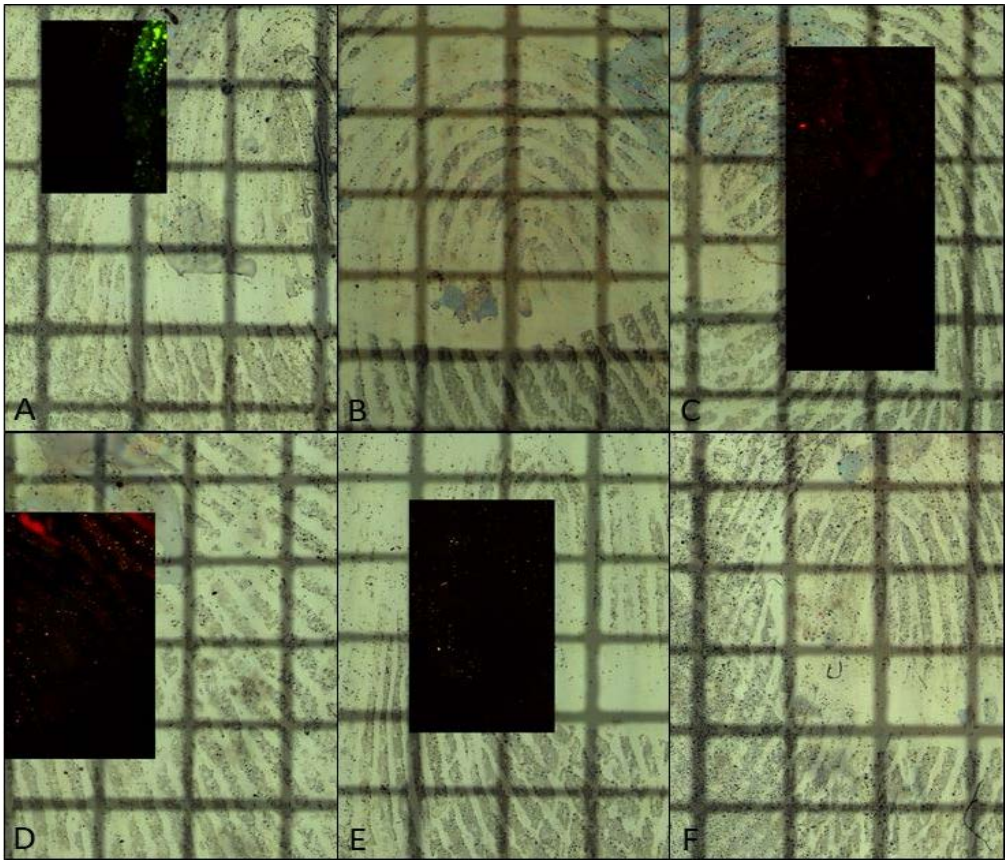


Figure 12: Fingermarks on glass microscope slides treated with 2% w/v (A) acridine orange, (B) methylene blue, (C) nile blue, (D) nile red, (E) rhodamine B and (F) toluidine blue in TEG
Fingermarks viewed with Leica DM4 M microscope at 50 times magnification; light areas indicate viewing under bright field, dark area indicate the presence of luminescence in in the treated area; where no dark area is present, no luminescence was observed.

As seen earlier with dyes in glycerol and chloroform on paper, no discernible fingermark ridges were visible under white light or luminescence mode using the Poliview® system (Figure 13; Table 3) and DNA and cellular material was not observed under the microscope.

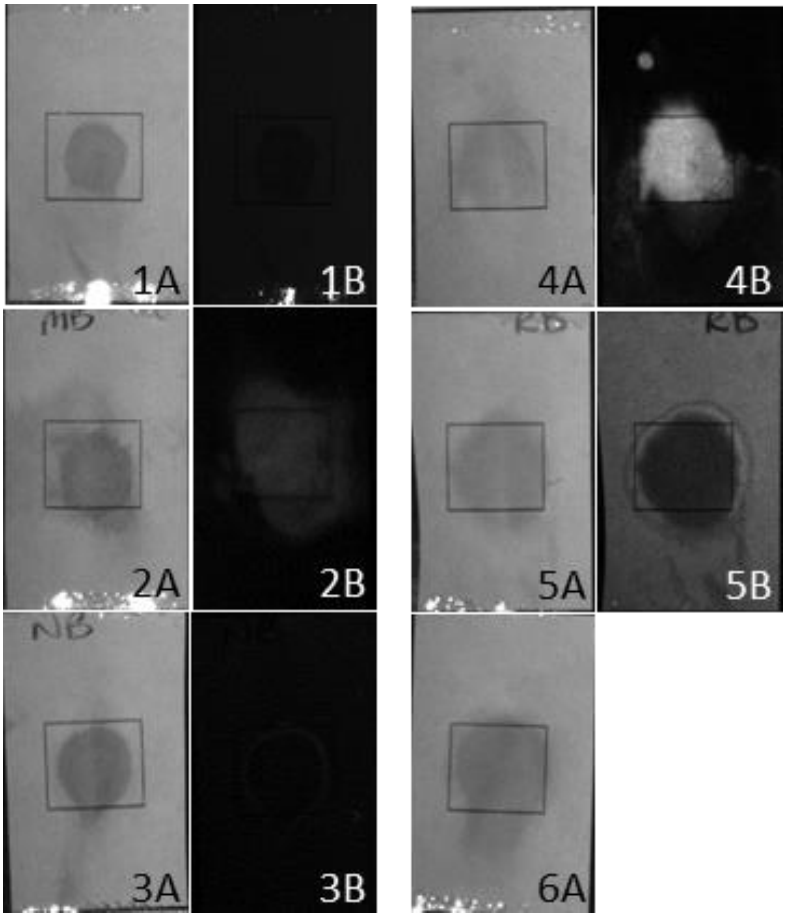


Figure 13: Fingermarks on copy paper treated with 2% w/v (1) acridine orange, (2) methylene blue, (3) nile blue, (4) nile red, (5) rhodamine B and (6) toluidine blue in TEG

Fingermarks viewed with the Poliview® IV System under (A) white light; and (B) under luminescence mode.

3.1.3.4 *Dyes in tris-HCl*

In tris-HCl, acridine orange, nile blue and nile red were not soluble at 2% w/v. Despite this, luminescent DNA or cellular material was still observed following treatment with acridine orange and slightly visible following nile blue treatment. Luminescence was also observed following rhodamine B treatment (Figure 14). Fingermark secretions appear to have been washed away following the application of the tris-HCl based dyes. However, faintly luminescent ridges were also apparent following acridine orange treatment.

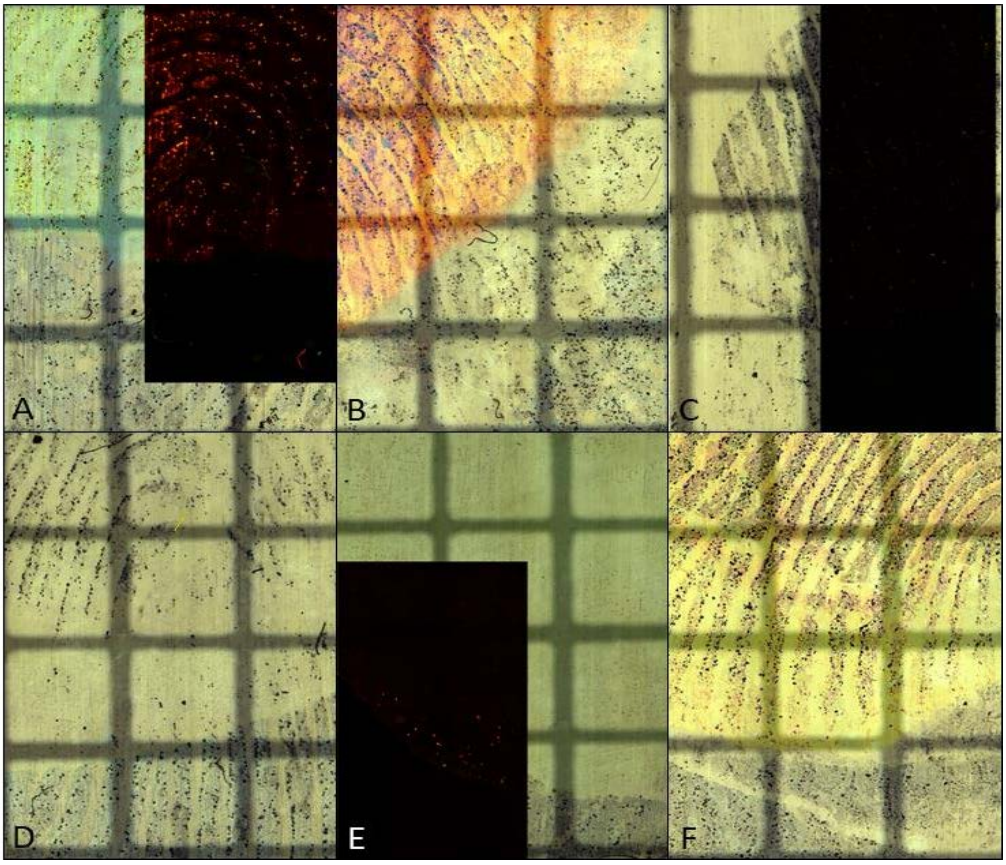


Figure 14: Fingermarks on glass microscope slides treated with 2% w/v (A) acridine orange, (B) methylene blue, (C) nile blue, (D) nile red, (E) rhodamine B and (F) toluidine blue in tris-HCl Fingermarks viewed with Leica DM4 M microscope at 50 times magnification; light areas indicate viewing under bright field, dark area indicate the presence of luminescence in in the treated area; where no dark area is present, no luminescence was observed.

As with all other solvents investigated, no discernible fingermark ridges were visible under the under white light or luminescence mode using the Poliview® system (Figure 15; Table 3. DNA and cellular material also was not observed under the microscope.

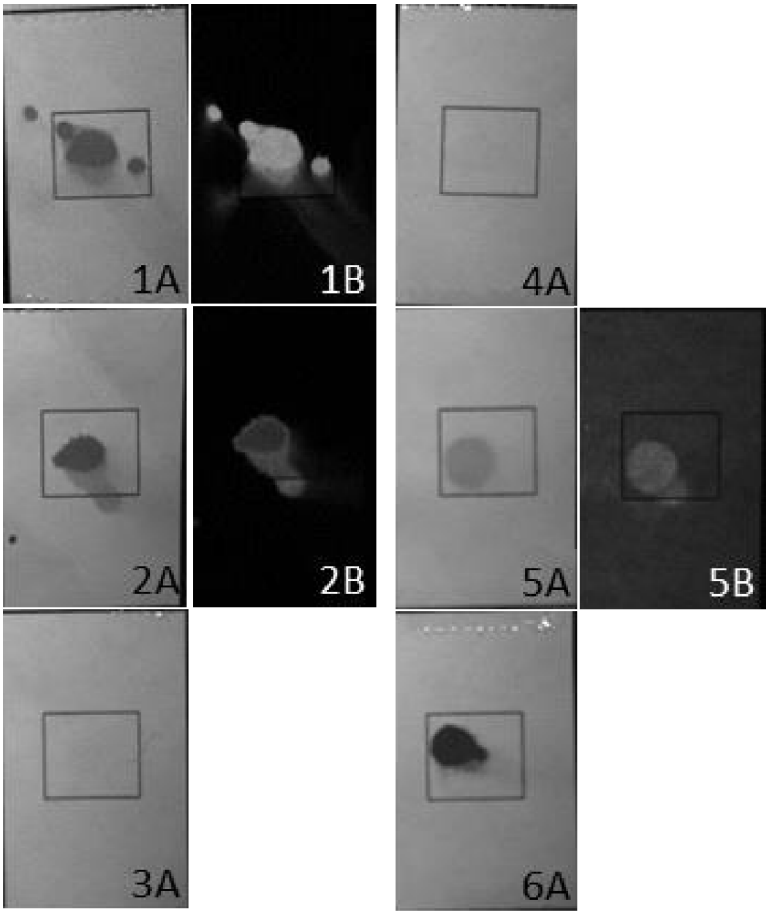


Figure 15: Fingermarks on copy paper treated with 2% w/v (1) acridine orange, (2) methylene blue, (3) nile blue, (4) nile red, (5) rhodamine B and (6) toluidine blue in tris-HCl

Fingermarks viewed with the Poliview® IV System under (A) white light; and (B) under luminescence mode.

3.1.4 Conclusions about Biological Stains

From this study, the reagents used had no fingermark or DNA enhancement effect on paper. These reagents were more successful on glass with acridine orange, nile blue, nile red and rhodamine B showing luminescent cells that were visible under fluorescent mode using a microscope. The least destructive solvent for use on fingermarks on glass was glycerol, however, no enhancement of the ridge detail was observed. Of the remaining three solvents, the most destructive depended on the dye used. Some fingermark ridge detail and cellular material was observed following application of 2% w/v acridine orange

in tris-HCl. However, this was weak and the rinse step may remove some of the viable DNA present in the deposit. The results of this study suggest that while the visualisation of DNA can be enhanced using DNA stains, the fingermark is not. This presents a challenge as the fingermark can be detected without magnifying equipment, while DNA detection requires magnification. It would therefore be logical to detect the fingermark first, then analyse the fingermark for the presence of DNA. Alternatively, if the fingermark cannot be visualised, for example, the source of the fingermark has weak secretions, then ideally a technique to increase the signal of the could be used to visualise DNA with minimal magnifying equipment. This will be further investigated in Section 3.2.

3.2 Whole-Genome Amplification: Multiple Displacement Amplification

3.2.1 Introduction

Whole-genome amplification (WGA) is an *in vitro* process for generating significantly increased quantities of DNA from minute starting quantities, for example, micrograms of product from nano- and pico-grams of starting material. A distinctive quality of this technique is the uniform regeneration of the DNA present in a specimen, rather than specific sections that traditional PCR methods result in. WGA is an isothermic procedure that is performed at 30°C, hence can be performed *in situ* as opposed to PCR techniques, which requires pre-extraction and purification prior to amplification as well as frequent changes in temperature (i.e. thermocycling) to achieve amplification [168].

Isothermic multiple displacement amplification (MDA)-based WGA utilises random hexamers to initiate the amplification at random locations of the genome to allowing complete replication of the template and phi29 (φ29) DNA polymerase rapidly strings together deoxynucleoside triphosphates (dNTP) polymerising complimentary strands of DNA from the template strand. As the φ29 polymerase from one complimentary strand meets the end of a second complimentary strand, the end of the second strand is displaced while the other polymerase continues polymerisation of its strand. Simultaneously, free hexamers bind complimentary locations on complimentary strands and φ29 polymerase commences polymerisation on the complimentary strands thus producing a multitude of copies of the template DNA (Figure 16) [168, 169].

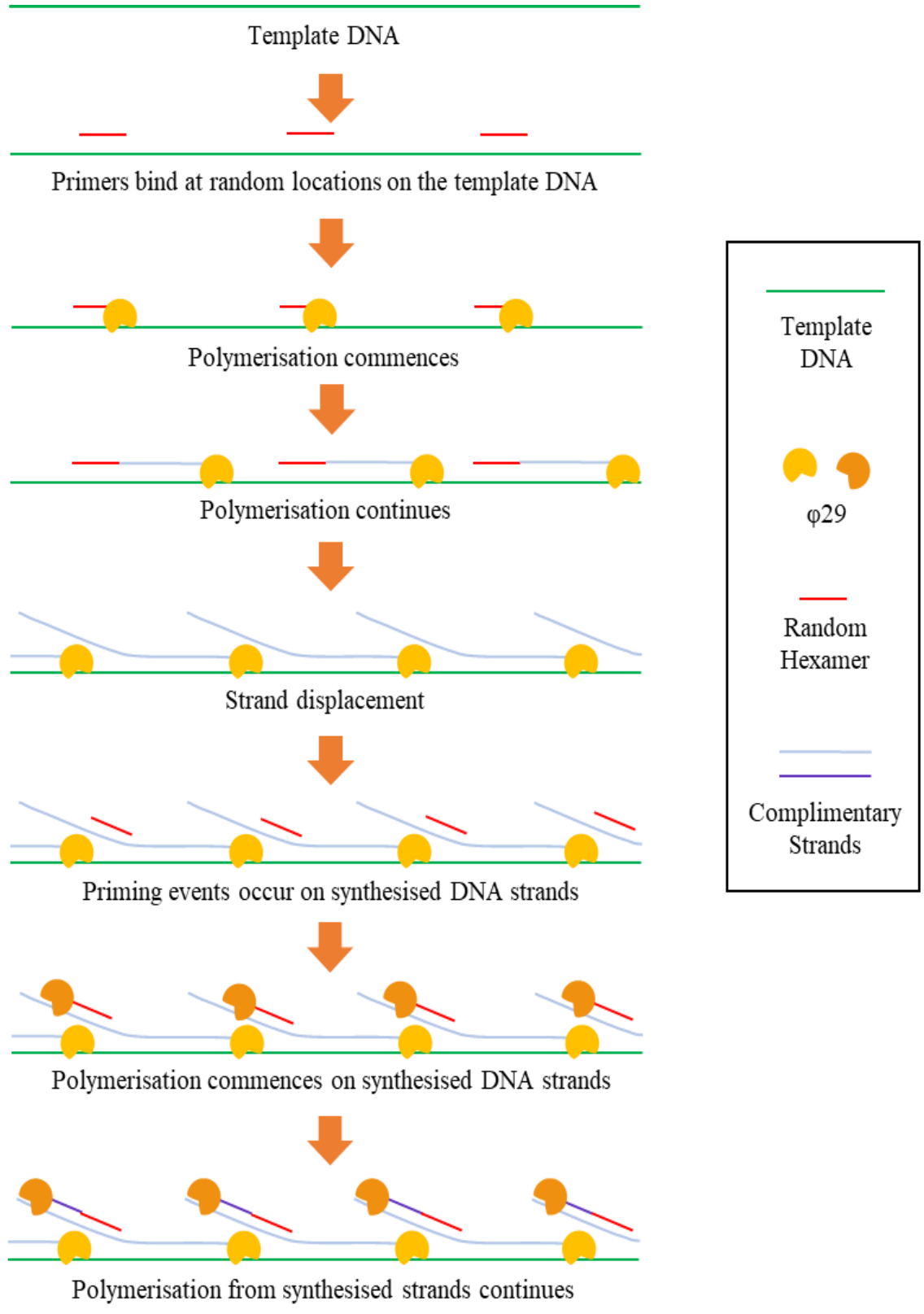


Figure 16: Schematic of the multiple displacement amplification process

MDA was first reported in 2002 [170] after the rolling circle amplification method was reported for the amplification of circular DNA such as that found in bacteriophages. This study [170] showed that similar components for the WGA of circular DNA could be used in the uniform amplification of linear DNA from humans. The result was a highly efficient amplification method [170, 171] that allowed for direct amplification of a number of biological specimens [170]; the generation of DNA products at least 10 kb in size [170]; with approximately locus representation of more than 100% [170, 171]. While Ballantyne *et al*. [172] report that MDA could potentially be used in forensic case work they also observed amplification bias, allele dropout and increased artefacts when the method was used. Hammond *et al*. [173] found that when the reaction proceeded in an emulsion with HFE-7500 (or the continuous phase) in bulk reactions and in picolitre (pL) droplets, amplification of the genome was more uniform. Rhee *et al*. [174] also reported better coverage of the genome when MDA was performed in nanolitre (nL) volumes. This would suggest that smaller reaction volumes would improve amplification due to less competition between the DNA templates for primer binding.

The use of the MDA technique is ideal for touch DNA as it can significantly boost the amount found in a few cells. In addition, if the continuous phase could match that used in fingermark detection, the technique may minimise degradation to both the DNA and fingermark traces. However, while the DNA is amplified, it cannot be detected using this method alone; a detection mechanism is still required to visualise and locate the DNA for further analysis. An advantage of using a detection mechanism in this technique is that the signal may grow as the DNA is amplified.

Hence, the aims of this study were to determine the feasibility of a MDA method for the detection of touch DNA and apply a fluorescent tag to the primers used in MDA to allow

for an *in situ* method for detecting touch DNA. To investigate this, a number of experiments were performed to answer the following questions:

Experiment 1: *Can MDA be applied to unpurified specimens?*

The objective was to determine if the MDA technique is capable of amplifying DNA that has not be extracted from cells. This presents a realistic scenario as, in the application of a dual detection technique, DNA cannot be collected and purified prior.

Experiment 2: *Could the formation of an emulsion and using a smaller volume assist in the amplification and detection of DNA?*

The objective of this experiment was to observe if amplification and detection of DNA from cells can be improved with the use of lysis buffers and similar techniques to droplet MDA.

Experiment 3: *Can MDA techniques be applied to porous and non-porous surfaces?*

The objective was to determine if the resulting MDA reagent from Experiments 1-3 could be applied to surfaces containing cells to allow for differentiation of the genetic material from the background.

3.2.2 Experiment 1: Can MDA be applied to unpurified specimens?

3.2.2.1 *Methods*

General Methodology

Experimentation was conducted on washed saliva cells to determine if the MDA reaction could be performed *in vitro* on low levels of DNA using an in-house preparation of the

MDA reaction mixture as described by Dean *et al*. [170]. Visualisation of the reaction was performed by running products on a 0.3% agarose gel and imaging under a gel reader.

Procedure

Collection of DNA

DNA was collected through saliva. This was considered the least invasive way to obtain a large amount of DNA what could be used as a spike for samples. Approximately 20 mL of saliva from one donor was collected in a 50 mL Falcon tube. 20 mL of PBS, prepared per Table 4, was added to the collected saliva before the mixture was shaken vigorously, then pelleted via centrifugation (bench top centrifuge, Rotina 380) for 10 minutes at 4,863 ×g. The supernatant was subsequently discarded and the washing process was repeated total of five cycles using 40 mL of PBS before being reconstituted in 10 mL of fresh PBS. The cell solution was then thoroughly mixed via vortex before ten 1 mL samples were aliquoted into 1.7 mL microtubes. A 100 µL sample from each tube was then extracted with QIAamp DNA Mini Kit using the *DNA Purification from Blood or Body Fluids (Spin Protocol) Protocol* [175] and the concentration of each was estimated using both the Plexor® HY System (Promega Corporation) and Quantifiler™ Trio DNA Quantification Kit (ThermoFisher Scientific) on a CFX96 Touch™ Real-Time PCR System (Bio-Rad Laboratories, Inc.).

Table 4: Preparation of PBS

Component	Concentration	Supplier	Grade
NaCl	137 mM	Sigma-Aldrich	American Chemical Society (ACS)
KCl	2.7 mM	Chem Supply	AR
Na_2HPO_4	10 mM	Chem Supply	AR
KH_2PO_4	1.8 mM	Chem Supply	AR

Application of MDA

The MDA reaction mixture was prepared according to Dean *et al*. [170] per Table 5. Firstly, the tris-HCl, MgCl, $(NH_4)_2SO_4$ were combined in water and sterilised via autoclaving at 121°C for 20 minutes. The required amount was then aliquoted into a 0.2 mL microtubes and before dNTPs, random hexamers and φ29 polymerase were added made to volume (50 μL) with sterile MilliQ water. Five tubes were prepared for initial experiments and cells were added to the concentration as described in Table 6.

Table 5: Preparation of MDA reagent

Adapted from Dean *et al*. [170].

Component	Concentration	Supplier	Grade
Tris-HCl	3.7 mM, pH 7.5	Sigma-Aldrich	ACS
KCl	50 mM	Chem Supply	AR
MgCl	10 mM	Merck Millipore	AG
$(NH_4)_2SO_4$	5 mM	Chem Supply	AR
dATP	1 mM	Thermo Scientific	99%
dTTP	1 mM	Thermo Scientific	99%
dGTP	1 mM	Thermo Scientific	99%
dCTP	1 mM	Thermo Scientific	99%
Random Hexamers	25 µM	Invitrogen®	50 µM
φ29 Polymerase	800 U / mL	Thermo Scientific	10 U / µL
MilliQ water	q.s. 100%	UTS	18.2 Ω, sterile

Table 6: Concentration of cells and DNA per 50 µL MDA reaction

Reaction	Number of cells	DNA concentration in 50 µL reaction (pg/µL)
Control	0	0
1	5	0.66
2	10	1.32
3	50	6.6
4	100	13.2

From each reaction 10 µL was sampled then stored at 4°C until analysis. The remaining samples were then incubated at 30°C for 18 hours in a shaking incubator (VorTemp

155™, Labnet International). At the conclusion of the incubation time, the reactions were terminated by heating to 65°C for 3 minutes. Samples were stored at 4°C until analysis.

In preparation for gel electrophoresis, 1x Trisaminomethane-borate-ethylenediaminetetraacetic acid (Tris-borate-EDTA; TBE) buffer was prepared per Table 7 with Tris, ETA and boric acid added to a vessel and dissolved with water. The buffer was then used to prepare the 0.3% agarose gel as shown in Table 8, where agarose powder was combined with the buffer and heated using a microwave until dissolved. Once cooled to approximately 60°C, Gel Red® (Biotium) was added and the mixture swirled to combine. The agarose mixture was then cast into a gel tray with the ends sealed with masking tape. A well comb was then inserted and all of the air bubbles were removed before the gel was allowed to set at ambient temperature. Once set, the comb and tape was removed and the gel and tray were placed into the Mini-Sub® Cell GT tank with PowerPac™ Basic Power Supply (Bio-Rad Laboratories, Inc.). The tank was filled with 1x TBE until the gel was covered.

Table 7: Preparation of 1x TBE buffer for electrophoresis

Component	Quantity	Supplier	Grade
Tris base	89 mM	Bio-Rad	99.8%
EDTA	2 mM	Sigma-Aldrich	≥ 99%
Boric acid	80 mM	Sigma-Aldrich	ACS
Milli-Q water	q.s. 100%	UTS	18.2 Ω

Table 8: Preparation of 0.3% agarose gel

Component	Quantity	Supplier	Grade
Agarose	0.24 g	Sigma-Aldrich	Molecular
1 x TBE	80 mL	Per Table 7	-
Gel Red®	8 µL (1x)	Biotium	10 000x

Prior to analysis, samples were prepared for loading as per Table 9. Thus 20 µL of each prepared sample was loaded to the wells of the agarose gel along with a 100 kb ladder and run for 8 minutes at 100V. The gel was then imaged under the under a gel reader (Molecular Imager® Gel Doc™ XR+ System with Image Lab™ Software, Bio-Rad Laboratories, Inc) using short wavelength UV.

Table 9: Preparation of samples for gel electrophoresis

Sample (µL)	Water (µL)	Loading buffer (µL)
10	7.5	2.5

3.2.2.2 *Results, discussion and conclusion*

As the 0.3% agarose gel contains a low agarose-to-buffer ratio, it is very weak. As a result it was destroyed during the transfer process from the tray to the gel reader prior to imaging. A gel was recast and electrophoresis was rerun using the remaining sample 18 hour reaction sample. 0 hour samples were omitted as none remained from the previous experiment. The gel was checked after 30 minutes, and 60 minutes then every 10 minutes thereafter, with the ladder (L) observed to have run to the other end of the gel after 80 minutes, indicating the electrophoresis had run to completion (Figure 17).

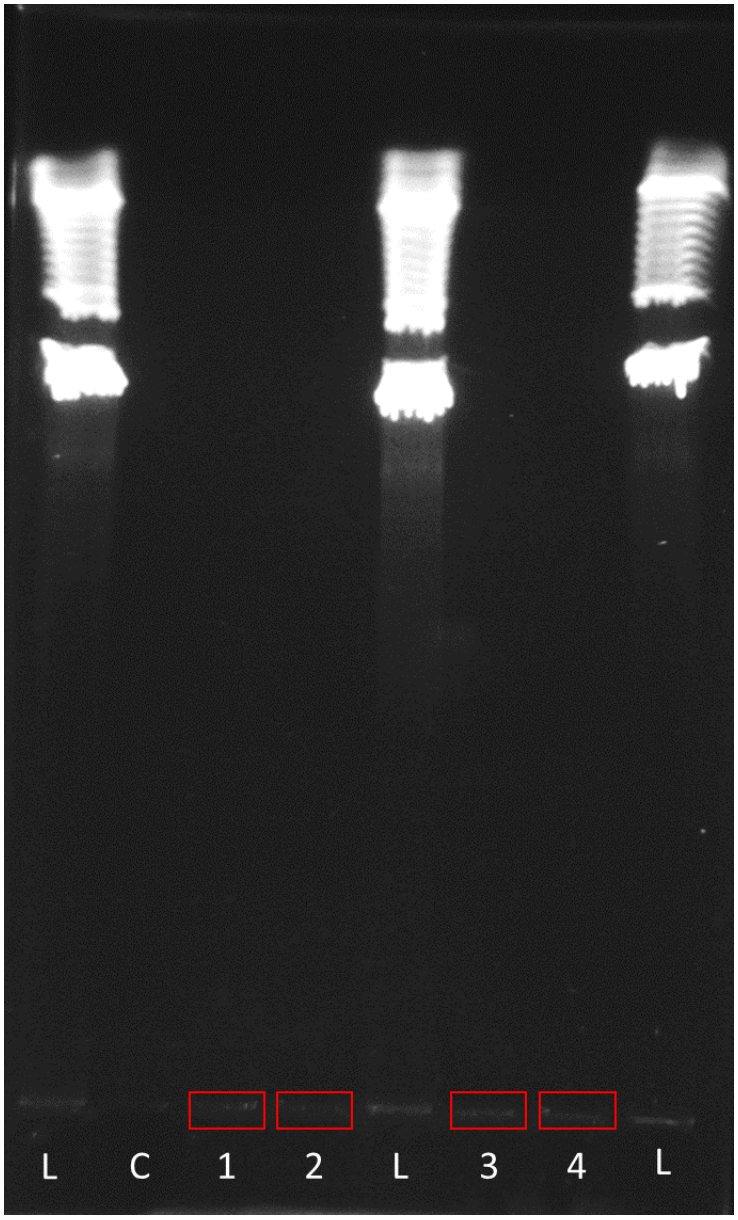


Figure 17: MDA reaction after 18 hours, run on 0.3% agarose gel and imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Red boxes indicate the presence of DNA from MDA reactions. L denotes 100 kb Ladder; Lane numbers correspond to reactions listed in Table 6.

The results indicate the presence of some DNA fragments from all reactions (1, 2, 3, 4) and none from the control (C) (Figure 17). However, the presence of DNA was observed as faint bands still within the loading wells only. It is difficult to ascertain if this DNA was a result of the reaction as 0 hour samples were not able to be run in this gel. However, due to the faintness of the bands it may indicate that the amplification was not very

efficient, possibly due to the lack of cell-free DNA. As the fragments did not migrate through the gel, this suggests that any amplification that occurred may have resulted in extremely large fragments (Figure 17).

The experiment was subsequently repeated. Again, during the transfer to the gel reader, this gel was also damaged. As mentioned earlier, the 0.3% agarose gel is weak and as 15-well-comb was used to cast the wells, the integrity of that portion of the gel was lacking resulting in a breakage. The remaining gel was salvaged and the results are as shown in Figure 18. Faint smears were observed for 18-hour samples and some 0-hour samples, that could indicate the MDA reaction proceeded. However, as the wells of gel were too small it was difficult to attribute the smears to the originating wells. Hence, the electrophoresis separation of 18-hour samples was repeated on an 8-well gel (Figure 19).

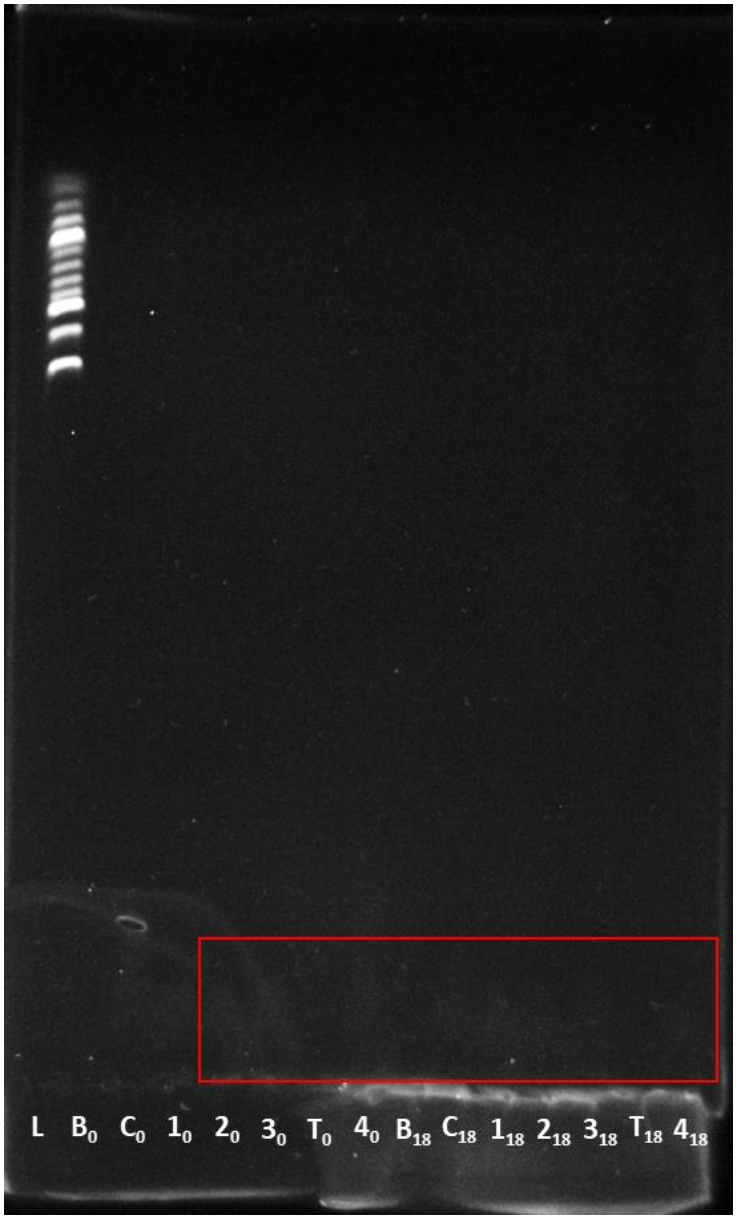


Figure 18: Rerun of 0 hour and 18-hour MDA reaction on 0.3% agarose gel imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Red box indicates the presence of smears. L denotes 100 kb Ladder; B denotes blank well loaded with water and loading buffer only; Lane numbers correspond to reactions listed in Table 6; subscript numbers denote sampling time.

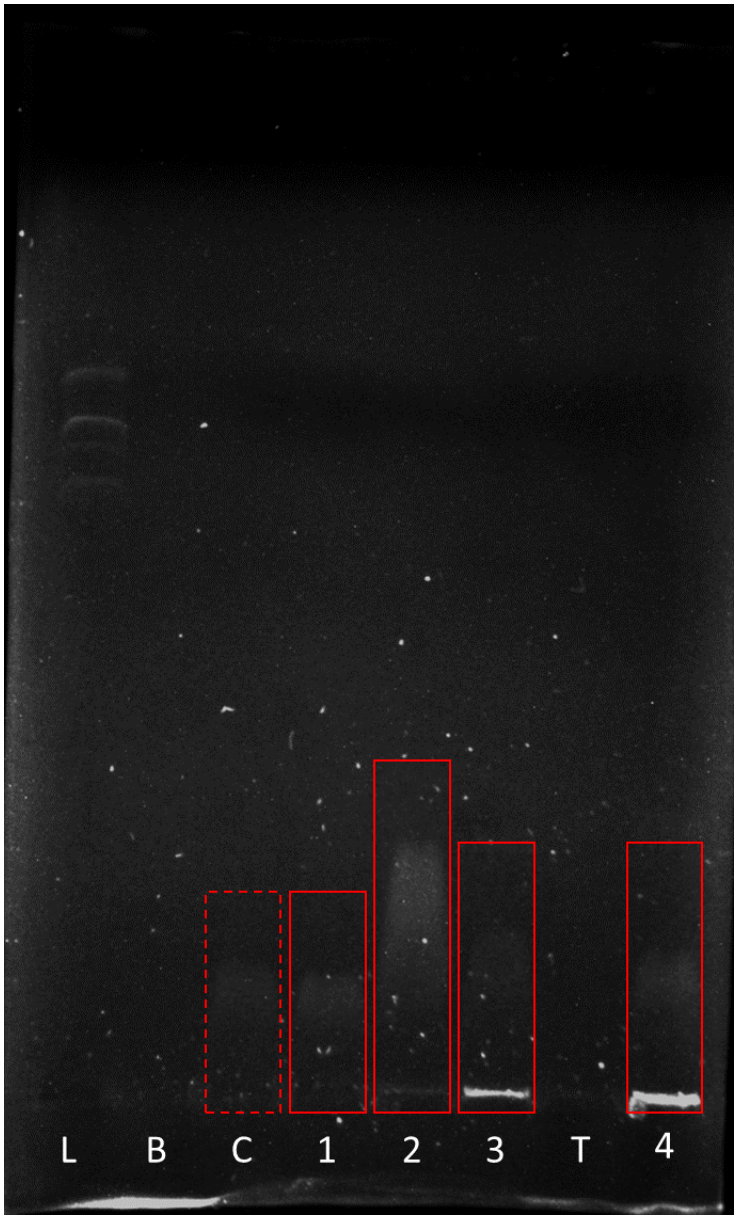


Figure 19: Reanalysis of 18-hour MDA reaction on 0.3% agarose gel imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Gel cast with 16 µL of Gel Red® (2x). Red boxes indicates the presence of smears and DNA containing wells. L denotes 100 kb Ladder; B denotes blank well loaded with water and loading buffer only; T denotes well loaded with a solution of 3.7 mM Tris-HCl and 6.6 ng/µL of DNA contained in saliva cells, incubated at 30°C for 18 hours. Lane numbers correspond to reactions listed in Table 6.

The reanalysis of MDA reactions was conducted on a 0.3% agarose gel with 16 µL (2x) Gel Red® to improve the enhancement of DNA present. Figure 19 shows the presence of definite smears from reactions 1, 2, 3, and 4. The presence of DNA was not observed in

the blank lane (B) showing that no DNA was present in the water or loading buffer. Additionally, the presence of DNA was not in the Tris-HCl buffer lane (T), indicating that amplification only occurs in the presence of the polymerase and dNTPs. This is observed in the control (C) where a faint smear was seen while no template DNA was added to the MDA reaction mix. This suggests that the reaction is very sensitive to contamination as all measures to minimise contamination were taken during this experiment. Lanes 1 and 2, with 0.66 pg/µL and 1.32 pg/µL of DNA respectively, showed some amplification. Lane 2 also showed the longest smear, implying that a range of large fragment sizes was generated. Lanes 3 and 4, with 6.6 pg/µL and 13.2 pg/µL of DNA respectively, showed fainter smears but had intense bands in the respective loading wells, indicating significant DNA amplification and large fragment sizes that would not migrate through the agarose matrix (Figure 19).

The results of this experiment signifies that the MDA reaction can be performed on DNA without prior extraction and purification. However, it also shows that the sufficient amount of DNA detecting dye needs to be applied before any visible reaction can be clearly observed. This may mean that if this technique is successful, a two-step application may be required in order to both amplify the DNA and then to detect the signal.

3.2.3 Experiment 2: Could the formation of an emulsion and using a smaller volume assist in the amplification and detection of DNA?

3.2.3.1 *Methods*

General Methodology

To determine if 'droplet' MDA techniques could be used to improve amplification and detection of DNA, a MDA reaction mixture from Rhee *et al*. [174] was adapted and applied to cells both with a "continuous phase" (to form an emulsion) and without. Following the reaction time, the products were run on a 0.3% agarose gel and imaged under a gel reader to determine how an emulsified MDA reaction compares to a conventional MDA reaction.

Procedure

Buffers used in Experiment 2 were prepared as described in Section 3.2.2.1. The droplet MDA reaction mixture was prepared per Table 10, adapted from Rhee *et al*. [174]. The MDA master mixture was prepared per Table 10. Tris-HCl, KCl, $MgCl_2$ and $(NH_4)_2SO_4$ were dissolved in sterile water before dNTPs, random hexamers, PEG, DMSO, Gel Red® (Biotium) and polymerase were added. The reaction mixture was then made to volume (300 µL) with water. Eight 20 µL aliquots of the reaction mixture were dispensed into 0.2 mL microtubes. To four of the aliquots, 20 µL of continuous phase (prepared per Table 11) was added while the remaining reactions were run 'neat' to allow for a comparison of how the emulsion phase impacts the reaction. The formulation for the continuous phase was based on that from Rhee *et al.* [174]. HFE-7100 was used in place of HFE-7500. These are both fluorinated solvents, however HFE-7500 was not able to be obtained for

this study, while HFE-7100 was available. Both HFE solvents are in the process of being phased out [176].

Table 10: Preparation of droplet MDA master mix

Adapted from Rhee *et al.* [174]; *Gel Red® was used in place of SYBR Green [174].

Component	Concentration	Supplier	Grade / Purity
Tris HCl	40 mM. pH 7.5	Sigma-Aldrich	ACS
KCl	50 mM	Chem Supply	AR
MgCl	10 mM	Merck Millipore	Analysis grade (AG)
$(NH_4)_2SO_4$	5 mM	Chem Supply	AR
dNTP (A, C, G, T)	0.5 mM (each)	Thermo Scientific	99%
Random Hexamers	25 µM	Invitrogen®	50 µM
φ29 Polymerase	1 U / µL	Thermo Scientific	10 U / µL
Poly(ethylene glycol) (PEG) 400	4% w/v	Sigma-Aldrich	-
Dimethyl sulfoxide (DMSO)	2% v/v	Sigma-Aldrich	≥ 99.9%
Gel Red® *	10x	Biotium	10 000x
MilliQ water	q.s. 100%	UTS	18.2 Ω, sterile

Table 11: Preparation of continuous phase for MDA emulsion

Adapted from Rhee *et al.* [174]; ǂPolysorbate 20 was used in place of PicoSurf®; *HFE-7100 was used in place of HFE-7500.

Component	Concentration	Supplier	Grade / Purity
Polysorbate 20ǂ	2% w/v	Chem-Supply	Laboratory reagent
HFE-7100*	q.s. 100%	3M	Engineered Fluid

Cells collected in Section 3.2.2.1 were used in this experiment. One microtube of cell solution was diluted to allow for 1 µL of the approximate required number of cells to be added to each MDA reaction as per Table 12. The reaction was incubated at 30°C for 18 hours following which the reactions were terminated by heating to 65°C for 3 minutes. The samples were then stored at 4°C until analysis. The samples were prepared for analysis per Table 9 and run on a gel prepared per Table 13 and imaged under the gel reader under short wavelength UV.

Table 12: Concentration of cells and DNA per MDA reaction

N denotes neat reaction (i.e. continuous phase not included in reaction); E denotes emulsion reaction (i.e. continuous phase included in reaction); C denotes control sample.

Reaction	Number of cells	DNA concentration per reaction (pg/µL)
NC	0	0
N1	20	6.6
N2	100	33
N3	200	66
EC	0	0
E1	20	3.3
E2	100	16.5
E3	200	33

Table 13: Preparation of 0.3% agarose gel for Experiment 2

Component	Quantity	Supplier
Agarose	0.24 g	Sigma-Aldrich
1 x TBE	80 mL	Per Table 7

3.2.3.2 *Results, discussion and conclusion*

As shown in Figure 20, little to no reaction product is observed from neat reactions (N1 – N3). These reactions differ from those in Experiment 1 by the addition of Gel Red® to the MDA reaction rather than to the gel and lower concentrations of dNTPs in the reaction while also including higher DNA concentrations than those from Experiment 1. Thus, there may have been insufficient dNTP as well as inefficiency at this volume of reaction. However, in emulsified reactions bands are observed for E2 and E3, at 16.5 pg/µL and 33 pg/µL, respectively. As a band is not observed for the emulsion control (EC), or E1 (3.3 pg/µL), where the concentration of DNA may be low, the luminescence does not come from the presence of HFE-7100 or polysorbate 20. Rather it can be attributed to the formation of the emulsion and an increase in efficiency when the reaction proceeds in a smaller volume due to a decrease in competition by the DNA for the core components of the replication process, resulting in more efficient replication [174].

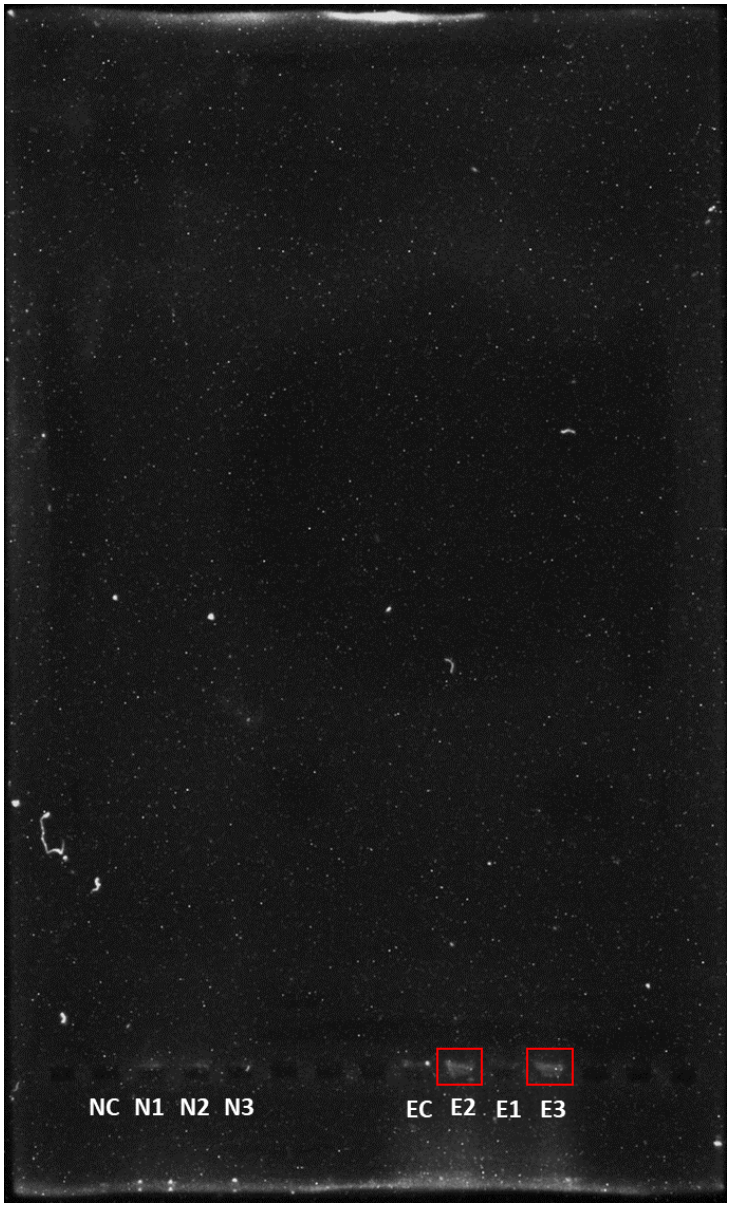


Figure 20: MDA reactions compared to "droplet" MDA reactions imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Samples are as per Table 12; N denotes neat reaction (i.e. continuous phase not included in reaction); E denotes emulsion reaction (i.e. continuous phase included in reaction); C denotes control sample. E1 and E2 are not in chronological order as E1 was aliquoted in the incorrect well due to oversight. E2 was subsequently aliquoted into the well preceding the E1 sample.

Hence, Experiment 2 shows that forming an MDA emulsion can result in increased amplification. Additionally, the compatibility of the MDA reaction mixture with HFE7100 is promising as it allows for the potential to combine this reagent with a

fingermark reagent, as HFE-7100 is a carrier solvent in a number of fingermark detection reagents for porous surfaces. While the method is successful *in vitro*, whether the technique can be applied to non-porous and porous surfaces needs to be determined.

3.2.4 Experiment 3: Can MDA techniques be applied to porous and non-porous surfaces?

3.2.4.1 *Methods*

General Methodology

Remaining sample from Experiment 2 were initially spotted onto glass and paper and visualised under the gel reader to determine if, post-reaction, the amplified DNA was visible on these surfaces. The optimal working concentration of Gel Red® (Biotium) was then determined to minimise background luminescence. Following this, cells were spotted onto glass and copy paper surfaces and the MDA reaction mixture was applied. The reactions were incubated with gentle agitation for 18 hours then visualised using the gel reader and Poliview® IV system to determine if DNA could be detected.

Procedure

The buffers used in Experiment 3 were prepared as described in Section 3.2.2.1 and MDA and MDA continuous phase were prepared per Table 10 and Table 11, respectively, as described in Section 3.2.3.1.

In this experiment the reacted solutions in Experiment 2 (Table 12) were gently vortexed before 2 µL of each solution was spotted onto copy paper and a glass microscope slide. The samples were allowed to dry under laminar-flow before being viewed under the gel reader under short wavelength UV. The concentration of Gel Red® (Biotium) in the

reaction mixture was then optimised. Firstly, the buffer mixture (MDA placebo) as described in Table 14 was prepared. To an aliquot of the buffer mixture, Gel Red® was added to a concentration of 10x then serial diluted down to a concentration of 0.01x per Table 15 and vortexed following each addition of Gel Red®. To each Gel Red® solution the equivalent amount of continuous phase prepared in Section 3.2.3.1, Table 11, was added. 1 µL of each final Gel Red® solution was spotted onto copy paper, allowed to dry, then imaged under the gel reader using short wavelength UV.

Table 14: Preparation of MDA buffer solution (MDA placebo)

Component	Grade	Concentration
Tris HCl	ACS	40 mM. pH 7.5
KCl	AR	50 mM
MgCl	AG	10 mM
$(NH_4)_2SO_4$	AR	5 mM
MilliQ Water	18Ω, sterile	q.s 100%

Table 15: Serial dilution of Gel Red® in MDA buffer solution

Gel Red® (GR) Conc.	Amount of Buffer (µL)	Amount of GR Solution
10x	990	10 µL GR 10,000x GR stock
1x	900	100 µL GR 10x
0.1x	900	100 µL GR 1x
0x	1000	0 µL GR

Once the concentration of Gel Red® was determined, the MDA placebo was prepared per Table 14 with the optimised amount of Gel Red®, then combined with the continuous

phase described in Table 11 (Section 3.2.3.1). 2 µL of extracted DNA at the concentrations specified in Table 16 were spotted onto glass slides and copy paper to determine the feasibility of the visualisation method.

Table 16: Concentration of DNA spotted onto copy paper and glass

Sample Number	Concentration of DNA per spot
Control	0
1	0.132 ng
2	0.66 ng
3	1.32 ng
4	6.6 ng
5	13.2 ng
6	66 ng
7	132 ng

The application of the an MDA mixture prepared per Table 10 with Gel Red® concentration as determined from Table 15 was then performed on paper surfaces to determine if it was possible for the reaction to proceed on this substrate. 0.66 ng of extracted DNA was applied to copy paper, in duplicate, before the 20 µL of MDA emulsified reaction mixture was applied on top. A blank sample, consisting of the 20 µL MDA emulsified reaction mixture, and a control (20 µL), consisting of the 0.66 ng of DNA in MDA placebo mixture and assessed alongside the MDA reactions. The reactions was performed in a glass petri dish, with the petri dish inserted into the VorTemp 1550™ Shaking Incubator at 30°C for 18 hours with gentle agitation. Results were then viewed under the Poliview® IV System (Polilight PL500, Peltier Cooled 12 bit CCD camera and

lens, with Poli-V®++ software, Rofin Australia) under the conditions for Gel Red® (ex: 390 nm; em: 610 nm).

3.2.4.2 *Results, discussion and conclusion*

Following the application of MDA reacted DNA to paper and glass, it was clear that the spotted samples were visible under short wavelength UV when imaged under the gel reader (Figure 21 and Figure 22). However, it should be noted that the control samples (NC and EC) did not contain any DNA and the DNA from neat reactions (N) and the lowest concentration emulsion sample (E1) were not visible when run on a agarose gel and imaged under the gel reader. Additionally, no difference in intensity of the luminescence was observed on paper (Figure 21). However on glass (Figure 22), while no difference in luminesce was observed in neat samples (N), luminescence tended to be found to the aqueous portions of the emulsified samples (E) where the dye would be localised. The results suggest that the concentration of Gel Red® dye is causing background luminescence. Hence, investigations into Gel Red® concentrations were performed in order to limit this and allow for an indication of DNA.

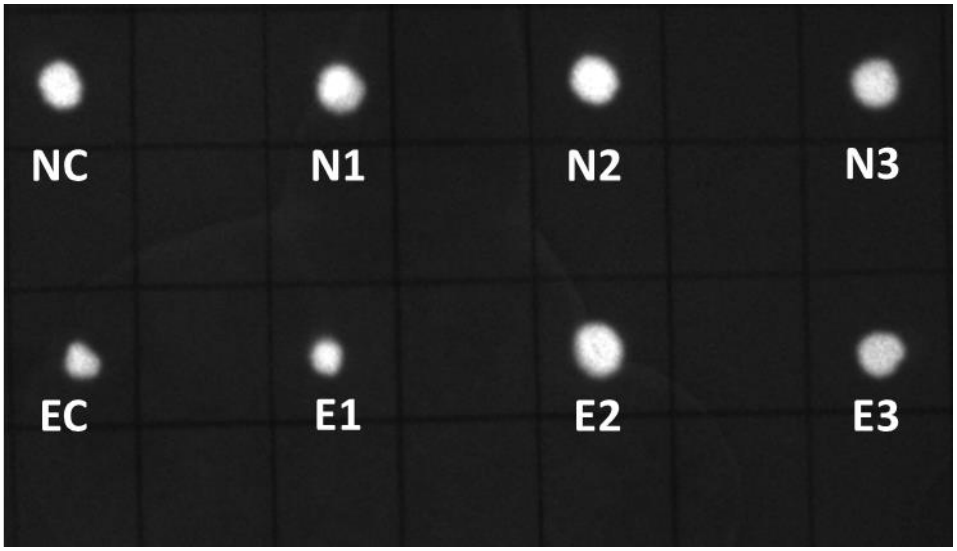


Figure 21: Reacted MDA reactions from Figure 20 spotted onto copy paper imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Samples are as per Table 12; N denotes neat reaction (i.e. continuous phase not included in reaction); E denotes emulsion reaction (i.e. continuous phase included in reaction).

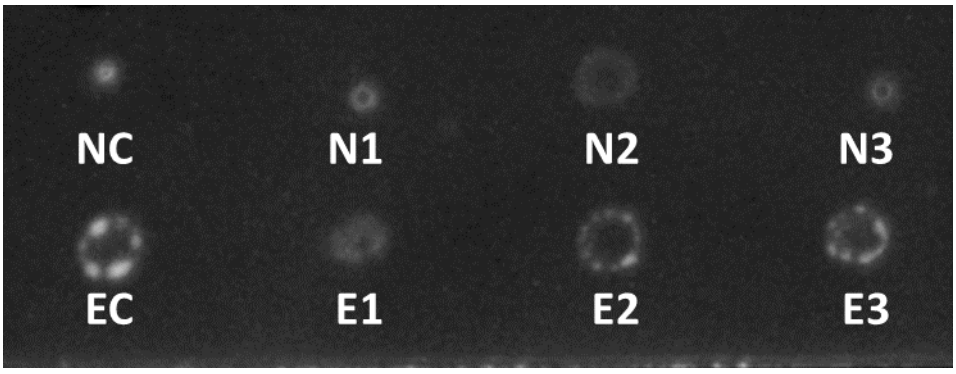


Figure 22: Reacted MDA reactions from Figure 20 spotted onto a glass microscope slide with the Molecular Imager® Gel Doc™ XR under short wavelength UV

Samples are as per Table 12; N denotes neat reaction (i.e. continuous phase not included in reaction); E denotes emulsion reaction (i.e. continuous phase included in reaction).

The MDA placebos with the various Gel Red® concentrations (Table 15) were spotted onto paper only as this showed the strongest luminescence and even distribution of luminescence. The results indicate that without DNA present in the sample, Gel Red®

was visible at 10x (as used in Experiment 2, Section 3.2.3) and 1x. Little to no luminescence is observed at 0.1x. This indicates the concentration of 0.1x Gel Red® should limit background luminescence.

Figure 23: MDA placebo with Gel Red® at concentrations between 0 and 10x spotted onto copy paper and imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

The MDA placebo was prepared to avoid wastage of the expensive active. It was thought that the application of the placebo to increasing concentrations of extracted DNA would increase in intensity as the Gel Red® in the mixture would interact with the DNA giving a luminescent reaction visible under short wavelength UV conditions. It was observed when DNA was spotted onto paper per Table 16 and MDA placebo applied, no spots corresponding to the deposition were seen when viewed under the gel reader Figure 24. However, using the Poliview® system to visualise the same samples, luminescent spots were observed for all DNA samples (Figure 25; 1-7). The intensity of sample 1 appears lower than that of samples 2 – 7, while little difference in intensity can be discerned between samples 2 – 7. It should also be noted that while the same experiments were conducted on glass microscope slides, no luminescent spots were observed on this surface when visualised using the Gel reader or Poliview® (images not shown as no distinctive areas are present on the substrate).

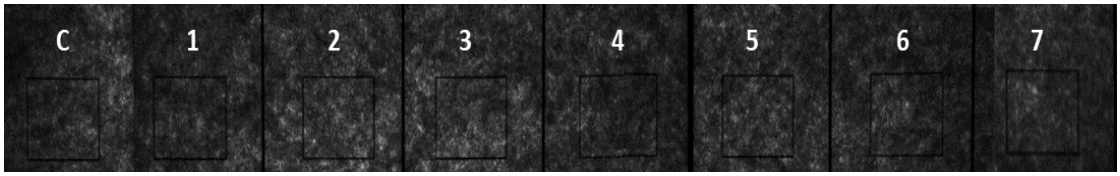


Figure 24: MDA placebo applied to DNA spots (Table 16) on copy paper imaged with the Molecular Imager® Gel Doc™ XR under short wavelength UV

C denotes Control and numbers indicate the concentration of DNA per Table 16.

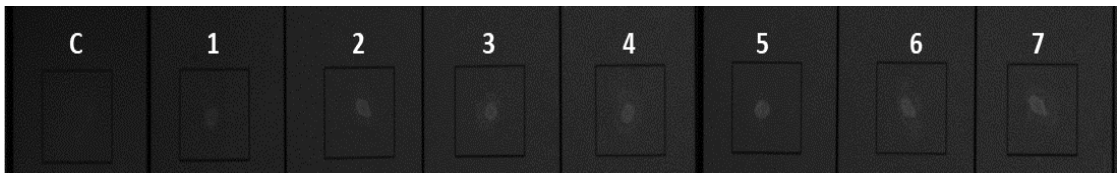


Figure 25: MDA placebo applied to DNA spots (Table 16) on copy paper imaged with the Poliview® IV System

Visualisation conditions: 390 nm excitation; 610 nm interference bandpass barrier filter. C denotes Control and numbers indicate the concentration of DNA per Table 16.

When the MDA emulsified reaction mixture was added to samples 1 and 2 with 0.66 ng extracted DNA, amplification of the DNA was expected with luminescence expected in these samples only. This concentration of DNA was expected to be low enough so that any luminescence could be attributed to amplification and high enough so that sufficient DNA was present in the paper substrate to allow amplification to occur. The luminescence of samples 1 and 2 are similar with sample 2 showing a reaction smaller area (Figure 26). However, the luminescence of the control sample with no MDA actives is also similar to samples 1 and 2. This would indicate that no amplification occurred and the luminescence is due to the original amount of DNA applied to the substrate.

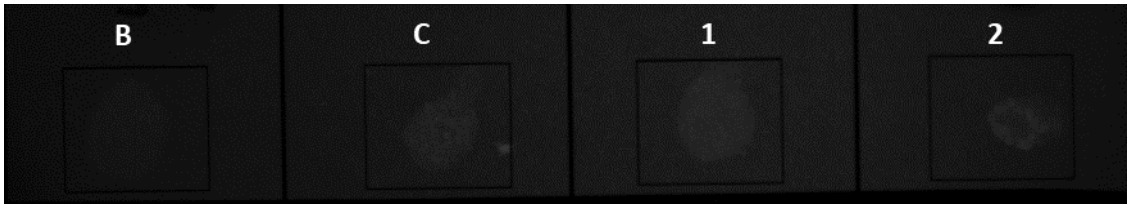


Figure 26: Assessment of MDA reaction on copy paper imaged with the Poliview® IV System Visualisation conditions: 390 nm excitation; 610 nm interference bandpass barrier filter. B denotes blank sample, C denotes Control sample and 1 & 2 denote duplicate samples of MDA emulsified reaction mixture applied to 50 ng of DNA.

3.2.5 Conclusions about MDA

The experiments performed in this Section have demonstrated that the MDA can be performed on cellular samples without prior extraction. It has also been seen that smaller volumes of MDA reaction and the formation of an emulsion can improve the detection capabilities of the technique. However, this technique is associated with a long incubation period (18 hours) and, due to the high cost of this method, experiments to combine MDA with a fingermark detection reagent were not conducted. While the method is capable of amplifying cellular DNA to allow for detection *in vitro*, it does not seem possible to perform these experiments and visualise the results *in situ*, even on extracted DNA.

In addition to this, this method is costly. The cost 20 µL of MDA reaction mixture is in excess of $ 60 (Australian dollars (AUD)). This figure covers only random hexamers, φ29 Polymerase and dNTPs only and does not account for DNA profiling process that will need to be conducted following a positive indication for DNA. DNA extraction with QIAamp DNA Minikit costs approximately 7 AUD per sample, quantification with Plexor HY costs approximately 4.5 AUD per sample with an additional seven standards required with any run (i.e. total 36 AUD) and profiling with PowerPlex® 21 incurs a cost of approximately 21 AUD per sample. Hence, it would be more cost effective and

informative to swab a suspected area of DNA and process for DNA and profiling than to apply MDA techniques prior. It is, however, recognised that cost efficiency does not necessarily capture the complete requirements as there are also benefits by being more selective and avoiding inundating forensic DNA laboratories with swabs that irrelevant or poor quality DNA traces.

These experiments have highlighted the extreme difficulties and costs associated with detecting DNA. In the detection of DNA, there is the difficulty of penetrating the cell to access cellular DNA. In addition, the DNA not only has to be distinguished, but the signal needs to be sufficient to be noticed with the use of relatively uncomplicated equipment. As observed in Section 3.1, a number of stains showed the presence of cells. While the presence of cells does not necessarily equate to DNA, it could perhaps give an indication of the presence of DNA and improve the rate of fuller DNA profiles. This was further investigated in Chapter 4.

Chapter 4. INVESTIGATION INTO DUAL DETECTION OF FINGERMARKS AND CELLS TO AID IN RECOVERY OF TOUCH DNA

4.1 One-Step Luminescent CAs for the Detection of Cells on Glass

4.1.1 Introduction

The detection of cells presents advantages over the detection of DNA in forensically relevant samples. The nucleus is housed within the cell, thus making cells more readily visible under magnification. Methods to visualise cells are also less specific than that for DNA, thus making cell detection techniques more affordable than that for DNA detection. A molecule that has gained increasing interest in the area of fingermark detection over recent years is *para*-dimethylaminobenzaldehyde (DMAB). Currently, DMAB is used in two commercial one-step luminescent CAs as the luminescent agent, while it has also been investigated as a fingermark detection reagent for porous surfaces.

In PolyCyano UV (Foster+Freeman) and PECA Multiband (BVDA) one-step luminescent CAs, DMAB is incorporated with the CA and fumed simultaneously. During fuming, it appears luminescent fingermarks are formed as the vaporised CA monomers

react with the fingermark residue building a polymer along the ridges. The vaporised DMAB is then attracted to the CA [177] and becomes trapped within the growing polymer [107]. Thus, in this respect, the DMAB is believed to have high affinity to the CA as the source of nitrogen, however, it may still have interactions with the cells present in the fingermark. DMAB has a boiling point of 176°C [178] while PolyCyano UV and PECA Multiband both require a temperature of 230°C to be vaporised. This suggests that some DMAB may vaporise prior to the bulk of the mixture, thus may access and interact with any cells present prior to the deposition of CA. This could render the cells visible under luminescence. Another one-step luminescent dye is Lumikit™. Little is known about the dye compound used in Lumikit™ ($C_4H_5ClN_4O$), with no information recorded about its physical and chemical properties other than it being a pungent powder [179]. However, along with PolyCyano UV, Lumikit™ claims to be compatible with DNA [118, 180]. It may be that Lumikit™ powder also has the potential to interact with cellular material and present luminescent cells.

Hence, the initial aim was to answer the following question:

Can one-step luminescent CAs be used to visually detect both fingermarks and sources of touch DNA on non-porous surfaces?

Studies have been completed to address how well PolyCyano UV, PECA Multiband and Lumikit™ perform in terms of fingermark development [111-113, 115, 181]. To investigate their potential for the detection of cells, fingermarks deposited on cells were fumed with the respective CA and examined under fluorescent microscopy.

4.1.2 Methods

General Methodology

Fingermarks were deposited onto glass microscope slides (gridded, uncoated, ProSciTech), viewed under microscope (Leica DM4 M, Leica Microsystems™ Pty Ltd), and left to age for approximately one day, prior to being fumed in a fuming chamber (MVC1000/D fuming cabinet, Foster+Freeman Ltd) with the respective one-step CA (PECA Multiband, BVDA; PolyCyano UV, Foster+Freeman Ltd; Lumikit™, Crime Science Technology). Following fuming, the fingermarks were visually examined to ensure CA development. Developed fingermarks were then viewed under fluorescent microscopy to determine the presence and luminescence of cells. To confirm the luminescence of the cells was due to the staining agent, an aliquot of cells on a glass microscope slide was fumed with the staining agent (DMAB, Fisher Scientific Co) only in a distillation column setup, then viewed under brightfield and fluorescent microscopy (Leica DM4 M with Leica LQ-HXP 120-L-Multipole external light source and Leica H3 filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm) Leica Microsystems™ Pty Ltd).

Procedure

Four donors ranging from weak to strong fingermark secretors were each asked to conduct their usual activities prior to depositing six natural fingermarks onto uncoated grid microscope slides, but refrain from washing their hands for at least 20 minutes before the deposition event. After deposition, the fingermarks were viewed at 50 times magnification under brightfield to determine the presence of cells prior to fuming. Following the initial visualisation, the fingermarks were left to age in a wooden box for

approximately 24 hours prior to being fumed with the respective one-step luminescent CA in the MVC1000 fuming chamber per the conditions listed in Table 17.

Table 17: Fuming conditions for one-step luminescent CAs in a MVC1000 fuming chamber

Conditions	PECA Multiband	PolyCyano UV	Lumikit™
Temp (°C)	230	230	120
Mass (g)	0.2	0.6	0.4 (0.5 % w/w)
Time (minutes)	15	15	15
Humidity (%)	80	80	80

After fuming, the fingermarks were viewed with ambient laboratory conditions to ensure the presence of CA. Once confirmed, the fingermarks were viewed again at 50 times magnification under brightfield then again under fluorescence with a H3 filter cube. The luminescence of cells was determined by comparison to images of the corresponding pre-fumed fingermark.

The cell solution prepared in Section 3.2.2.1 was used to investigate whether the staining agent had an affinity to the cells. The cell solution was firstly thawed on ice, then gently vortexed before a 10 µL aliquot of the solution was dispensed onto a clean glass microscope slide, in duplicate. The slides were gently shaken to disperse the solution, then allowed to dry overnight in a plastic container, protected from light. To fume, 0.05 g of DMAB was dispensed into a round bottom flasks, samples were placed into a distillation column before being connected to the round bottom flask then a glass stopper placed over on the top end of the column. The round bottom flask was then heated to 180°C for 30 minutes to vaporise and disperse the DMAB. Cells were then viewed at

100 times magnification under brightfield, then again under fluorescence with a H3 filter cube.

4.1.3 Results, discussion and conclusion

Cellular material was visible in pre-treated fingermarks visualised under brightfield as indicated by the red arrows (Figure 27; A1 – C1). It should be noted that these arrows only indicate a few of the cells present in the fingermark image. Cells can be seen to generally be present on the deposited ridges of the fingermark and can still be seen under brightfield following fuming (Figure 27; A2 – C2). When viewed under fluorescence (Figure 27; A3 – C3), these cells appear to have up taken the staining agent in the CAs and become luminescent.

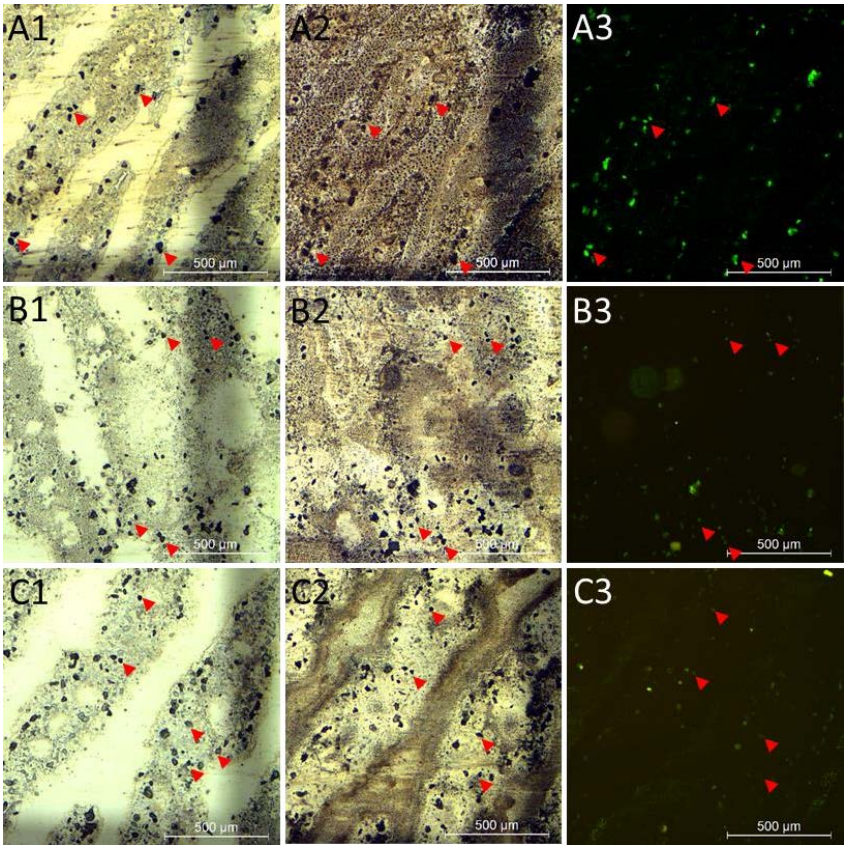


Figure 27: Luminescent cells following one-step luminescent treatment of fingermarks on glass surface

One-step luminescent CA treatments: PECA Multiband (A), PolyCyano UV (B), Lumikit™ (C); Fingermarks viewed at 50 times magnification: untreated (1), fumed with the respective one-step luminescent CA (2) both viewed under bright field (incident light), then under fluorescence using H3 filter cube (3): imaged with Leica DM4 M microscope.

Following fuming with PECA Multiband (Figure 27; A3) cells presented with strong luminescence, requiring a short exposure time (ex: 160 millisecond; aperture: 9; intensity: 20). Cells were visible following PolyCyano UV fuming (Figure 27; B3) however luminescence was weaker and increased exposure time was required (ex: 1 second; aperture: 9; intensity: 20), while cells were weaker still after being fumed with Lumikit™ (Figure 27; C3) (ex: 1.5 seconds; aperture: 9; intensity: 20).

There appears to be little affinity between the staining agent in Lumikit™ and cells, with these being difficult to visualise. Again, little information is available on the $C_4H_5ClN_4O$

staining agent in Lumikit™ for an accurate conclusion on the dye to be made. The intense luminescence in PECA Multiband treated cells could be due to the concentration of DMAB being 15% [182], compared to 5% in PolyCyano UV [117], where the luminescence of the cells was notability weaker than that of the former. However, it should be noted that three times as much PolyCyano UV (0.6 g) is recommended in a fuming cycle compared to PECA Multiband (0.2 g) thus in each fuming cycle the same about of DMAB should be present. Due to the smaller amount required in PECA Multiband it may be possible that the smaller surface area of the mixture and higher concentrations of DMAB, more of the dye is vaporised before the CA allowing earlier access to the cells. Alternatively, less CA in the PECA Multiband fuming cycle may result in less obstruction by the CA when viewing the samples under luminescence mode.

To determine if the luminescence of the cells was due to DMAB, fuming with the staining agent alone was conducted. Initially, fuming was conducted in the MVC1000 fuming chamber with 0.03 g DMAB (the equivalent amount of DMAB in each fuming cycle of PECA Multiband and PolyCyano UV), for 15 minutes at 230°C, 80% relative humidity (RH). However, luminescent cells were not visible following staining. It was thought that the ratio between the MVC1000 fuming chamber and the small amount of DMAB may have rendered it ineffective. The amount of DMAB was increased to 0.05 g, however the same result was obtained, while it was noticed that the fuming chamber became yellow in the interior, suggesting that DMAB was adhering to residual CA in the chamber, despite being thoroughly cleaned prior to use.

Fuming experiments were then conducted using laboratory glassware, where 0.05 g of DMAB was fumed in a round bottom flask. The round bottom flask was connected to a stoppered Soxhlet extractor and samples were placed through the percolator to area where the thimble would sit of the extractor. Little to no luminescence was observed following

30 minutes of fuming at 180°C. It was unclear whether the vaporised DMAB was carried through the distillation tube and remaining at the top of the extractor as no condenser was connected, or if the vaporised DMAB cooled at the top of the extractor, thus the condensed DMAB did not make contact with the cells on the way back down the percolator.

Thus, the process was repeated with a distillation column in place of the Soxhlet extractor. This allowed for direct contact between the DMAB vapour and the cellular material deposited on the glass slide. From Figure 28 luminescence can be seen from cells following the fuming process. However, this luminescence was weak in comparison to that seen following treatment PECA Multiband and PolyCyano UV (Figure 27) despite the higher relative concentration of DMAB used and the smaller fuming volume. This suggests that the CA plays a role in enhancing the luminescence of the DMAB treated cells when fumed, possibly by attracting DMAB molecules to the CA polymer as it forms over cells.

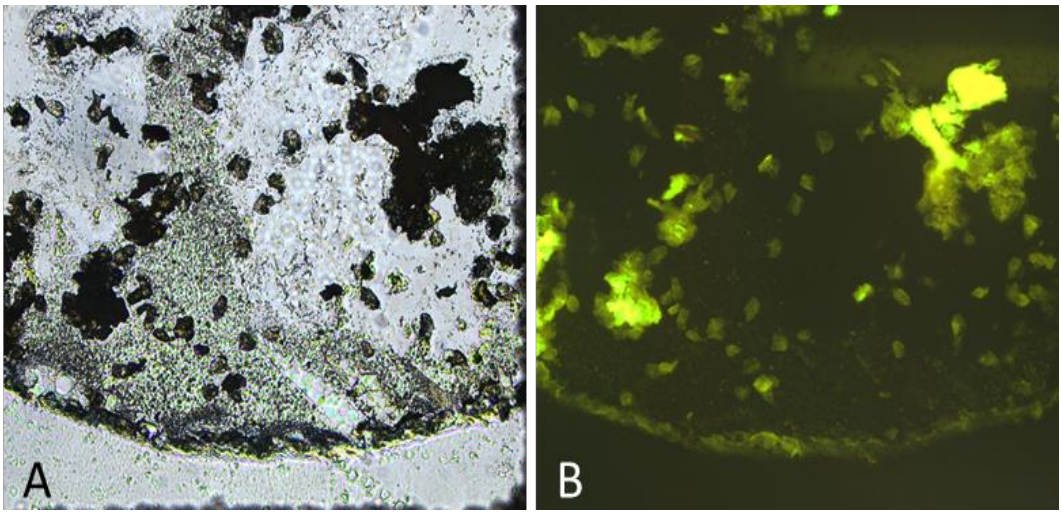


Figure 28: Cells deposited on a glass microscope slide fumed with DMAB in a distillation column set-up viewed at 100 times magnification under brightfield (A) and fluorescence mode with H3 filter cube (B)

While PECA Multiband gave the most intense luminescence of cells, at the time of these investigations, this product was not widely available and only a sample was able to be obtained. Instructions for use were obtained through personal communications from BVDA International BV [182, 183] and no claims regarding DNA compatibility were made by the manufacturers at the time of the investigation, nor at the time of writing. Hence, investigation into the compatibility of PECA Multiband with DNA processing was not continued in Chapter 5, while the compatibility of PolyCyano UV and Lumikit™ with DNA has been stated [117, 118] and, thus, was further evaluated.

The ability of these one-step luminescent CAs to develop fingermarks was not investigated as part of this project as their fingermark development capabilities have been reported previously [111-113, 115]. DMAB has been shown to indicate the presence of cells on glass when fumed and the resulting luminescence appears assisted by the presence of CA. Its use to detect fingermarks on paper was reported by Fritz *et al*. [184], however it was outside the scope of the study to determine its impact on touch DNA. The ability of DMAB to detect cells deposited on paper by touch will be described in Section 4.2.

4.2 The Use of DMAB for the Detection of Cells and Fingermarks on a Paper Surface

4.2.1 Introduction

The traditional use of DMAB is in the field of histology where it is employed to detect primary and secondary amines [185]. Following a condensation reaction with DMAB, amines form a Schiff base which is visible due to the colour it imparts [185]. Thus, in theory, DMAB has excellent potential as a fingermark detection reagent as each

fingerprint is composed of approximately 250 ng of amino acids [126]. Amino acids from the fingerprints are therefore transferred onto the surface of contact, where the fingermark ridges will contain a number of amines that can facilitate a reaction with DMAB. Fritz *et al*. [184, 186] investigated the use of this compound for the detection of fingermarks however the luminescence of the detected fingermarks was inferior to those treated with IND-Zn.

While the formulation prepared by Fritz *et al*. [184] does not provide the luminescent intensity of IND-Zn, it may in fact be able to indicate the presence of cellular material. To investigate this, fluorescent microscopy was used alongside the Poliview® system to determine the presence of cells and fingermark development following the DMAB treatment on copy paper.

This study was divided into three experiments with the aim of answering the following questions:

Experiment 1: *Does the DMAB reaction that occurs within cells* in vitro *still occur when cells are located on a porous substrate?*

When used in histology, typically DMAB is applied to cell specimens on non-porous surfaces. The objective here was to determine if this molecule was still effective in detecting cells when applied to a porous surface via the wet contact formulation reported by Fritz *et al*. [184].

Experiment 2: *Can the visualisation of cellular material within a fingermark be enhance with the use of an extraction buffer?*

When the cell is intact, it may be difficult for DMAB to penetrate and make contact with the amine components of the cell when on paper. Hence, the objective was to determine

if the solution reported by Fritz *et al*. [184] could be enhanced for cellular detection when in combination with an extraction buffer.

Experiment 3: *Can the dual detection of fingermarks and DNA on a porous substrate be enhanced by combining DMAB with another fingermark detection reagent?*

The luminescence of the DMAB-fingermark has been reported to be weaker than that of IND-Zn. It may also be difficult to distinguish the fingermark from cellular components when DMAB is used alone, thus the objective of this experiment was to determine if the addition of an existing fingermark detection reagent could assist in the differentiation of fingermarks and cells.

4.2.2 Experiment 1: Does the DMAB reaction that occurs within cells *in vitro* still occur when cells are located on a porous substrate?

4.2.2.1 *Methods*

General Methodology

Charged and natural fingermarks deposited onto copy paper (Carbon Neutral 20 % Recycled A4, STAPLES®) then treated with a DMAB solution prepared per Fritz *et al*. [184]. DMAB-treated fingermarks were allowed to develop in the dark for 48 hours. The treated fingermarks were imaged under fluorescent microscopy 30 minutes (Leica DM4 M microscope with Leica LQ-HXP 120-L-Multipole external light source and Leica H3 filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm), Leica Microsystems™ Pty Ltd) post-treatment to visualise the cells in the fingermark. After 3 hours and 48 hours, fingermarks were imaged with the Poliview® IV System (Polilight PL500, Peltier Cooled 12 bit CCD camera and lens, with Poli-V®++ software, Rofin Australia) under fluorescent mode to record the fingermark.

Procedure

Two charged and two natural fingermarks were deposited onto white copy paper with printed grids by a medium fingermark secretor. Fingermarks were charged by rubbing the fingertips behind the ear region to ensure the presence of cells as the 'shedder status' of the donor was unknown. Samples were left on a laboratory bench for 1 hour before they were then immersed with the DMAB reagent until wet. The DMAB reagent prepared per the wet contact solution described by Fritz *et al*. [184] to the concentrations indicated Table 18.

Table 18: Preparation of wet contact DMAB [184] for treatment of copy paper

Components added in numerical order

	Component	Concentration	Supplier	Grade
1	DMAB	0.4% w/v	Fisher Scientific Co	Certified ACS
2	Ethyl acetate	8.8% v/v	Chem Supply	AR
3	Acetic acid	1.2% v/v	Chem Supply	AR
4	HFE7100 (methoxy-nonafluorobutane)	q.s. 100%	3M™ Novec™	Engineered Fluid

Following treatment, samples were left to air dry (< 1 minute), then adhered to a glass slide with sticky tape on the edge of the substrate before imaging with a microscope under fluorescence mode using an H3 filter cube. Microscopy imaging of the samples at 100 times magnification began 30 minutes post-treatment, and was completed approximately 120 minutes post treatment. The image was then digitally stitched together. Following microscopy imaging, samples were viewed with the Poliview® system under fluorescence mode (ex: 470 nm; em: 590 nm) 3 hours post treatment and again 48 hours post treatment.

4.2.2.2 *Results, discussion and conclusion*

After 30 minutes post-treatment, cells were apparent under fluorescent microscopy for the charged fingermarks (Figure 29), however, difficult to detect in the natural fingermark. The solution reveals both cells and possible fingermark residue with high nitrogen content. The areas of significant luminescence from the charged fingermark viewed under microscopy correspond to areas of luminescence on the treated fingermark when viewed with the Poliview® system 3 hours post-treatment (Figure 30: A). Thus,

cells are visible when imaged with the Poliview® system, however difficult to detect. Using the Poliview® system, the fingermark is very faintly visible under fluorescent mode for the charged fingermarks, and not visible in the natural fingermarks 3 hours post-treatment (Figure 30: 1). No brown ridges, as reported by Fritz *et al*. [184] were visible under ambient laboratory lighting for either of the different fingermarks. It is noted that Fritz *et al*. [184] used heat treatment in their experiments and the brown ridges may be a result of the acceleration of the procedure.

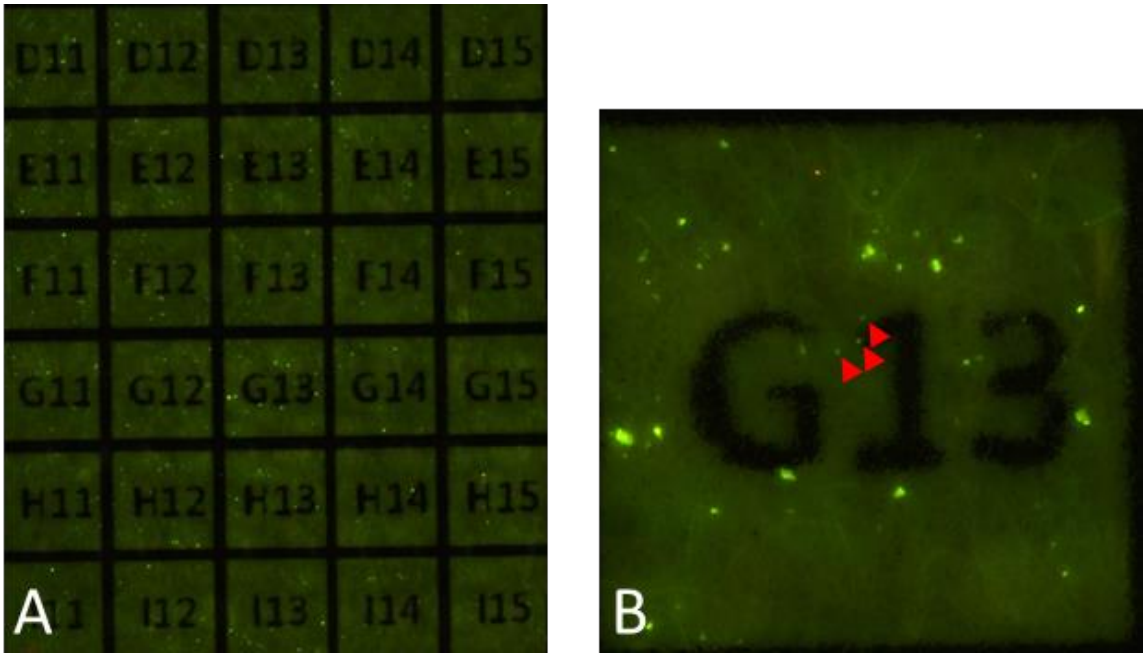


Figure 29: Cells from a representative charged fingermark treated with DMAB solution prepared per Fritz *et al.* [184] 30 minutes post-treatment

Red arrows indicate some luminescent cells. (A): Image viewed under fluorescence using H3 filter cube imaged with Leica DM4 M microscope at 100 times magnification; (B): close up of an area containing cells.

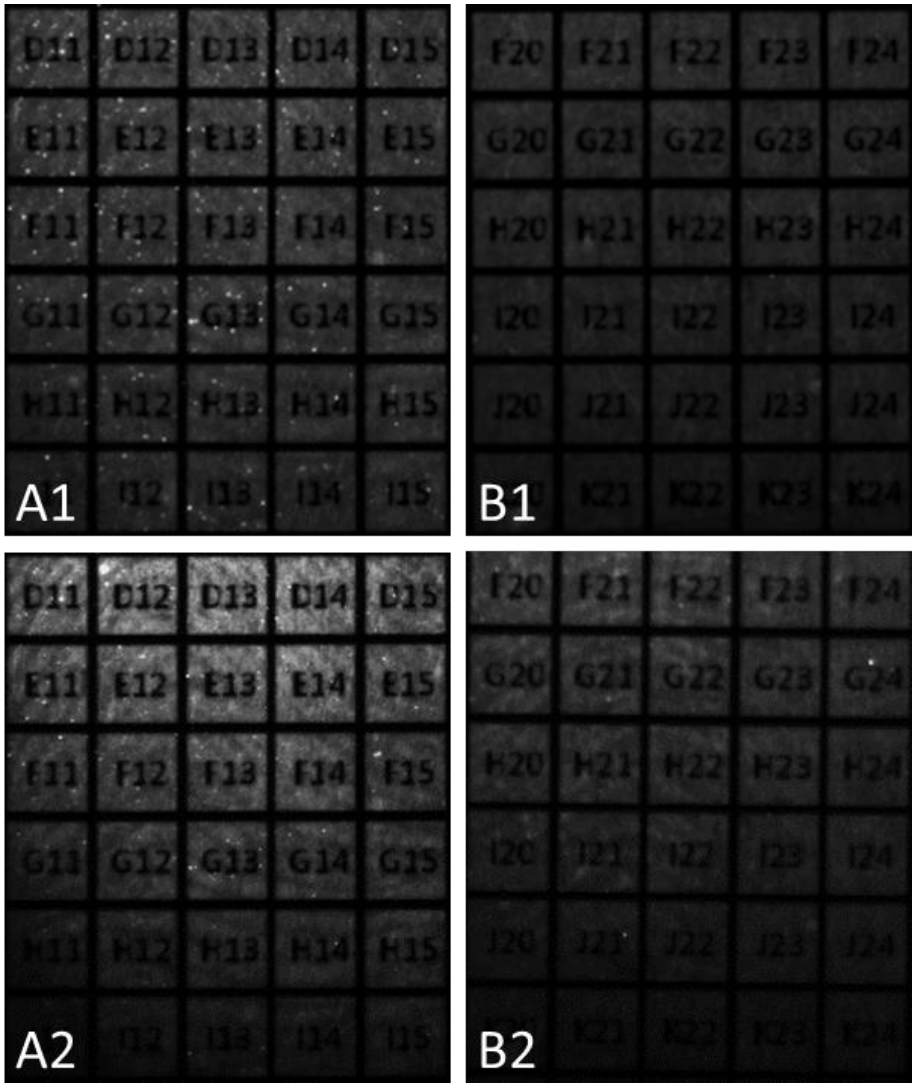


Figure 30: Representative charged (A) and natural (B) fingermarks treated with DMAB solution prepared per Fritz *et al.* [184] viewed with the Poliview® IV System (ex 470; em 590)
Samples were imaged 3 hours (1) and 48 hours (2) post-treatment.

No brown ridges were observed 48 hours post-treatment under ambient conditions. When viewed again with the Poliview® system at this time point, both charged and natural fingermarks became more apparent under fluorescent mode, than the 3 hour time point, however the luminescence of the cells began to fade despite being stored in a dark cupboard. This suggests that at a macro level there is a trade-off between cell and fingermark visualisation. Fingermarks treated with NIN and IND-Zn typically require the application of heat to catalyse the reaction that may otherwise take hours. It appears that

this reagent has similar properties in this respect, however heating was not conducted in these samples to both minimise the loss of cells to the heat press process and the dehydration of the cells that may cause the cells to shrink and expel or become devoid of the staining reagent. Potentially, the use of a heat and humidity controlled chamber could be used to avoid damage to the cells due to the heat alone. Under real time conditions, it appears that DMAB reacts rapidly with cells, which may be facilitated by the moisture in the cell, with this luminescence fading with time. Whereas the reaction with the fingermark becomes more prominent over time, and, at a macro level, this appears to obscure the luminescence of the cells.

This study has indicated that the DMAB solution reported by Fritz *et al*. [184] allows for the detection of cells and fingermarks when viewed under luminescent conditions. However, cells were difficult to detect due to their size and the luminescence of fingermarks was weak using this DMAB solution. As a result, experimentation was conducted to increase the visibility of cellular material and enhance the prominence of the fingermark secretions.

4.2.3 Experiment 2: Can the visualisation of cellular material within a fingermarks be enhance with the use of an extraction buffer?

4.2.3.1 *Methods*

General Methodology

To enhance the visibility of cells, the DMAB formulation was combined with an aqueous extraction buffer. It was expected that during treatment, the cell would lyse and the

contents would allow for a larger visible surface area when reacted with DMAB. Two charged fingermarks were treated with the solution, allowed to dry, then imaged using fluorescent microscopy (Leica DM4 M microscope with Leica LQ-HXP 120-L-Multipole external light source and Leica H3 filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm), Leica Microsystems™ Pty Ltd) and with the Poliview® system (Polilight PL500, Peltier Cooled 12 bit CCD camera and lens, with Poli-V®++ software, Rofin Australia) under luminescent mode.

Procedure

This experiment utilised the same equipment as listed in Section 4.2.2.1. Similarly, materials listed in Section 4.2.2.1 were also use in this experiment, and suppliers and quality of the materials can be found in the aforementioned section.

Three charged fingermarks were collected as described in the Procedure of Section 4.2.2.1 from a single donor. The DMAB solution was prepared per the components and concentrations listed for solution A in Table 19. The extraction buffer was prepared with Tris (ACS , Sigma-Aldrich), EDTA (ACS, Sigma-Aldrich) and sodium dodecyl sulfate (SDS; ACS, Sigma-Aldrich) per that of solution B in Table 19. The solutions were combined in a 50:50 ratio and well mixed before samples were immersed in the treatment solution until wet.

Table 19: Preparation of combined DMAB and extraction solution for treatment of copy paper

Components added in numerical order per solution.

Solution		Component	Concentration	Final Concentration in Combined Solution
A	1	DMAB	0.4% w/v	0.2% w/v
	2	Ethyl acetate	8.8% v/v	4.4% v/v
	3	Acetic acid	1.2% v/v	0.6% v/v
	4	HFE7100	q.s. 100%	q.s. 50%
B	1	Tris	0.12% w/v	0.06% w/v
	2	EDTA	0.29% w/v	0.155% w/v
	3	SDS	0.2% w/v	0.1% w/v
	4	Milli-Q water	q.s. 100%	q.s. 50%

Samples were then allowed to air dry in the dark overnight (approximately 18 hours). Following this, samples were adhered to a glass slide with sticky tape on the edge of the substrate before imaging with the microscope under fluorescence mode using an H3 filter cube at 100 times magnification then digitally stitched together. Following microscopy imaging, sample were viewed with the Poliview® system under fluorescence mode (ex: 470 nm; em: 590 nm), then again 3 days post-treatment.

4.2.3.2 *Results, discussion and conclusion*

It should be noted that the DMAB-extraction buffer solution was not stable, showing precipitation as soon as combined. However, the samples were able to be immersed in the solution, and it was able to soak through the substrate. The substrate was found to be fragile when wet and the addition of the aqueous phase also required a longer drying time than that seen in Experiment 1 (Section 4.2.2). Luminescence was observed after the

drying time (approximately 18 – 21 hours post-treatment) under both fluorescent microscopy and the Poliview® system (Figure 31).

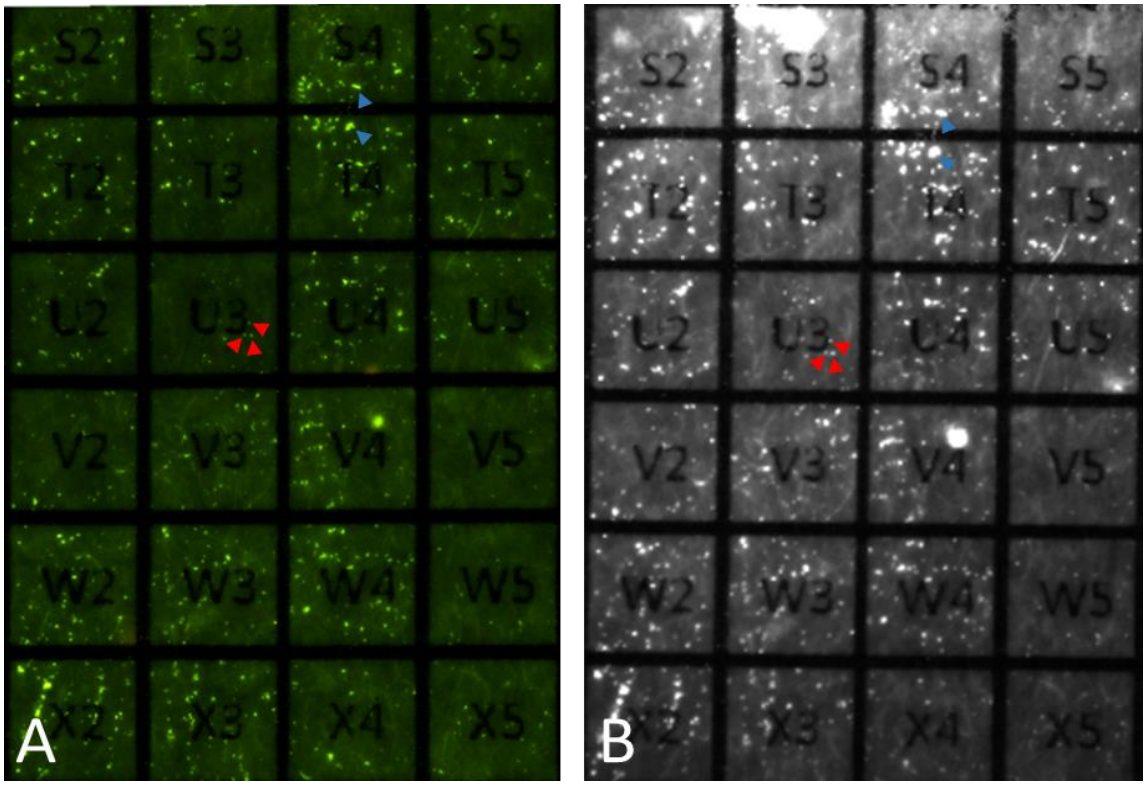


Figure 31: Representative fingermark treated with 50:50 DMAB : Extraction Solution (A) viewed with a Leica DM4 M microscope at 100 times magnification and (B) the Poliview® IV System (ex: 470 nm; em: 590 nm)

Red arrows indicate some of the cells present in the sample; blue arrows indicate some of the areas of proteinaceous material.

Treatment with this DMAB solution containing an extraction buffer was observed to allow for the detection of a significant amount of cellular material. These were not visible under ambient lighting conditions (i.e. no brown or other coloured stains were observed). However, under luminescence, cellular material was detected (Figure 31). This material was observed at 50 times magnification, and again using the Poliview® system as indicated by the red arrows. It appears that the charged fingermarks contained a high amount of proteinaceous material, as by the blue arrows in Figure 31. This could either indicate contaminants from the charging process or a by-product of the use of the

extraction buffer, where the cells are lysed and cell material is released to which DMAB binds, creating a larger visible surface area.

In Figure 31 A, it can be seen that there are some smaller areas of luminescence as indicated by the red arrows, this may indicate some cells that have partially lysed as these areas are also visible under fluorescent mode of the Poliview® system also indicated by the red arrows in Figure 31 B. While some semblance of fingermark ridges was observed, this is likely a result of cells being transferred from the ridges to the substrate (Figure 31). No fingermark ridges resulting from fingermark secretions were visible up to 3 days post-treatment. This suggests that the concentration of DMAB in the treatment solution may not have been strong enough to detect the fingermark. Alternatively, it could indicate that the aqueous fraction of the treatment solution may dissolve and wash away some of the water-soluble fraction of the fingermark residue.

This experiment has indicated that the detection of cells can be enhanced by the addition of an extraction buffer to the DMAB solution. However, this comes at the expense of the fingermark secretions. The addition of an aqueous fraction to the HFE-7100 carrier also resulted in precipitation of the solution, longer drying times and increased impact on the substrate. Thus, Experiment 3 (Section 4.2.4) investigates the potential of combining a fingermark detection reagent with DMAB to enhance detection both fingermark secretions and cells.

4.2.4 Experiment 3: Can the dual detection of fingermarks and cells on a porous substrate be enhanced by combining DMAB with another fingermark detection reagent?

4.2.4.1 *Methods*

General Methodology

To increase the luminescent of the fingermark and allow for enhanced differentiation of developed cells and fingermark secretions, the DMAB solution was separately combined with NIN and IND-Zn. Groomed fingermarks deposited on copy paper were treated with the solutions, then visualised using fluorescent microscopy and the Poliview® system.

Procedure

Eighteen charged fingermarks were collected as described in Section 4.2.2.1 onto copy paper (Carbon Neutral 20 % Recycled A4, STAPLES®). Three of the fingermarks were treated with DMAB-1,8-Diazafluoren-9-one (DFO) solution prepared per Table 20, three of the fingermarks were DMAB-NIN solution prepared per Table 21 and the three fingermarks were treated with the DMAB-IND solution prepared per Table 22. Three of the remaining fingermarks were treated with DFO solution, another three with NIN solution and the final three with IND-Zn solution, all prepared per Stoilovic and Lennard [187]. Fingermark samples were treated by immersion into the respective solution until wet then allowed to air dry under ambient laboratory conditions then heat treated in an oven at 130°C for approximately 3 minutes. Approximately 15 minutes following heat treatment, samples were then attached to a glass microscope slide and examined under bright field and fluorescent mode with H3 filter cube using a microscope and as well as with the Poliview® system under fluorescent mode.

Table 20: Preparation of combined DMAB-DFO solution for treatment of copy paper

Components added in numerical order; DFO concentration as suggested in the NCFS Manual [187].

	Component	Concentration	Supplier	Grade / Purity
1	DMAB	0.40% w/v	Fisher Scientific Co	Certified ACS
2	DFO	0.07% w/v	Sirchie	100%
3	Methanol	5.50% v/v	Chem Supply	AR
4	Ethyl acetate	8.80% v/v	Chem Supply	AR
5	Acetic acid	1.20% v/v	Chem Supply	AR
6	HFE7100	q.s. 100%	3M™ Novec™	Engineered Fluid

Table 21: Preparation of combined DMAB-NIN solution for treatment of copy paper

Components added in numerical order; NIN concentration as suggested in the NCFS Manual [187].

	Component	Concentration	Supplier	Grade / Purity
1	DMAB	0.40% w/v	Fisher Scientific Co	Certified ACS
2	NIN	0.46 % w/v	Chem Supply	ACS
3	Ethanol	5.50% v/v	Chem Supply	AR
4	Ethyl acetate	8.80% v/v	Chem Supply	AR
5	Acetic acid	1.20% v/v	Chem Supply	AR
6	HFE7100	q.s. 100%	3M™ Novec™	Engineered Fluid

Table 22: Preparation of combined DMAB-IND solution for treatment of copy paper

Components added in numerical order per solution; IND and $ZnCl_2$ concentrations as suggested in the NCFS Manual [187].

	Component	Concentration	Supplier	Grade / Purity
1	DMAB	0.40% w/v	Fisher Scientific Co	Certified ACS
2	IND	0.06% w/v	Casali Institute	-
3	Ethyl Acetate	8.80% v/v	Chem Supply	AR
4	Acetic Acid	1.20% v/v	Chem Supply	AR
5	$ZnCl_2$	0.02% w/v	Sigma-Aldrich	Reagent
6	Ethanol	0.40% v/v	Chem Supply	AR
	HFE-7100	q.s. 100%	3M™ Novec™	Engineered Fluid

4.2.4.2 *Results, discussion and conclusion*

Fingermarks treated with DFO showed luminescent fingermarks under the Poliview® system, with limited luminescence of the fingermark observed under the microscope using an H3 filter cube (Figure 32: A). Additionally, luminescent cells were not observed following DFO treatment suggesting that DFO cannot enter into cells to react with those amino acids. Using DMAB-DFO, the fingermark residue was observed using the Poliview® system (Figure 32: B1). This was also faintly observed under fluorescence mode on the microscope. Cellular material was also visible under these conditions (Figure 32: B2; B3).

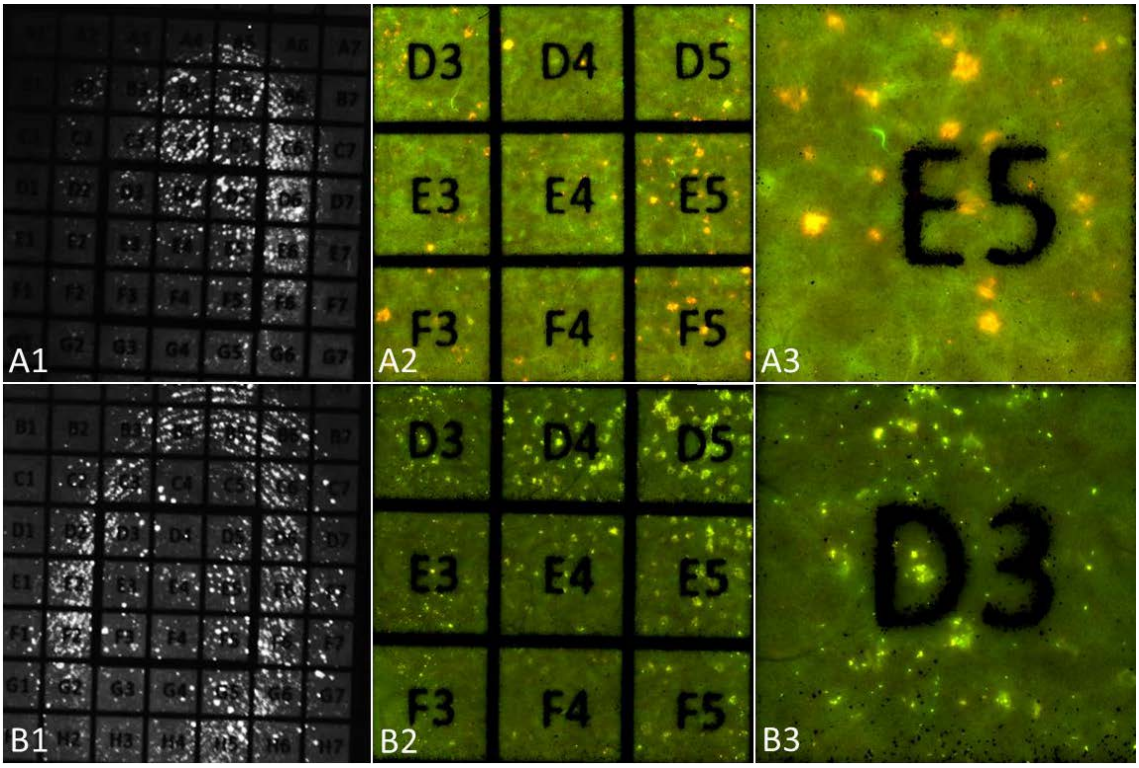


Figure 32: Representative fingermark treated with (A) DFO solution and (B) DMAB-DFO solution viewed under (1) Poliview IV system (ex 555 nm; em 610 nm) and (2) fluorescent mode using a Leica DM4 M microscope and (3) close up of (2)

Samples viewed under the microscope were at 50 times magnification and digitally stitched together.

Treatment of fingermarks with the DMAB-NIN solution produced purple fingermarks characteristic of Ruhemann's purple as compared with NIN treatment (Figure 33). These purple ridges began to present approximately 5 minutes following both treatments and became more pronounced following heat treatment. When imaged with under the microscope, the purple colour of both NIN and DMAB-NIN treatment is attributed to the ridges with cells being slightly darker (Figure 33). No luminescence was observed in any of the fingermark secretions or cells following with DMAB-NIN. This suggests that ninhydrin has a higher affinity for amino acids than DMAB rather than the DMAB reaction being masked. This is because if the reaction by DMAB with amino acids was masked by that of ninhydrin, it may still be detected under luminescence.

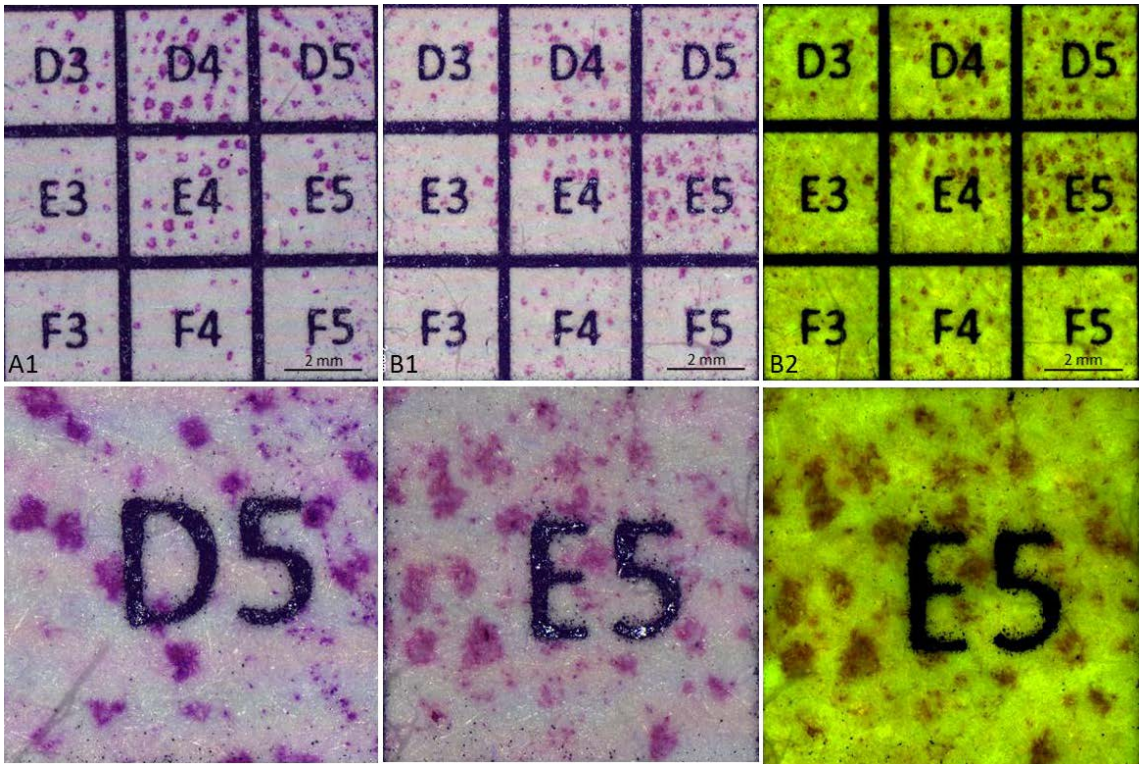


Figure 33: Representative fingermark treated with (A) NIN solution or (B) DMAB-NIN solution viewed under (1) bright field and (2) fluorescent mode using a Leica DM4 M microscope
Samples viewed at 50 times magnification and digitally stitched together; bottom images are close-ups of the corresponding area in the images at the top.

When treated with IND-Zn solution, fingermarks were visible under fluorescent mode using the Poliview® system (Figure 34: A1). Incomplete ridge detail, as compared to the Poliview® image was observed under fluorescent mode using a microscope, however no cellular material was visible (Figure 34: A2; A3). After treatment with the DMAB-IND solution, fingermark secretions and cells were visible under fluorescent mode, at a lower intensity than observed following IND-Zn only treatment (Figure 34: *c.f.* A1 and B1). Following the DMAB-IND treatment, cells were visible using the microscope only (Figure 34: B2; B3), that presented as luminescent spots that can be differentiated from the background under fluorescent microscope conditions. This indicates that following a single application of the DMAB-IND solution to paper both fingermarks and cells can be detected at a macro level and micro level, respectively.

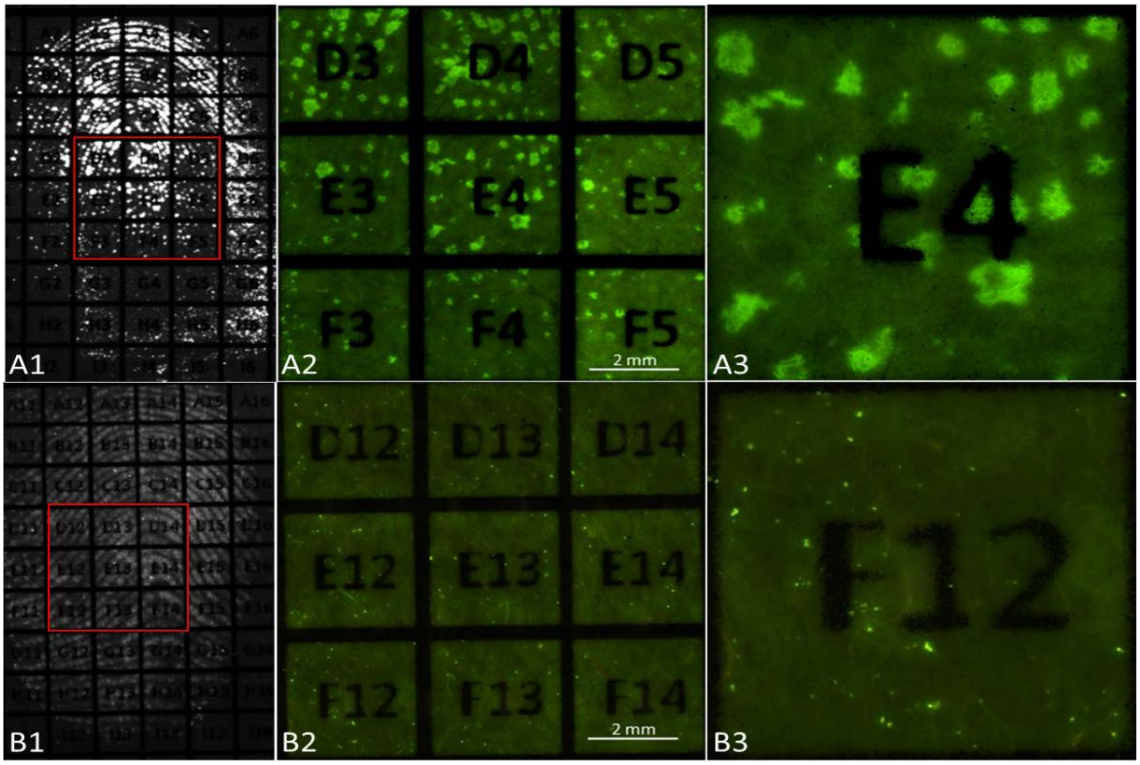


Figure 34: Representative fingermark treated with (A) IND-Zn and (B) DMAB-IND solution viewed under (1) luminescence mode using the Poliview® IV System (ex: 505; em: 555); (2) under fluorescent microscopy using H3 filter cube with a Leica DM4 M microscope; and (3) a close up (2) Samples viewed at 50 times magnification and digitally stitched together.

4.2.5 Conclusions about DMAB

These studies suggest the use of DMAB alone for fingermark detection is not as effective as existing fingermark reagents and its use does not indicate cells at a macro level. When DMAB is prepared in a solution that can be absorbed into paper, it can be used to detect cells on the absorbed surface, where IND-Zn and DFO alone cannot. While NIN shows fingermarks and cells, it is difficult to differentiate the two. As DMAB undergoes a reaction with amino acids giving off luminescence, these cells can be detected using an ALS and microscopy techniques. When DMAB is combined with DFO and IND-Zn in solution, fingermarks are enhanced by IND-Zn, while cells are still detected at a micro level by DMAB. These combinations appears to have significant potential for detecting

and preserving both fingermarks and touch DNA. Further, in respect to touch DNA, the detection capabilities of this reagent may allow for the increased detection of alleles in profiling. As IND-Zn is becoming the leading technique for the detection of fingermarks on porous surfaces, the use of this reagent in conjunction with DMAB will be examined further. Optimisation of the concentrations of DMAB and IND-Zn and the impact of the reagent on DNA will be explored in Chapter 6.

Chapter 5. DNA RECOVERY FROM A NON-POROUS SURFACE FOLLOWING ONE-STEP LUMINESCENT CA TREATMENT

The work detailed in this chapter has been published under the title: "Impact of one-step luminescent cyanoacrylate treatment on subsequent DNA analysis" in Forensic Science International 2018 May; 286:1-7 (doi: 10.1016/j.forsciint.2018.02.015) [188]

5.1 Introduction

Non-porous surfaces do not allow for the absorption of fingermark residue, instead, the residue remains on the contact surface and degrades over time [1]. Fingermark detection methods for non-porous surfaces differs from that of porous surfaces as the inherent nature of these surface types means that different residues are retained, thus the target for each surface type is different[1]. Similarly, DNA has been found to behave differently according to the surface [66], as part of the fingermark residue, it is absorbed into porous substrates, while remains on the surface for non-porous substrate. Because fingermarks

on non-porous surfaces are exposed to the external environment, they can be easily destroyed. They are also susceptible to both organic and aqueous solutions [1]. This limits the available techniques for processing fingermarks on non-porous surfaces as organic solvents are generally required for DNA dyes to permeate the cell membrane and aqueous buffers are generally used to maintain cells and cellular material.

CA fuming of fingermarks is a well-established technique for fingermark development on non-porous surfaces and recommended as a pre-treatment for the preservation of touch DNA [84]. Previous analyses suggest that DNA profiling is not inhibited by the CA treatment, while care must be taken with extraction methods to ensure PCR inhibitors are removed to improve the process [62, 74, 82, 106]. This technique does not require the application of a solution for latent fingermarks to be revealed, however contrast can be increased following CA-treatment, when the fingermark residue, including touch DNA, are encased in the protective polymer.

Recently, one-step luminescent CAs have been marketed as an alternative to the conventional two-step process that is often required to increase contrast between the developed mark and the background. These one-step luminescent CAs all incorporate a luminescent staining agent with a liquid CA monomer or a solid CA polymer. One-step luminescent CAs were first reported in 1993 by Weaver and Clary [107], who successfully combined CA monomer with a styryl dye to produce a technique for simultaneously developing and staining latent fingermarks. They speculate that as the CA and dye are vaporised, the fumes of the dye become incorporated within the developing CA polymer in a specific manner [107]. This was confirmed by *Prete et al*. [110], who further determined that CA polymer is closely linked with the dye molecule and this affinity results in the selective luminescence of developed fingermarks.

Since the Weaver and Clary's [107] advent of one-step luminescent CA, the number on the market has slowly grown, with both polymer and monomer formulations both available. Weaver continued optimisation work on a one-step luminescent CA [108] and it is believed the polymer, CN-Yellow (Aneval Inc.), is the marketed product from this work. Other well-known one-step polymers include PolyCyano UV (Foster+Freeman) and PECA Multiband (BVDA). Lumicyano™ (Crime Science Technology) appears to be the only marketed one-step luminescent monomer, which also requires a two-step preparation prior to fuming.

The fluorophore in CN-Yellow is believed to be solvent yellow 43 [108]. Weaver [108] reported this dye to show affinity for the CA polymer as well as having a similar sublimation temperature to that of solid CA. Few studies have reported on the use of CN-Yellow with luminescence of CN-Yellow found to be poor. Groeneveld *et al*. [114] was unable to optimise the application of the product for use in their study and Khuu *et al*. [115] failed to observe contrast between the developed fingermark and the background. This could in part be due to the vaporisation temperature that is achievable by standard fuming chambers. For example, the Foster+Freeman MVC™1000/D fuming chamber used by Khuu *et al*. [115] is capable of a maximum temperature of 230°C, while the required sublimation temperature required for CN-Yellow was 250°C. As CA development of the fingermark was achieved with fuming at 230°C, this would suggest that this temperature was insufficient to vaporise the dye, hence luminesce was not observed in both cases [114, 115].

PolyCyano UV uses 5% DMAB as the fluorophore in a solid CA formulation [180]. DMAB is a staining agent employed in histology to detect amines [185]. It has been reported to show reliable affinity for the CA polymer when used in a two-step process (i.e. CA fuming followed by vapour staining with DMAB) and offers enough luminescent

contrast to allow for differentiation of the ridge detail from the background [177]. Hahn and Ramotowski [109] investigated the use of PolyCyano UV using a modified Foster+Freeman MVC^{TM}1000/D fuming chamber. In their modified chamber, the CA separated from the heating plate until the required temperature was achieved to avoid slow heating of the polymer. As the MVC^{TM}1000/D fuming chamber begins its fume time count down as soon as the heating begins extra time would be required in order to allow for adequate development of latent fingermarks. In this study they found comparable results between PolyCyano UV and the conventional technique with R6G stain, however state that more ridge detail was observed following the two step method [109]. Chadwick *et al*. [111] and Khuu *et al*. [115] found similar results in their studies, with CA development of the two techniques being comparable, while luminescence of R6G was superior to that of PolyCyano UV on a range of non-porous and semi-porous surfaces. Stewart *et al*. [116] also observed stronger fluorescence when CA was post-stained with BY40. These results were achieved without modification of the respective fuming chambers, suggesting that slow heating of the product does not degrade the dye. Considering PolyCyano UV and the MVC^{TM}1000/D fuming chamber were both developed by Foster+Freeman, it is likely that slow heating of the polymer was taken into account during development.

PECA one-step luminescent CAs have only made their debut in recent years and are not widely available, hence few studies describe their efficacy against the convention method. In PECA Multiband, the formulation includes CA polymer with DMAB at a concentration of 10-15% as the staining agent [182], while PECA Fluor Extra contains the polymer with 7-14% DMAB [189]. Khuu *et al*. [115] observed PECA Multiband to give inferior luminescence when compared to the conventional method with R6G post-staining. Stewart *et al*. [116] had similar results when comparing to BY40 post-staining.

They also noted that PECA developed ridges were of a slightly higher quality than conventional fuming. However, due to the higher dye content, excess DMAB appears to adhere to some substrates, causing come background luminescence, as well as the interior of the fuming chamber causing it to be tinted yellow [115, 116].

The first generation Lumicyano™ was available as a prepared CA monomer solution containing 1% dye, and superseded by the Lumikit™, which requires a two-step preparation: the addition of a solid dye ($C_4H_5CN_4O$) [179] to the liquid monomer to a recommended concentration of 5% prior to fuming. The use of the different Lumicyano™ preparations has been compared to the conventional CA process. Prete *et al*. [110] found the first generation Lumicyano™ effective at developing latent fingermarks on a range of non-porous and semiporous surfaces, however the luminescence of the dye is weaker than the use of a post-stain (BY40) following the use of the conventional technique. Farrugia *et al*. [113] and Stewart *et al*. [116] came to the same conclusion when they conducted a comparison study on a number of different plastic bags with BY40 providing superior luminescence than the one-step method. Farrugia *et al*. [113] also found less background interference when using Lumicyano™, and that luminescence could be improved by the use of BY40 post-stain. When using the Lumikit™ to fume fingermarks on used plastic bags and other semiporous surfaces, Farrugia *et al*. [112] found superior quality CA development than conventional CA, reporting 20% more development when the Lumikit™ was used, however the luminesce was still inferior. Hence, post-staining was still required in order to successfully detect all fingermarks. The higher rate of development following the application of Lumikit™, may in fact be a coincidence, as this study was a pseudo-operational trial, the actual number of deposited fingermarks was unknown. It may be that the surfaces treated with Lumikit™ had a higher number of fingermarks at the start of the trial than others. Khuu *et al*. [115] observed better detection

of aged fingermarks on various semi-porous surfaces when using the Lumikit™ which could also account for the results observed by Farrugia *et al*. [112] as the age of the fingermarks would be unknown. As with other studies, conventional CA post-stained with R6G offered superior luminescence [115].

Farrugia *et al*. [181] reported enhanced fingermark detection following "double-fuming" of Lumikit™ at 5 % w/w concentration and attributed this to both the solution and the dye as an increase was also reported following secondary treatment with the respective Lumikit™ component. This study [181] also found that separate but simultaneous fuming of 8% w/w Lumikit™ with 8% weight of dye in a single fuming cycle functions as a time saving alternative to their "double-fuming" technique. However, as they reported comparable results between single fuming of 5% w/w and 8% w/w Lumikit™ without co- or double-fuming, it can be suggested that the availability of the extra dye in the simultaneous co-fuming test is able to be incorporated in more CA ridge developments. This would also account for the increased detection of fingermarks following BY40 post-treatment of Lumikit™ exposed marks [112, 113, 181], that is, a number of Lumikit™ treated fingermarks are developed with CA however, the dye is not incorporated. Additional dye in the form of Lumicyano™ dye or BY40 post treatment can assist in the visualisation of those developed fingermarks. It is difficult to gain a true conclusion from these results of Farrugia *et al*.'s [181] study, as, again, this was a pseudo-operational trial and the true number of deposited fingermarks is unknown; the number of fingermarks detected cannot be definitively compared across treatments – only the quality of development can be compared across the different treatment trials. Additionally, no statistical analysis is reported in the study [181] to establish the significance of the difference between treatments. Hence, it cannot be concluded that one treatment is

superior to another without a controlled study and the application of the appropriate statistical analysis.

The detection of hand-marks on fabrics has also been shown to be possible with Lumikit™, with Beerman *et al.* [190] able to detect fresh and aged marks on a range of fabrics with Lumicyano™ dye concentrations of 8 – 10% w/w. The ridge quality of the marks was poor, generally achieving average results of less than 1.5 on a modified University of Lausanne (UNIL)/ UK Centre for Applied Science and Technology (CAST) scale following treatment, where less than 1/3 of the mark showed continuous detail [190]. The size of the fabric weave was found to also impact on the quality of development, where a larger weave resulted in poorer ridge detail [190]. Poor development could also be due to the porous nature of this surface, where the water-soluble constituents of the fingermark absorbed into the fibres [1], hence the CA developing the diffused residue, rather than distinct ridge detail as often seen on non-porous surfaces resulting in poor ridge quality. The use of BY40 post-stain was also found to be detrimental to the visualisation of Lumikit™ treated marks on white silk, obscuring any distinguishable marks [190]. On other tested substrates no impact, or no positive impact was observed from the addition of BY40 [190]. This is likely due to the dye absorbing into the substrate and limiting contrast of the developed mark with the background. The Lumikit™ comes with a scoop for the dye. Overall, the reported results for 8-10% Lumicyano dye treated marks on polyester and silk showed averages from approximately 1 to 1.5 on the modified scale [190]. The user guide [118] provides a table to approximate the weight based on the size of the scoop; it could be likely that the 1% increment used in this comparison study from 8-10% may not prove significant if the manufacturers' recommendations allow for such an error when using the scoop to approximate weight.

When a larger difference in dye concentration is used, a more significant difference is observed. A comparison of first generation, Lumicyano™ (1% fluorophore; mock preparation) to Lumikit™ (recommended use at 4% w/w fluorophore) was conducted [191]. The finding suggests that the higher the dye concentration, the better the visualisation of fingermarks up to 21 days following deposition and across a number of non-porous substrates [191]. The study [191] indicates that lower levels of dye is encrusted in the CA development for Lumicyano™ than Lumikit™ as less dye is available. This resulted in poor contrast when fumed with the 1% treatment while a 4% treatment provided a time saving advantage as the marks were more visible, resulting in decreased time required for detection [191].

5.2 Objectives

These studies have shown that while one-step luminescent CA fuming techniques develop fingermarks, a limitation was that their luminescence was generally inferior to conventionally fumed fingermarks post-stained with either R6G [111, 115] or BY40 [110, 112, 113, 116] or with increased presence of dye [181, 191]. However, as this technique is capable of encasing the fingermark residue, it should offer a preservation effect for touch DNA similar to that of conventional CA fuming. As this method reduces the need for post-treatment, it also reduces the chance of loss and contamination that can be associated with staining. Additionally, the DMAB dye used in the PolyCyano UV and PECA one-step products is used in biological work as a cell-staining agent, thus allows for the detection of cellular material and allow for an indication of the presence of DNA (see Section 4.1). The objective of this study was to determine whether the use of the one-step luminescent CAs, PolyCyano UV and Lumikit™, for fingermark detection is

detrimental to subsequent DNA analysis processes. Another objective was to determine how the one-step luminescent CAs compare to conventional CA techniques in terms of quantity and quality of recovered DNA; determine if one-step CAs can be used to locate genetic materials. While PECA Multiband was found give superior luminescent cells than the other two one-step luminescent CAs, at the time of experimentation, this product was not widely available with only sample obtained, instructions for use were obtained through personal communications and no claims were made towards its compatibility with DNA processing. As such, testing of this product was not conducted with the other one-step luminescent CAs.

5.3 Method

5.3.1 General Methodology

The impact of the one-step luminescent CAs on DNA analysis was assessed on natural and spiked fingermarks deposited on glass microscope slides (Premium Glass Microscope Slides, Livingstone International) per the procedure shown in Figure 35. Traces were deposited on the substrate, before being treated with the respective CA treatment (PolyCyano UV, Foster+Freeman Ltd; Lumikit™, Crime Science Technology; Cyanobloom, Foster+Freeman) in a fuming chamber (MVC1000/D fuming cabinet, Foster+Freeman Ltd). The treated specimens were then visualised (Poliview® IV System: Polilight PL500, Peltier Cooled 12 bit CCD camera and lens, with Poli-V®++ software, Rofin Australia) and development was recorded prior to DNA recovery, extraction (QIAamp DNA Mini Kit, QIAGEN) and quantification (Plexor® HY System, Promega Corporation;). Following this, DNA profiles were generated (PowerPlex® 21

System, Promega Corporation) from the recovered samples. Quantitation and profiling results from each treatment were then compared to assess the impact of each CA fuming technique on the respective DNA analyses.

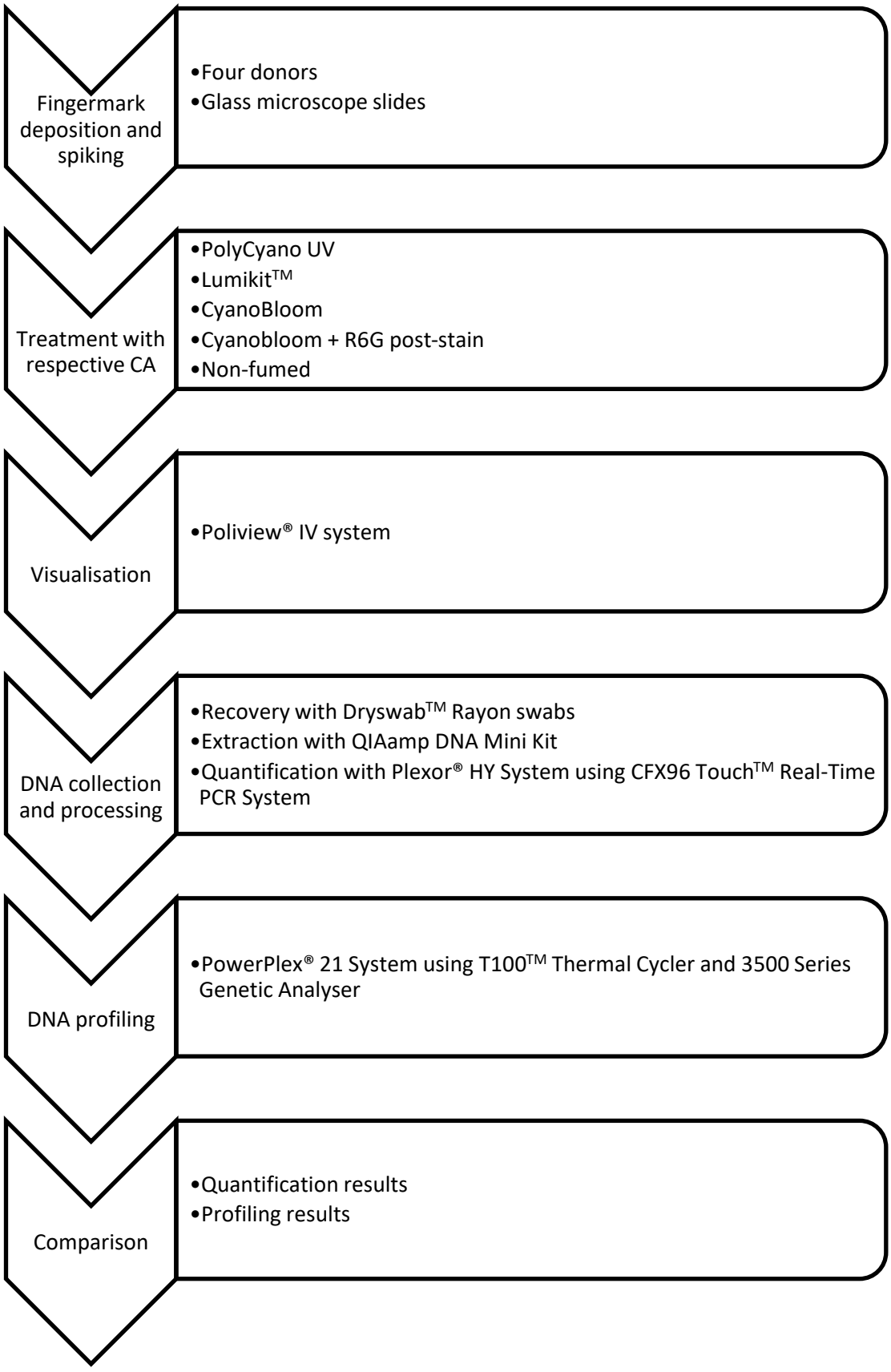


Figure 35: Overall procedure for assessing impact of one-step luminescent CA treatment on DNA analysis

5.3.2 Procedure

5.3.2.1 *Fingermark collection and ageing*

Glass microscope slides were prepared for fingermark deposition by decontamination under laminar-flow with 10% bleach solution, thoroughly rinsed with sterile Milli-Q water, allowed to dry and packaged before being autoclaved. Four donors (two males; two female) were used in this study, ranging from weak to strong fingermark secretors. For each set of CA treatments, each donor gave four natural, single finger depositions on separate glass microscope slides. Fingermarks from each donor were deposited using the index and middle fingers of the right and left hands to allow for similar sized fingermarks. Five sets of fingermarks were collected, with one set being used for each CA treatment and one set collected as non-fumed controls. Donors were requested to conduct their usual activities prior to depositing fingermarks. They also refrained from washing their hands for at least 20 minutes prior to deposition, and allowed at least 20 minutes between the depositions of each set of fingermarks to represent realistic deposition conditions.

Two of the fingermarks collected for each set from each donor were spiked with a volume of cell solution equivalent to 20 ng of DNA. Spikes were allowed to dry prior to ageing and CA treatment. The cell solution was derived from approximately 20 mL of saliva from one independent female donor who did not donate any fingermarks to the study nor work in any of the laboratories in which this study utilised, to minimise contamination of results. The saliva specimen was washed in PBS (prepared per Table 4), pelleted and the supernatant discarded for total of five cycles before being reconstituted in 10 mL of fresh PBS. The cell solution was then thoroughly mixed before ten 1 mL samples were aliquoted into 1.7 mL microtubes. The concentration each was estimated using both the

Plexor® HY System and Quantifiler™ Trio DNA Quantification Kit (ThermoFisher Scientific) on a CFX96 Touch™ Real-Time PCR System (Bio-Rad Laboratories, Inc.). Spiked specimens were aged with the natural non-spiked fingermarks in the dark at ambient laboratory temperature and relative humidity for 14 days.

5.3.2.2 *CA fuming and visualisation*

Following the ageing period, each set of fingermarks and along with a blank glass microscope slide, i.e. a decontaminated microscope slide with no fingermark, were fumed with the respective CA treatment in a MVC 1000 fuming chamber according to Table 24. The chamber was firstly set to reach 80% humidity before CA could be vaporised. For consistency, all samples were exposed to CA fumes for 10 minutes to allow for a similar amount of CA build up on each donor's respective fingermark for each treatment and limit bias on the results. All samples were imaged using the Poliview® IV system, initially under white light, then under respective optimal luminescent conditions for each treatment per Table 24 within one hour of fuming, with the exception of R6G post-stained samples. Cyanoblooom samples requiring R6G post-staining were left to cure overnight before post-treatment as per Stoilovic and Lennard [187] to allow the CA development to harden. R6G stain was prepared according to Table 23 [187] and gently applied with a disposable pipette to avoid developed ridges breaking off from the surface during staining. Excess stain was then gently rinsed off with sterile Milli-Q water and specimens were allowed to dry before being visualised under the respective conditions (Table 24). Fingermark processing was conducted in a clean but non-sterile environment to represent realistic conditions with respect to this trace.

Table 23: Preparation of Rhodamine 6G [187]

Component	Quantity	Supplier	Purity
Rhodamine 6G	0.01% (w/v)	Sigma-Aldrich	95%
Isopropanol	10% (v/v)	VWR	99.9%
Methyl Ethyl Ketone	15% (v/v)	Chem-Supply	99.5%
Water	q.s. 100%	UTS	MilliQ

Table 24: Fuming conditions used in the MVC1000 fuming chamber and visualisation conditions used with a PL500 Polilview® system

CB denotes Cyanobloom; CB+R6G denotes Cyanobloom with R6G post-stain; PC denotes PolyCyano UV; LC denotes Lumikit.

Visualisation: excitation wavelength; interference bandpass barrier filters for each respective CA; *Values adjusted for use in this study per operating instructions [118].

Conditions	CB	CB+R6G	PC	LC
Temp (°C)	120		230	120
Mass (g)	0.5		0.6	0.4* (0.5 % w/w)
Time (minutes)	10		10	10
Humidity (%)	80		80	80
Visualisation (nm)	White light only	Ex 490; Em 555	Ex 350; Em 450	Ex 350; Em 555

5.3.2.3 *DNA processing and analysis*

Extraction

A buccal swab was obtained from each donor as a reference prior to extraction of touch DNA from treatment groups. Following the visualisation of the treated fingermarks, traditional steps for collecting and processing DNA [56] were followed in this study. Under a decontaminated laminar-flow cabinet, the treated and control specimens were processed in batches according to the treatment applied. Specimens were swabbed using the double swab technique [36]. Sterile Milli-Q water was used to wet the initial swab (Sterile Dryswab^{TM} Rayon, Medical Wire & Equipment, UK) with this swab tip being used to remove the CA-development from the glass surface. As much of the CA residue was collected as possible onto the wet swab. A dry swab was then applied over the surface of the slide, going over any wet spots from the initial swab as well as collecting any remaining residue. The tips of both swabs were then transferred to 2 mL microtubes.

The touch DNA collected, as well as the reference samples, were extracted with QIAamp DNA Mini Kit using the "DNA Purification from Buccal Swabs (Spin Protocol)" protocol [175], as summarised in Figure 36. Firstly, 400 µL PBS was added to each microtube. Then 20 µL of QIAGEN Protease Stock Solution and 400 µL of Buffer AL was added to each sample and vortexed immediately for approximately 15 seconds. The samples were then incubated at 56°C for 10 minutes, following which they were briefly centrifuged to remove vapour from the inside of the lid. Following this, tube were opened and 400 µL of ethanol (96%) was then added to each sample and vortexed and centrifuged. From this, 700 µL of each sample was then carefully added to a QIAamp Mini spin column within a 2 mL collection and spun down at 6000×g for 1 minute. The filtrate was then discarded and the QIAamp Mini spin column was placed in a fresh 2 mL collection tube. Again, up

to 700 µL of each sample was applied to the corresponding QIAamp Mini spin column and spun down at 6000×g for 1 minute before 500 µL of Buffer AW1 was carefully added and spun down at 600×g for 1 minute. The filtrate was discarded and the QIAamp Mini spin column was placed into a fresh collection tube. 500 µL of Buffer AW2 was carefully added to the QIAamp Mini spin column and centrifuged at 20 000×g for 3 minutes. The QIAamp Mini spin column was then placed into a fresh microtube and centrifuged at 20 000×g for 1 minute remove Buffer AW2 carryover. The QIAamp Mini spin column was then placed into a fresh 1.5 mL microtube and 150 µL of Buffer AE was added. The samples were incubated at ambient temperature for 1 minute before being centrifuged at 6000×g for 1 minute to elute purified DNA from the column.

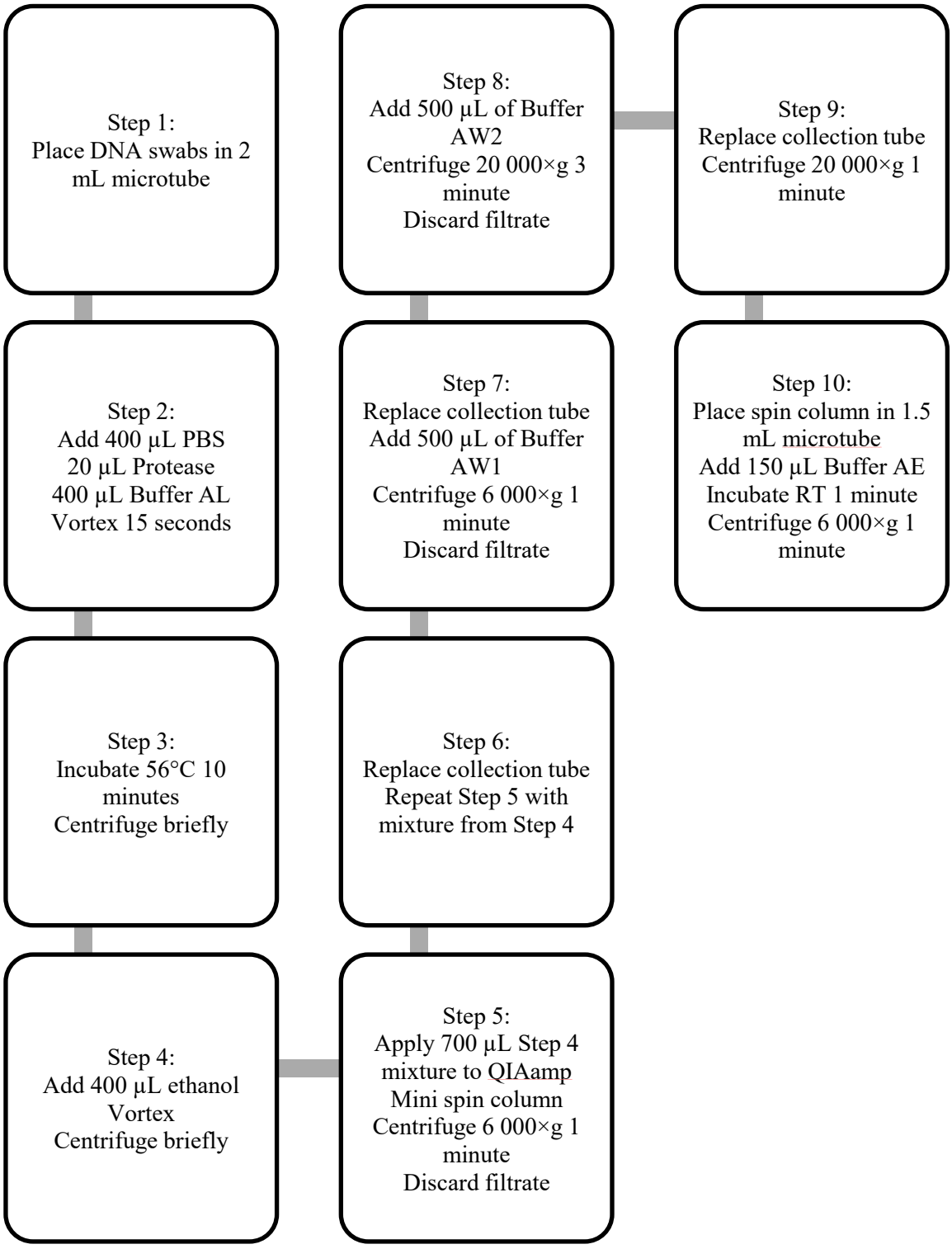


Figure 36: Workflow for purification of DNA from swabs using QIAamp DNA Mini Kit [175]

Quantitation

Purified DNA was stored at -20ºC between DNA extraction and quantitation, and quantitation and profiling. Samples were prepared for DNA quantitation with Plexor® HY System (Promega Corporation) [192] in duplicate as summarised in Figure 37. Firstly, the Plexor® HY Male Genomic DNA Standard was thawed then vortexed for 10 seconds and kept on ice. Serial dilutions of the Plexor® HY Male Genomic DNA Standard were prepared per Table 25 with each dilution vortexed before a fresh tip was used to take an aliquot for the next dilution. The extracted DNA samples, Plexor ® HY 2x Master Mix, Plexor® HY 20x Primer/IPC Mix and Water, amplification grade were all thawed on ice. Plexor ® HY 2x Master Mix and Plexor® HY 20x Primer/IPC Mix were vortexed for 10 seconds to mix. The reaction mixture was prepared by combining the Plexor® HY 2x Master Mix, Plexor® HY 20x Primer/IPC Mix and Water, Amplification Grade in multiples corresponding to the number of samples and standards, i.e. “reactions”, as shown in Table 26. The reaction mixture was vortexed to mix before 18 µL was added to reaction wells of a Hard-Shell® 96-Well PCR plate (low profile, thin wall, skirted, white/clear, Bio-Rad Laboratories, Inc). 2 µL of TE^{-4} buffer (10 mM Tris-HCl, pH 8; 0.1 mM EDTA) was added to negative control wells, while 2 µL of unknown extracted DNA was added to remaining reaction wells, in duplicate. Plates were sealed with optical grade Microseal® 'B' PCR Plate Sealing Film (adhesive, optical, Bio-Rad Laboratories, Inc.) before being centrifuged to collect contents into the bottom of the wells and undergoing thermal cycling.

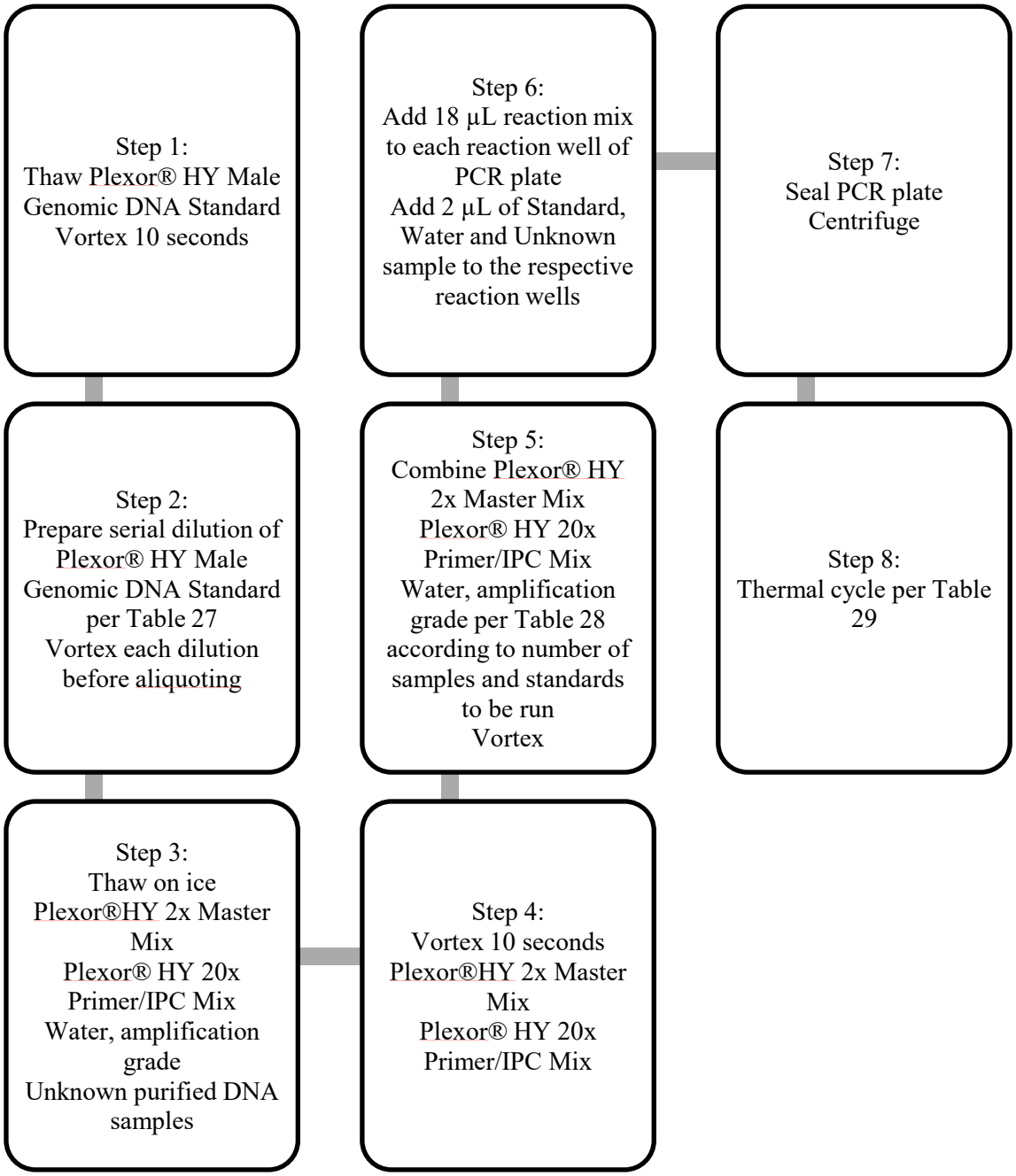


Figure 37: Workflow for quantification of DNA using Plexor® HY System [192]

Table 25: Serial Dilution of Plexor® HY Genomic DNA Standard

Standard	Concentration	Volume of DNA	Volume of TE^{-4} buffer
1	50 ng/µL	Plexor® HY Male Genomic DNA Standard	0 µL
2	10 ng/µL	10 µL Standard 1	40 µL
3	2 ng/µL	10 µL Standard 2	40 µL
4	0.4 ng/µL	10 µL Standard 3	40 µL
5	0.08 ng/µL	10 µL Standard 4	40 µL
6	0.016 ng/µL	10 µL Standard 5	40 µL
7	0.0032 ng/µL	10 µL Standard 6	40 µL

Table 26: Preparation of reaction mixture for quantification of DNA with Plexor® HY System

Component	Volume required per reaction
Plexor® HY 2x Master Mix	10 µL
Plexor® HY 20x Primer/IPC Mix	1 µL
Water, Amplification Grade	7 µL
Final Volume	18 µL

Samples were quantitated on a CFX96 Touch™ Real-Time PRC System (Bio-Rad Laboratories, Inc.) [193] using the thermal cycling program outlined in Table 27 with a melt-curve generated following the completion of the program to enable the data to be processed using Plexor™ Analysis Software – Forensic v1.6.0 (Promega Corporation).

Table 27: Thermal Cycling Program used to quantitate touch DNA with the Plexor® HY System

Step	Temperature	Time	Number of cycles
Initial Denaturation	95°C	2 minutes	1 cycle
Denaturation	95°C	5 seconds	38 cycles
Annealing and Extension	60°C	35 seconds	

Profiling

Prior to amplification and DNA profiling, quantification results were used to dilute samples as required to allow for 15 µL of template DNA sample (≤ 33.3 pg/µL) to be added to each reaction (≤ 0.5 ng DNA). Samples showing DNA concentrations ≤ 33.3 pg/µL were not diluted and used neat. DNA Profiling was performed with PowerPlex® 21 System (Promega Corporation) with samples prepared for profiling per the "Amplification of Extracted DNA" protocol [194] as summarised in Figure 38. PlowerPlex® 21 5x Master Mix and PlowerPlex® 21 5x Primer Pair Mix were completely thawed on ice, centrifuged then vortexed for 15 seconds to homogenise. PCR amplification mixture was prepared per combining the PlowerPlex® 21 5x Master Mix and PlowerPlex® 21 5x Primer Pair Mix corresponding to the number of reactions as shown in Table 28. Water, Amplification Grade was not required in these experiments as the DNA concentrations of samples was initially low. The amplification mixture was vortexed to homogenise, before 10 µL of the mixture was aliquoted into each reaction well of a PCR plate (Multiplate™ 96-Well PCR Plates, low profile, unskirted, white, Bio-Rad Laboratories, Inc.). To the aliquots, 15 µL of each diluted DNA or neat DNA sample

was added to the reaction to give a final concentration of ≤ 5 ng per reaction. 15 µL of TE^{-4} buffer was used a negative control, while 2800M Control DNA was diluted and added to a reaction well to a final concentration of 0.5 ng as a positive control. The plate was sealed and centrifuged to bring contents to the bottom of the wells and remove air bubbles. After the reactions were prepared, samples were amplified on a thermal cycler (T100™ Thermal Cycler, Bio-Rad Laboratories, Inc.) using the protocol shown in Table 29.

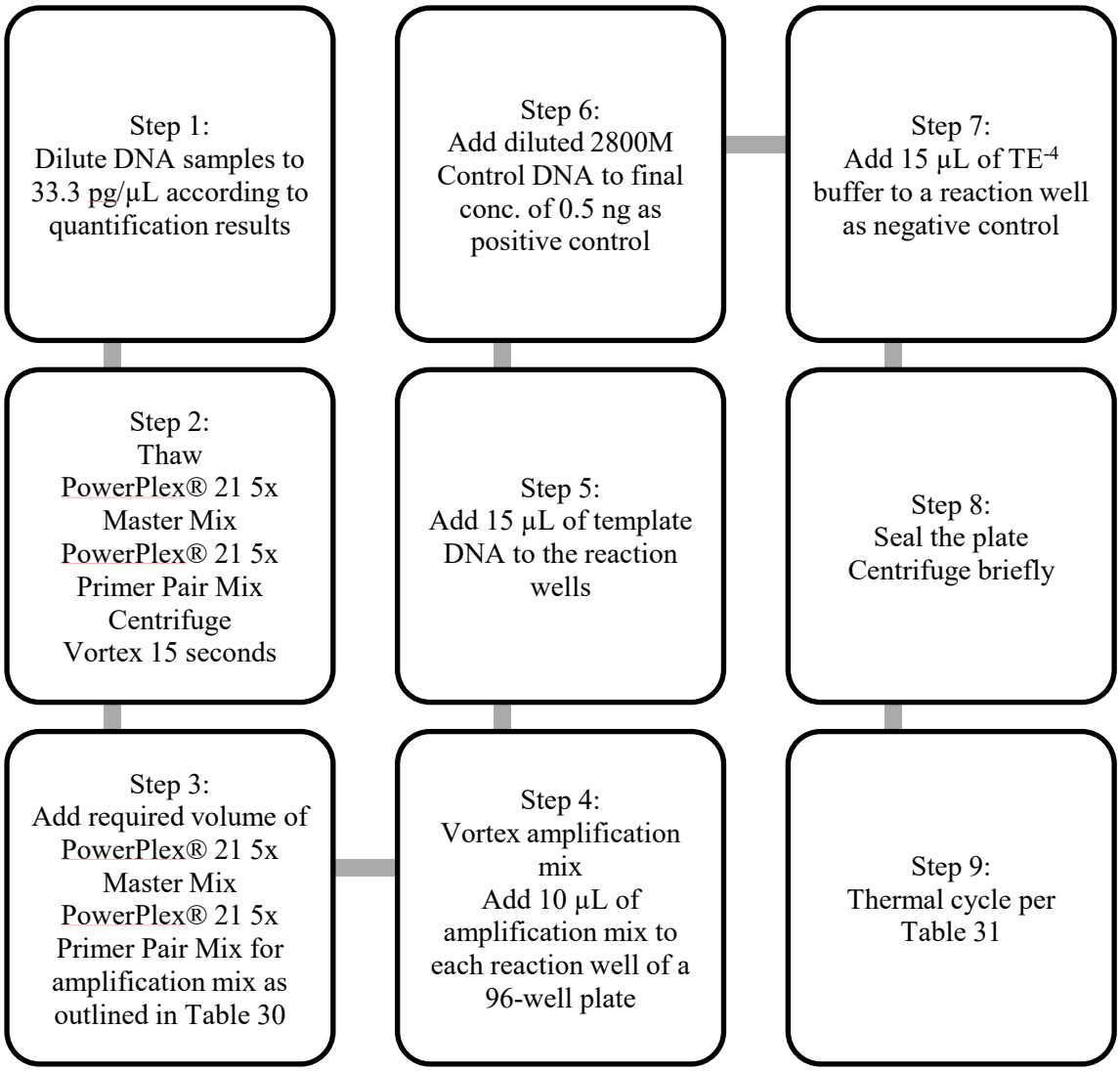


Figure 38: Workflow for amplification of DNA using PowerPlex® 21 System [194]

Table 28: Preparation of amplification mixture for profiling of DNA with Powerplex® 21 System

Component	Volume required per reaction
PlowerPlex21® 21 5x Master Mix	5.0 µL
PlowerPlex21® 21 5x Primer Pair Mix	5.0 µL

Table 29: Thermal cycling protocol for amplification of DNA using PowerPlex® 21 System on BioRad T100™ Thermal Cycler

Step	Time	Temperature	Number of cycles
Initialisation	1 minute	96°C	1
Denaturation	10 seconds	94°C	
Annealing	1 minute	59°C	30
Extension	30 Seconds	72°C	
Final extension	10 minutes	60°C	1
Hold	∞	4°C	Soak

Following amplification, samples were prepared for STR analysis on a Applied Biosystems Hitachi 3500 Series Genetic Analyser. Firstly, WEN Internal Lane Standard (ILS) 500 and Hi-DI™ formamide were thawed on ice, then a loading cocktail was prepared by combining WEN ILS 500 and Hi-DI™ formamide in amounts corresponding to the number of samples as per Table 30. The cocktail was vortexed before 10 µL was added to each sample well of a 96-well plate. 1 µL of amplified sample was then added to each sample well, with one well per column having 1 µL of PowerPlex® 21 Allelic Ladder Mix in place of amplified sample. The 96 well plate was then covered with a

septum and placed centrifuged to remove air bubbles, then denatured at 95°C for 3 minutes before being immediately chilled on ice for at least 3 minutes. Plates were then placed into a plate retainer and base, then loaded onto a genetic analyser for separation and data collection using the 3500 Series Data Collection Software 3 (3500 Series Genetic Analyser, Applied Biosystems; Hitachi) and processed with GeneMapper® *ID-X* Software (Applied Biosystems).

Table 30: Preparation of loading cocktail for profiling of DNA with Powerplex® 21 System

Component	Volume required per sample
WEN ILS 500	0.5 µL
Hi-Di™ Formamide	9.5 µL

5.4 Results and Discussion

5.4.1 Visual Examination

Treated fingermarks showed the development of CA covered ridges following the respective treatments. PolyCyano UV treated fingermarks showed a slight yellow tinge to the developed ridges, while ridge development following Lumikit™ treatment was white. Developed fingermarks were also visible under the respective luminescent conditions used in Table 24 (Figure 39). Results corresponded to previous studies [111, 115] with R6G post-stained samples providing more luminescent fingermarks than one step luminescent CA treated fingermarks. As samples were all fumed for 10 minutes, some background development occurred, however care was taken to ensure minimal background development was sampled when collecting touch DNA.

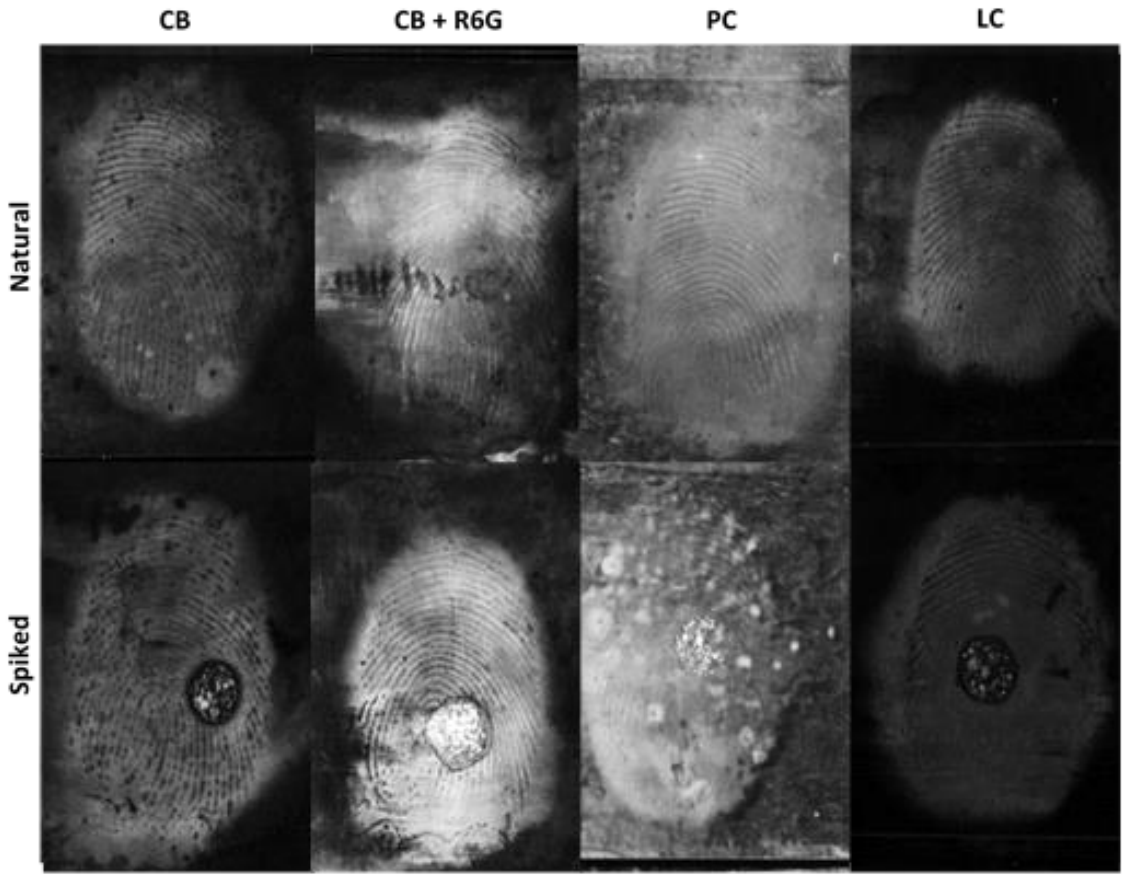


Figure 39: Representative fingermarks on glass developed with the respective CA treatment

Fingermarks were imaged under their respective visualisation luminescent conditions (Table 24).

It is interesting to note that the area of where cells spikes were dispensed on the fingermark showed distinct spotty development encompassed by an area of no development. The area of no ridge development can be attributed to the PBS used to suspend the cells after washing. It is highly likely that this aqueous phase interacted with the parts of the fingermark that it was in contact with dissolving the fingermark residue and thus there was no interaction with the CA monomer. The areas showing spotty development correspond to cells from the cell solution. The membrane of epithelial cells is rich in amino acids and proteins such as with carboxyl (COOH) and amine functions groups [195]. Epithelial cells, such as saliva and skin cells, contain filaggrin, which degrades into a number of amino acids that function to maintain the integrity of the cell. Among these is pyrrolidone carboxylic acid, making up approximately one-quarter of the

free amino acids found in the epidermis and oral mucosa [195, 196]. Additionally, inside, and on the cytoplasmic membrane of the cornified cell a number of amino and carboxyl rich proteins exist. Involucrin, expressed in during the differentiation of epithelial cells, is deposited in the cytoplasmic face of the cell membrane though interactions with transglutaminase and other membrane bound proteins before and as the cell is cornified [197]. This protein is abundant in the cell and contains a primary amine end and a carboxyl end (Figure 40). As the assembly of a cornified envelope begins before the nuclear components are destroyed, the detection of this molecule or associated functional groups may indicate the presence of DNA.

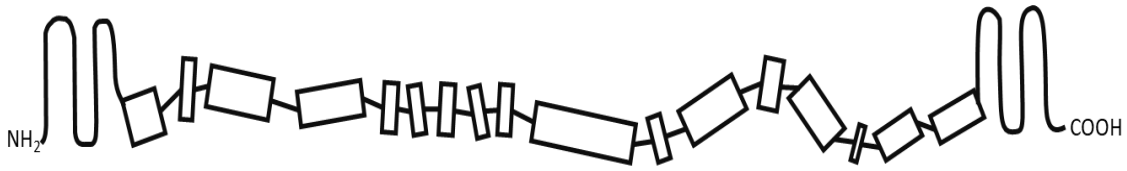


Figure 40: Model of human involucrin secondary structure

Adapted from Eckert *et al.* [197]

Wargacki *et al*. [198] found that CA polymerisation initiates preferentially at COOH functional groups and will also initiate at amine groups, thus the cell membrane promotes the polymerisation of CA vapours. Thiburce *et al*. [199] reported a similar finding where CA polymerisation occurred on the cells of diluted bloodstains where they suggest that either the components of the cell surface and/or the internal heme group, bearing an abundance of COOH groups, causes the initiation of CA polymerisation. The initiation of CA polymerisation on cell surfaces maybe assisted by the moisture level in the cells. Blank [200], noted, in the cornified layer (Figure 1) of the skin cells typically contain approximately 20% moisture, suggesting that saliva cells may be more hydrated due to the environment of the oral mucosa. Similarly, saliva cells suspended in PBS would contain a higher level of moisture. Ageing of cells may result in slower degradation than

that of fingermarks on non-porous surfaces as important CA polymerisation initiators are more protected from the effects of erosion [201].

5.4.2 Quantitation of Recovered DNA

The total amount of DNA recovered from natural fingermarks was found to be low and vary both within the treatment groups and between treatments (Figure 41). At least one outlier was found for each treatment group, except Lumikit™, which had no outliers. As the outliers were between 9 and 204% greater than the average recovery of the respective treatment group with the outlier value removed (Table 31), it was concluded that contamination contributed to the higher DNA amounts seen in these samples. To avoid skewing of the results, outliers were removed from the calculation of average, standard deviation and standard error as well as ANOVA. As such, there was no significant differences between the treatment groups as determined by one-way ANOVA ($F(4,30) = 2.55$, $P = 0.059$).

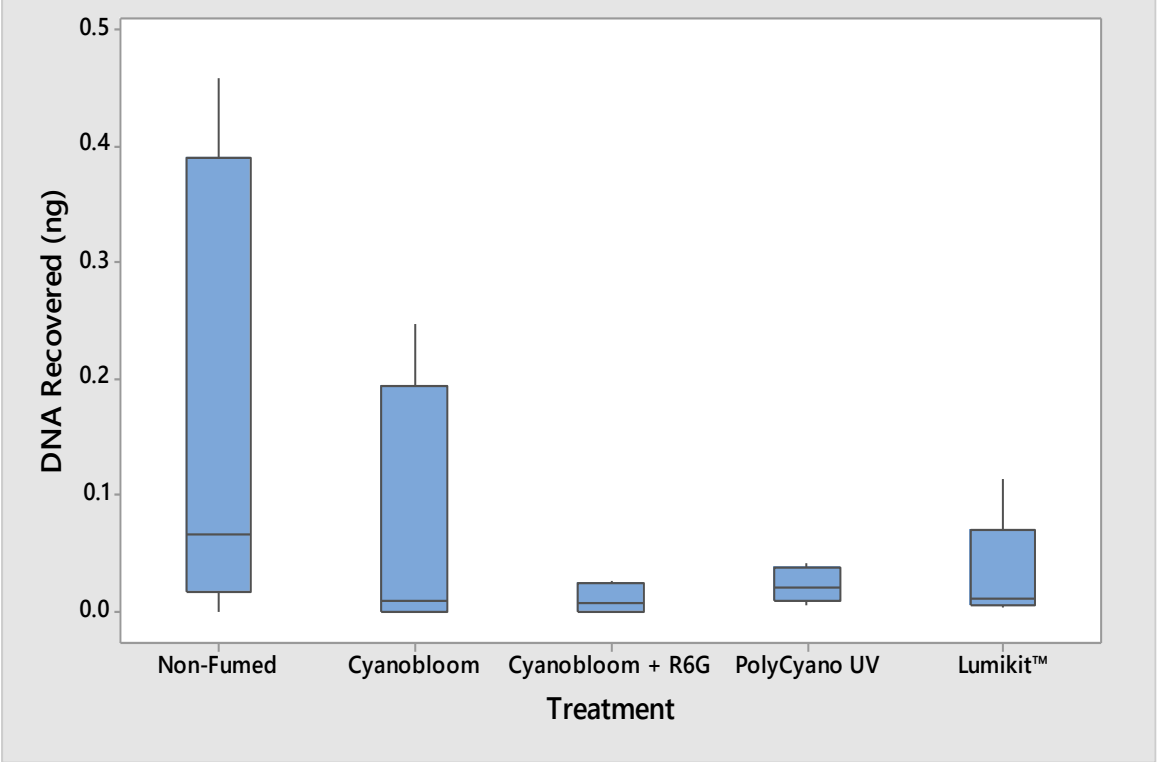


Figure 41: Amounts of DNA recovered from natural fingermarks developed with the respective CA treatments

Extraction of DNA was performed using QIAamp DNA Mini Kit. Quantitation of DNA in samples was performed using Plexor® HY.

Table 31: DNA recovered from natural non-fumed and CA treated fingermarks

Each line for each donor relates to the separate fingermark depositions collected for the respective treatment; * denotes outliers; SD denotes standard deviation; SE denotes standard error; Outliers were removed from the calculation of average, SD and SE.

Donor	DNA recovered (ng)				
	Non-Fumed	Cyanobloom	Cyanobloom + R6G	PolyCyano UV	Lumicyano™
1	0.009	0.017	0.000	0.017	0.012
	0.052	0.000	0.026	0.023	0.088
2	0.000	1.505*	0.004	2.967*	0.003
	0.457	0.000	0.000	0.041	0.011
3	0.037	0.000	1.595*	0.013	0.005
	0.189	0.032	0.017	0.004	0.113
4	1.069*	0.000	0.000	0.008	0.009
	0.080	0.246*	0.008	0.025	0.014
Mean	0.12	0.01	0.01	0.02	0.03
SD	0.16	0.01	0.01	0.01	0.04
SE	0.06	0.01	0.00	0.00	0.02

DNA was recovered from all spiked samples following each of the treatments with the total amounts varying from 3.0 – 20.8 ng of total DNA (Figure 42; Table 32). Spiked samples were included to provide a more consistent and genuine result as the nature of touch DNA is quite unpredictable. Analysis of variance showed a significant difference between at least two of the means (ANOVA: $F(4,35) = 2.64$, $P = 0.04$). Fisher-Hayter pairwise comparisons at $\alpha = 0.05$ was subsequently conducted to determine which treatments showed statistically different results (Table 33). The comparisons showed a

significant difference between the mean recoveries of PolyCyano UV and Cyanobloom+R6G. This suggests that the application of the R6G post-stain either dissolves some of the CA and washing away the DNA, or causes some of fragile regions of the CA polymer to break off taking the trapped DNA with it. While PolyCyano UV provides weaker luminescence, the observations suggest that it provides superior recovery of DNA compared to post-staining with R6G. This may be due to the lack of post-treatment allowing DNA to be retained within the polymer until collection. It may also be that DNA is more easily extracted PolyCyano UV than the CA used in Cyanobloom + R6G, as it is believed that the fumes of the dye are incorporated into the developing CA polymer [107], this may present a weaker polymer and allow for a higher amount of DNA recovery in those samples.

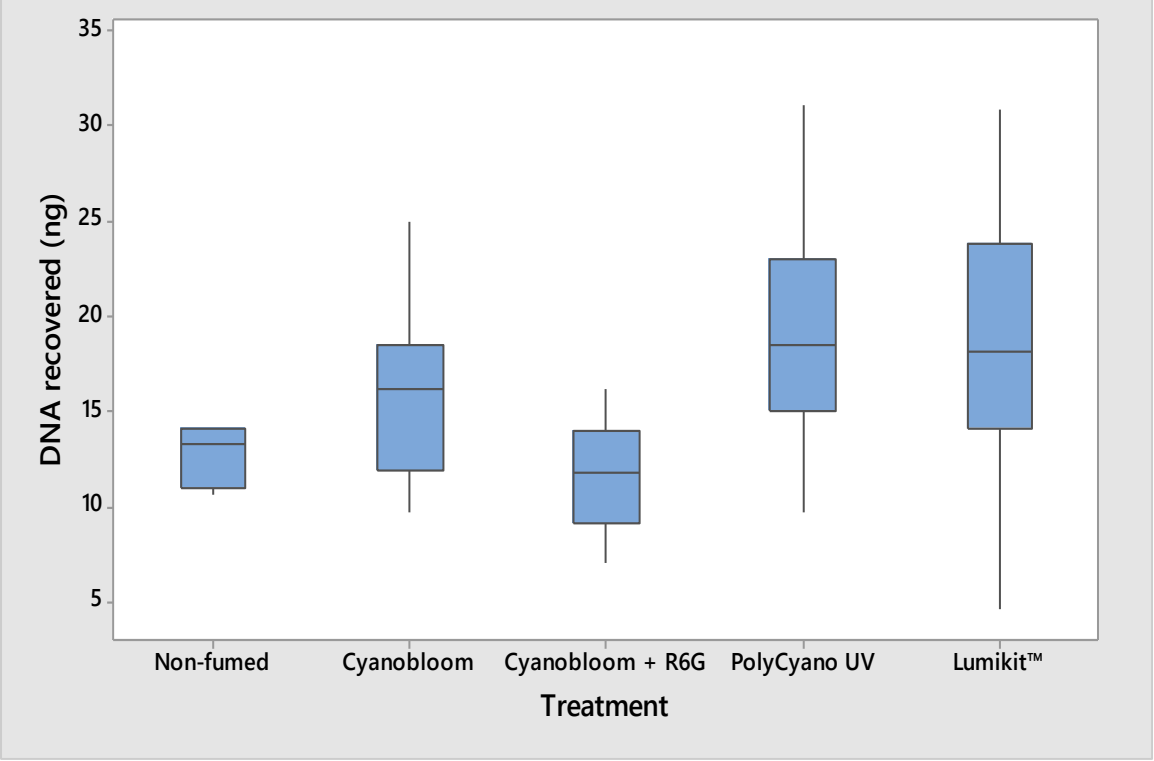


Figure 42: Amounts of DNA recovered from spiked natural fingermarks developed with the respective CA treatments

Extraction of DNA was performed using QIAamp DNA Mini Kit. Quantitation of DNA in samples was performed using Plexor® HY.

Table 32: DNA recovered from spiked non-fumed and CA treated fingermarks

Each line for each donor relates to the separate fingermark depositions collected for the respective treatment; SD denotes the standard deviation; SE denotes the standard error.

Donor	DNA recovered (ng)				
	Non-Fumed	Cyanobloom	Cyanobloom + R6G	PolyCyano UV	Lumicyano™
1	12.9	14.9	8.9	23.3	15.4
	5.7	13.7	9.9	9.6	24.3
2	13.5	18.6	16.2	22.3	30.8
	14.2	9.7	11.4	18.1	18.2
3	23.3	11.3	7.0	31.2	18.1
	12.1	18.0	13.0	14.5	4.6
4	10.6	17.3	12.1	16.5	13.7
	13.8	25.0	14.2	19.0	22.7
Mean	13.3	16.1	11.6	19.3	18.5
SD	4.9	4.8	3.0	6.5	7.8
SE	1.7	1.7	1.0	2.3	2.8

Table 33: Fisher-Hayter pairwise analysis of DNA quantity recovered following CA treatment and processed with Plexor® HY System

D_{FH}: 5.0727; α = 0.05; * denotes significant difference between mean DNA recovery following the respective treatments.

Variance summary								
Groups	**Count**	**Sum**	**Average**	**Variance**	**Cyanobloom**	**Cyanobloom + R6G**	**PolyCyano UV**	**Lumikit™**
Non-Fumed	8	70.813	8.852	10.595	1.8611	1.1238	4.0166	3.4576
Cyanobloom	8	85.702	10.713	10.299		2.9848	2.1556	1.5966
Cyanobloom + R6G	8	61.823	7.728	3.907			5.1404*	4.5814
PolyCyano UV	8	102.946	12.868	18.567				0.5590

Low recovery was observed in all samples across the treatments. This could be due to a number of factors. As CA is a fingermark enhancement technique, the donors in this study were chosen based on the amount of fingermark secretions they deposit rather than their DNA shedding status. As such, results for the natural fingermarks (Figure 41, Table 31) the results may be genuine with these particular donors sloughing low quantities of touch DNA, indicating that they are poor shedders. It could also be due to the donors conducting their usual activities between depositing fingermarks. While this is a realistic scenario, the touch DNA from donors may have been lost due to contact with other surfaces prior to the collected deposition [31].

DNA and cells have high affinity for rayon [202], however, there is no mechanism for the release of the genetic material from the swab. This could account for the low recovery seen in non-fumed samples but would also affect the collection of all other samples. Verdon et al. [70] also reported rayon swabs to be relatively inefficient for DNA recovery from touched glass and more suitable for biological fluids, which have higher amounts of DNA, from porous substrates. However, the CA polymer may aid in the recovery of genetic material, with DNA and cells being trapped in the CA polymer making it easier visualise an area of touch and to swab and retrieve cells from the glass surface due to both the fragility and visibility of the polymer. The size of the CA polymer also makes it less prone to becoming trapped within the fibres of the swab. While this resulted in higher amounts of DNA being collected into the vessel, in some cases, the genetic material may still be trapped within the CA polymer that is discarded during the extraction process. This could account for the variability in the recovery of DNA in these samples.

Additionally, a loss of DNA may have occurred during the extraction process with a previous study [203] showing approximately 35 – 60% recovery from a similar manual extraction process, depending on the collection mechanism. The fingermark detection

method may also have played a part in the low recovery. It is possible that some DNA was still encased within the CA polymer following the extraction process, which may also account for the slightly higher recovery of non-fumed samples compared to CA treated samples.

5.4.3 Profiling of Recovered DNA

Analysis of the negative control sample did not show the presence of any alleles above the threshold of 50 RFU (Appendix. Electropherograms, Figure 62). This shows that any contamination in test samples is not a result of the amplification and loading steps. Peaks were not reported for any alleles in the non-fumed control, however some small spikes with some semblance to allele peaks, less than the peak height threshold of 50, were visible in the electropherogram (Appendix. Electropherograms, Figure 63: amelogenin, D3S1358, D1S1656, D6S1043, D13S317 and Penta E). As peaks were not seen the fumed blank slides (Appendix. Electropherograms, Figure 64 to Figure 67), this potentially indicates that small amounts of DNA may be trapped in CA and discarded during extraction steps. Every care was taken to minimise the risk of contamination; however, it must be noted if the peaks resulting from the non-fumed control (Appendix. Electropherograms, Figure 63) are genuinely from DNA, then the source if it was not able to be identified. The pattern did not correspond to that of any of the fingermark donors used in this study, the spike donor, nor the individual who conducted the experimental work. It may indeed be minute cross contamination from any piece of equipment or instrument used in the experiment or from other lab users who were in the vicinity at the time. None of the fumed control samples showed any peaks nor patterns similar to that seen in the non-fumed control, however two anomalies were seen in the PolyCyano UV sample (Appendix. Electropherograms, Figure 66: CSF1PO and THO1) where a single

small peak was observed at these alleles. Overall, the results of the fumed controls indicate that the results derived from touched and spiked touch specimens are unlikely to be the result of contamination during the fuming, visualisation or DNA recovery stages.

Natural fingermark samples from this study were profiled, however, due to the low concentration (0 – 0.5 ng/μL), partial profiles were returned from only two samples while remaining samples returned no profiles. Similarly, informative profiles were not able to be achieved from fingermark donors of spiked samples, however, 100% of the spike donor's alleles were called for each treatment as shown by the representative electropherograms (Appendix. Electropherograms, Figure 68 – Figure 72). When comparing the amount of DNA recovered from natural samples and spiked samples (Figure 41, Figure 42), it was expected that the spike donor DNA would be the primary contributor to the spiked samples. This was confirmed when the percentage of alleles called was determined (Figure 43), with 100% of spike donor alleles being called while only 1 – 12% of alleles from individual fingermark donors being called.

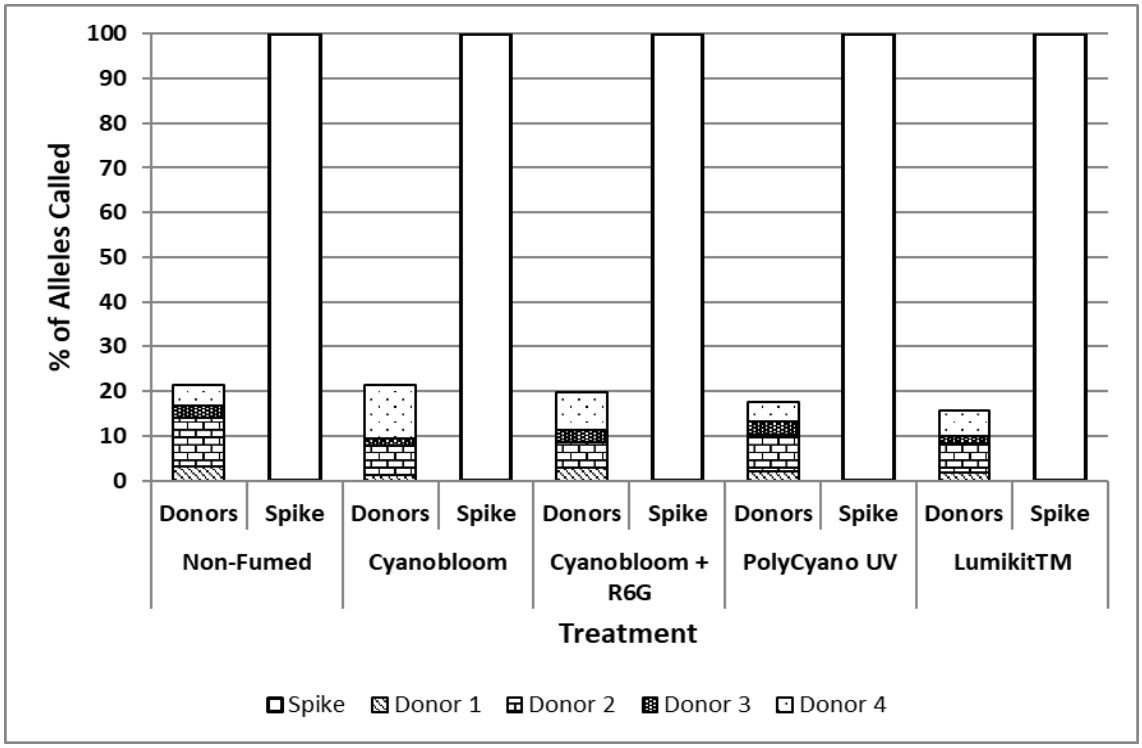


Figure 43: Percentage of alleles called from spiked fingermark samples following the respective CA treatment

Alleles were identified following STR analysis with PlowerPlex21® using HID software.

While there is a slight variation in the percentage of fingermark donor alleles called (Figure 43), this can be attributed to the intra- and inter-donor variation rather than the different treatments as no significant difference was observed between the treatments (ANOVA: $F(4,19) = 0.13$, $P = 0.97$). Touch DNA has been found to be highly variable. Not only is DNA shed differently among individuals, but within the same individual, DNA recovery varies from day-to-day and from hand-to-hand, being impacted by a variety of factors [62]. The amount of DNA deposited also decreases significantly following an initial touch [31]. As donors in this study donated fingermarks from their only the index and middle fingers, over the course of a day and during their usual activities, the amounts of DNA recovered from the fingermark donors were expected to be both low and inconsistent.

As observed in Figure 68 to Figure 72 (Appendix. Electropherograms), as the fragment size increased, the peak height decreased. This trend is not observed in the positive control (2800M DNA, Promega) where the peak height does not decrease drastically as the fragment size increases (Appendix. Electropherograms, Figure 73). An analysis of peak height and fragment size of the recovered DNA was therefore conducted to determine if any degradation was due to the CA treatments (Figure 44). The equation of the trend lines is presented in Table 34. Only alleles corresponding to the spike donor were used in this analysis to avoid skewing the results, while 0.5 ng of input DNA (2800M Control DNA) was used as the positive control per the recommended protocol [194]. One-way analysis of covariance (ANCOVA) of the spiked non-fumed and all spiked CA treated samples was conducted to determine if there were any significant difference between the respective treatments on the peak height of recovered DNA controlling for the locus size. A significant difference was found between at least two of the treatments ($F(1,4) = 539.91$, $P = 0.000$) (Table 35).

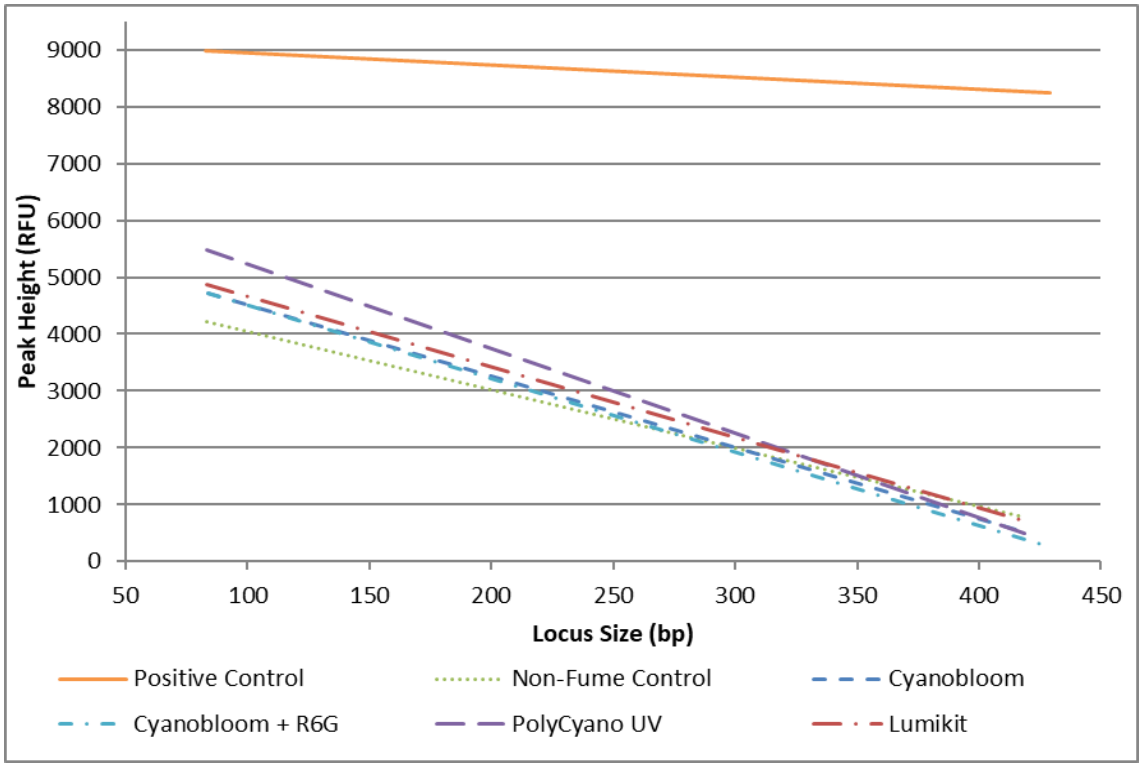


Figure 44: Peak height trends of identified allele from spike donor DNA recovered following the respective CA treatment

DNA was extracted with QIAamp DNA Mini Kit and profiled with PlowerPlex21® using HID software.

Table 34: Equations of peak height trends of identified allele from spike donor DNA recovered following the respective CA treatment

Treatment	Trend Line Equation
Positive Control	y = -2.1146x + 9159.7
Non-fume	y = -10.258x + 5078.6
Cyanobloom	y = -12.545x + 5764.3
Cyanobloom + R6G	y = -12.949x + 5802.8
PolyCyano UV	y = -14.865x + 6727.3
Lumikit™	y = -12.411x + 5901.2

Table 35: ANCOVA of spiked non-fumed and treatment samples

DF denotes degrees of freedom; SS denotes sum of squares; MS denotes mean squares.

Source	DF	Adjusted SS	Adjusted MS	F Ratio	P Value
Locus Size	1	2108147301	2108147301	539.91	0.000
Treatment	4	56844436	14211109	3.64	0.006

Fisher LSD post-hoc analysis of these treatments (Table 36) found there was no difference between the means of the two one-step luminescent treatments, while there was also no difference between Lumikit^{TM}, Cyanobloom, Cyanobloom + R6G and non-fumed samples. This indicates that a significant difference existed between PolyCyano UV treated samples and that of Cyanobloom, Cyanobloom + R6G and non-fumed samples. This suggests the application of PolyCyano UV has some degradation effect on the sample, having the steepest declining slope of all treatments (Table 34, Figure 43). As no significant difference was found between Lumikit^{TM} and PolyCyano UV (Table 35), it is perhaps likely that a minor degree of degradation has occurred following that treatment. The degradation caused by these treatments may be due to the formation of the CA, where the dye of the one-step luminescent CAs are incorporated into the CA polymer. This may produce a more porous CA structure on the ridges and cells and DNA may begin to fragment and degrade as it is not as protected from the environmental conditions. This could also explain why the R6G dye did not adversely impact the quality of DNA compared to non-fumed controls and Cyanobloom treatment as genetic material was more fully encased with on the CA and less likely to have direct contact with the dye. When dealing with the minute amounts of DNA found in touch DNA, the effects of these treatments may be more severe, especially in the case of PolyCyano UV.

Table 36: Fisher LSD analysis of significant difference between treatments

Means that are grouped by a letter are not significantly different.

Treatment	N	Mean	Groupings		
Positive Control	39	8645.75	A	-	-
Non-Fumed	242	2717.51	-	B	-
Cyanobloom	242	2877.80	-	B	-
Cyanobloom + R6G	277	2822.97	-	B	-
LumikitTM	279	3045.48	-	B	C
PolyCyano UV	278	3308.75	-	-	C

Difference of Treatment Levels	Difference of Means	SE	Individual 95% CI	T-Value	P-Value
Cyanobloom + R6G - Cyanobloom	-55	180	-408, 298	-0.30	0.761
Lumikit - Cyanobloom	168	180	-185, 520	0.93	0.351
Non-Fume - Cyanobloom	-160	186	-525, 205	-83.83	0.389
PolyCyano UV - Cyanobloom	431	180	78, 784	2.40	0.017
Positive Control - Cyanobloom	5768	353	5075, 6461	16.34	0.000
Lumikit - Cyanobloom + R6G	223	174	-118, 563	1.28	0.200
Non-Fume - Cyanobloom + R6G	-105	180	-459, 248	-0.59	0.558
PolyCyano UV - Cyanobloom + R6G	486	174	145, 827	2.80	0.005

Difference of Treatment Levels	Difference of Means	SE	Individual 95% CI	T-Value	P-Value
Positive Control - Cyanobloom + R6G	5823	350	5136, 6509	16.64	0.000
Non-Fume - Lumikit	-328	180	-681, 25	-1.82	0.068
PolyCyano UV - Lumikit	263	173	-77, 603	1.52	0.126
Positive Control - Lumikit	5600	350	4914, 6286	16.01	0.000
PolyCyano UV - Non-Fume	591	180	238, 944	3.29	0.001
Positive Control - Non-Fume	5928	353	5236, 6621	16.79	0.000
Positive Control - PolyCyano UV	5337	350	4651, 602	15.26	0.000

It is also of note that the positive control was significantly different from all other treatment samples. The positive control used in this study was 2800M control DNA from PowerPlex® 21 System. This DNA sample is kept frozen at -20^{o}C when not in use and hence shows very little degradation (Table 34, Figure 44), while the CA treated and non-fumed samples were all aged for 14 days prior fuming and DNA collection. The latter resulted in much steeper trend lines than the positive control. This indicates that the ageing of the samples has a significant impact on the degradation of DNA [67] and this is more severe than the application of the one-step treatments. Hence, in order to maximise the amount of meaningful information that can be extracted from such traces, they need to be located and processed promptly as information can be lost over time.

5.5 Conclusions

In this study, DNA analysis of natural fingermarks and spiked fingermarks was conducted following the application of various CA techniques. The results of the DNA analyses were evaluated with the aim of determining the impact that one-step luminescent CAs, PolyCyano UV and Lumikit™, have and how they compare to traditional CA fuming, and with R6G post-stain. Fingermarks were detectable following each of the treatments. Visualisation of treated spiked fingermarks indicates that large concentrations of cells are developed by CA and can be more easily visualised following treatment when viewed under an alternative light source. Visualisation of natural fingermarks shows that cells originating from touch are also developed by CA however as they are encompassed within the fingermark residue, require the use of fluorescent microscopy to clearly identify these sources of DNA. DNA from both touch samples and spiked samples were also recoverable and quantifiable following the use of PolyCyano UV and Lumikit™ as well as conventional CA methods.

The results of this study indicate that while all CA treatments evaluated caused a degree of DNA degradation, the effect of PolyCyano UV on DNA was more significant compared to the other CA methods used in this study. The impact of PolyCyano UV may be more significant on decreased DNA quantities resulting in allele drop out at larger fragment sizes. Conversely, the use of Lumikit™ is comparable to both PolyCyano UV as well as conventional CA techniques and non fumed samples. This may suggest that it degrades DNA to a lesser degree than PolyCyano UV. It is recommended, however, that users employ their own discretion as to the technique that will maximise recovery and exploitation of individual traces.

Ideally, a larger scale study on lower known quantities of DNA should be conducted to compare the treatments at amounts representative of touch DNA as this may be more indicative of a realistic scenario, however with the error involved in working with small quantities of DNA would have to be considered. Critical factors that would need to be carefully assessed in future studies include differences due to donor inter- and intra-variations that is seen in touch DNA as these variations present difficult parameters to control for; and the effects of the DNA extraction method and the losses due to the extraction procedure and other DNA processing methods.

Chapter 6. DUAL DETECTION ON A POROUS SURFACE FOLLOWING TREATMENT WITH A DMAB-IND REAGENT

The work detailed in Section 6.4 of this Chapter has been published under the title: "Detection of Latent Fingermarks and Cells on Paper" in Forensic Science International 2020 April; 309:110185

(doi: 10.1016/j.forsciint.2020.110185) [204]

6.1 Introduction

Unlike the detection of fingermarks on non-porous surfaces, the detection of those on porous surfaces cannot use techniques that target surface residues [1]. On these types of surfaces, the water-soluble constituents, such as amino acids, urea and salt, are absorbed into the substrate over time, while the water-insoluble component remains on the surface and evaporates (Figure 4). Typically, the techniques used for the enhancement of fingermarks on non-porous surfaces rely on physical properties of the fingermark to create contrast with the background [1]. As the constituents of the fingermark are rapidly absorbed or evaporated on porous surfaces, the use of techniques such as fingermark powdering and CA fuming are ineffective as is it difficult to access the fingermark residue

with in the porous surface. Thus, when applied, these detection molecules become trapped within the pores of the porous surface and fail to distinguish the fingermark from the background.

Most techniques for revealing fingermarks on porous surfaces require the enhancement agent to be absorbed into the item to allow for interaction with the fingermark residue contained within the substrate. Ideally, a reagent for detecting genetic material on porous surfaces should also be able to absorb into the substrate. Studies have indicated that DNA is more readily transferred to porous substrates, and more persistent in these surfaces [70, 205, 206]. It is likely that the rough and fibrous nature of these surfaces is able to absorb and scrape off more DNA than non-porous surfaces and maintain hold on cells and other sources of DNA within the pores.

As previously discussed in Section 2.2.3, techniques currently used for fingermark detection on porous surfaces primarily target the amino acids found in the fingermark residue [1]. The most common techniques are PD, for porous substrates that have been in contact with a high levels of moisture, and DFO, NIN, and IND (/-Zn) for items that have not been wetted [1]. Studies have shown that the use of DFO and NIN to detect fingermarks reduces the amount of recoverable DNA while having no notable impact on STR analysis [33, 74, 75, 80]. Due to the superior detection capabilities of IND-Zn, the use of this technique has become increasingly common, widely superseding the use of DFO and NIN [129, 187, 207]. Similar to DFO and NIN, treatment with IND and IND-Zn has not shown an adverse impact on STR analysis when conducted promptly following treatment. However, the quality of the profile is seen to decline with time following treatment [73, 76, 81]. While treatment with IND or IND-Zn on porous surfaces is particularly useful for detecting amino acids in the fingermark residue, it is difficult visualise touch DNA or differentiate cellular material from the fingermark when the

treatment is applied. Hence, to allow for further exploitation of the traces, a complementary reagent could be used to assist in detecting the genetic component of the residue.

Reagents to detect DNA are not routinely used in the examination of exhibits, rather indicators for types of biological stains are employed. Current research into the detection of DNA focuses on the use of DNA stains such as SYBR Green I, GelGreen and Diamond^{TM} Nucleic Acid Dye on non-porous surfaces [141, 148-150, 152, 154]. As discussed in Section 2.4.2, the use of these techniques is not economical when locating touch DNA on exhibits and in some cases is the reagent is prepared at toxic levels and may hinder DNA analysis processes. Additionally, the minute size of the DNA molecule presents a challenge for visualisation as their presence may be overlooked even with the use of specialised equipment. Hence, targeting sloughed cells or components of sloughed cells may present a cost-effective method for detecting genetic material.

Following the success of incorporating DMAB into CA to create an efficient method of enhancing fingermarks on non-porous surfaces, interesting trials have been conducted with DMAB for the detection of fingermark residue deposited on porous surfaces. DMAB is used in the detection and investigation of nitrogen compounds such as amines in histological work [185]. The molecule reacts with primary amine groups to produce imines in acidic conditions producing a colour change. In their work, Fritz *et al*. [184] determined that DMAB could be used to develop fingermarks on porous surfaces. Initial tests used 1% DMAB in ethanol. This solution was applied to fingermarks on paper before being heat pressed, revealing slightly brown marks that were luminescent at 490 nm excitation with 550 nm emission filter [184]. Investigations then commenced into optimal formulations for wet and dry contact methods (Table 37) [184]. Fritz *et al*. [184] report the wet contact method for detecting fingermarks on paper was more successful than the

dry and used a similar treatment process to that of IND-Zn; however, it lacked sensitivity when compared to IND-Zn [184, 187]. In further work by this group [186], the dry contact formulation (Table 37) was reassessed against NIN under a wide range of conditions. DMAB was again found to be less sensitive especially on fingermarks more than 2 weeks old, but did show improved efficiency on semi-porous surfaces.

Table 37: Wet and Dry contact formulation of DMAB and IND-Zn used by Fritz *et al*. [184]

Components	DMAB		IND-Zn	
	Wet	Dry	Wet	Dry
DMAB	0.4% v/w	4% w/v	-	-
IND	-	-	0.08% v/w	0.149 % w/v
Ethyl Acetate	8.8% v/v	qs 100 %	9% v/v	6.9% v/v
Acetic Acid	1.2% v/v	-	1% v/v	-
$ZnCl_2$	-	-	0.016% w/v	0.004% w/v
Ethanol	-	-	0.4% v/v	0.1% v/v
Dichloromethane	-	-	-	2.97% v/v
HFE-7100	qs 100%	-	qs 100%	qs 100%

While DMAB shows some fingermark development potential, its enhancement of fingermarks is inferior to that of well-established methods such as IND-Zn and NIN. Due to its immense capability to detect nitrogen species, it could instead be used to detect cellular material in the fingermark deposit as cells are rich in nitrogen containing molecules. Nitrogen contributes to 15.09% of the dry weight of the skin cells on the cornified layer [208], with amines being particularly abundant [26, 195-197, 209]. This may facilitate a luminescent reaction between DMAB and cellular components allowing for cells to be visualised.

Due to the size of DNA itself, it cannot be readily visualised without the use of complex molecular techniques that may be additionally costly and further delay analysis processes. Cells are vastly larger than DNA being from 30 μm^3 (sperm cells) to 4,000,000 μm^3 (egg) or generally between the range of 10 – 100 μm in diameter [210]. Cornified cells of the hand are approximately 35 μm, while on other regions of the body can be up to 44 μm [211]. While some microscopy equipment may be required, the cell presents a much larger area for detection than DNA with the nucleus being approximately 0.01 μm^3 or up to 10 μm in diameter and DNA only occupying 0.3% of this space [210]. Thus, not only do cells offer a much larger target for visualisation, this also eliminates the need for dyes to penetrate both the cell and nucleus to detect DNA. Hence, the detection of cellular material may provide for an indication for the likelihood of detecting viable DNA.

DMAB is used in some one-step luminescent cyanoacrylate products [180, 182, 189] with it noted that cells spiked on to fingermarks were visible along with the developed fingermark following treatment when treated with the PolyCyano UV (see Section 5.4.1). As DMAB has shown compatibility with the IND-Zn base formulation (Table 37) it appears possible to combine these reagents to obtain a solution dual acting solution.

6.2 Aim and Objectives

The aim of this study was to determine feasibility of a combined DMAB and IND-Zn solution for the dual detection of latent fingermarks and cellular material. Thus, the use of this solution should enhance fingermarks and provide an indication of the presence of cellular material on paper, where the positive indication of cellular material can be further tested for the presence of DNA. The objective was to optimise the concentration of each

active component, then compare the optimised reagent to IND-Zn treatment as well as untreated controls to evaluate the ability of the reagent to detect fingermark and cell and how it compares against the other treatments with regard to DNA profiling. The quality of the DNA profiles from treated samples was also assessed with time following treatment to determine the impact of the reagent on DNA.

6.3 Optimisation of the Active Concentrations

6.3.1 Methods

6.3.1.1 *General methodology*

The experimental design is summarised in Figure 45. The optimal concentrations of DMAB (ACS, Fisher Scientific Co.) and IND (Casali Institute) were determined for the DMAB-IND reagent using the recommended IND-Zn base formulation [187] with HFE-7100 (3M^{TM} Novec^{TM}) as the carrier solvent. Concentrations of DMAB trialled ranged from 0.1 – 0.6% w/v with 0.06% w/v IND, while concentrations of IND trialled were from 0.03 – 0.08% w/v with 0.4% w/v DMAB. Briefly, fingermarks deposited on copy paper (Carbon Neutral 20 % Recycled A4, STAPLES®) were treated separately with the formulations at the different concentrations. Treated fingermarks were then compared to one another to determine which formulation was the least detrimental to both the visualisation of the fingermark and cells. Once the optimal concentration was determined, the reagent was prepared with these concentrations according to Stoilovic and Lennard [187], with Solstice® Performance Fluid (PF; Honeywell) substituted for HFE-7100 as the carrier solvent, and the results of treatment were compared to that of the same concentration reagent prepared with HFE-7100.

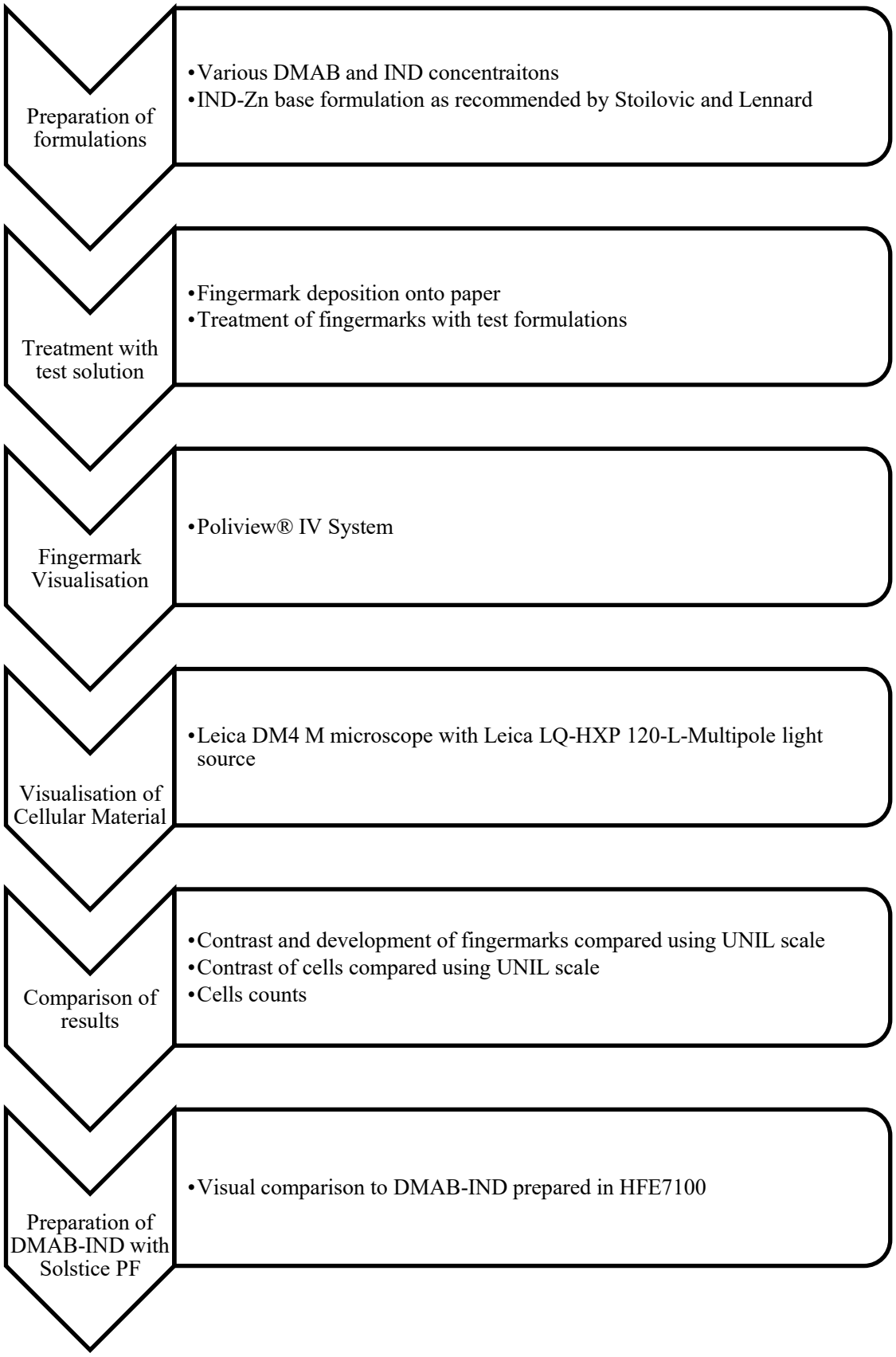


Figure 45: Workflow for the optimisation of DMAB and IND concentrations for the DMAB-IND combined reagent

6.3.1.2 *Preparation of DMAB-IND reagent at different concentrations*

DMAB-IND combined reagent was prepared at various concentrations per Table 38. Eleven different solutions were compared, with six of these solutions containing DMAB at 0.4% w/v while the concentration of IND varied between 0.03 – 0.08% w/v; the remaining five solutions contained IND at 0.06% w/v while the DMAB concentration varied between 0.1 – 0.6% w/v. The constant concentrations in these solutions represent the concentrations recommended for fingermark detection in wet contact solutions [184, 187].

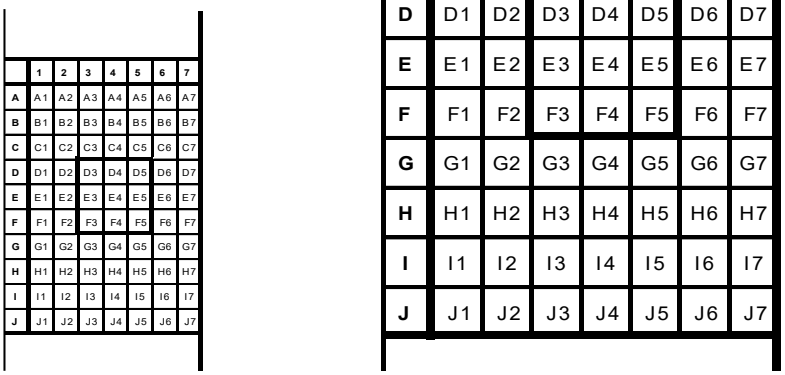
Table 38: Preparation of DMAB-IND solutions at different active concentrations

[§]working solution of $ZnCl_2$ and ethanol was prepared separately; *refers to DMAB and IND; [ǂ]individual solutions prepared per concentration combination of the actives; suppliers and quality of reagents listed in Table 22.

Order of Addition	Component	Concentration [ǂ]		Amount required / 100 mL	
		DMAB	IND	DMAB	IND
1	Active *	0.40% w/v	0.08% w/v	0.40 g	0.08 g
			0.07% w/v		0.07 g
			0.06% w/v		0.06 g
			0.05% w/v		0.05 g
			0.04% w/v		0.04 g
			0.03% w/v		0.03 g
		0.10% w/v	0.06% w/v	0.10 g	0.06 g
		0.20% w/v		0.20 g	
		0.30% w/v		0.30 g	
		0.50% w/v		0.50 g	
		0.60% w/v		0.60 g	
2	Ethyl Acetate	12.48% v/v		12.48 mL	
3	Acetic Acid	0.52% v/v		0.52 mL	
4[§]	$ZnCl_2$	0.016% w/v		0.016 mL	
	Ethanol (absolute)	0.40% v/v		0.40 mL	
5	HFE-7100	qs 100%		qs 100 mL	

6.3.1.3 *Procedure*

Six donors each deposited 11 natural fingermarks (n = 66) onto separate 36.3×26.4 mm^2 grids printed on copy paper, consisting of 3.3 mm^2 squares per grid (Figure 46). Following deposition of fingermarks, one of each of the fingermarks from each donor were immersed into each of the DMAB-IND solution (n = 6 for each different concentration) until wet, removed from the solution and allowed to air dry. Once dry, samples were placed between fresh paper towel and briefly heat pressed together at 120°C. Samples were then visualised using the Poliview® system (Polilight PL500, Peltier Cooled 12 bit CCD camera and lens, with Poli-V®++ software, Rofin Australia) and the optimal viewing conditions were determined.



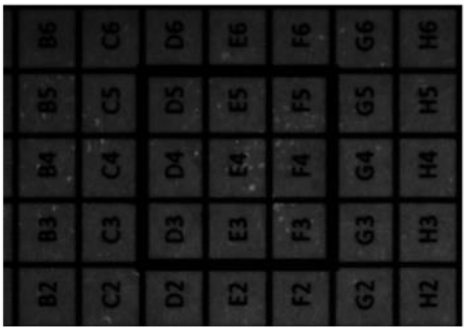
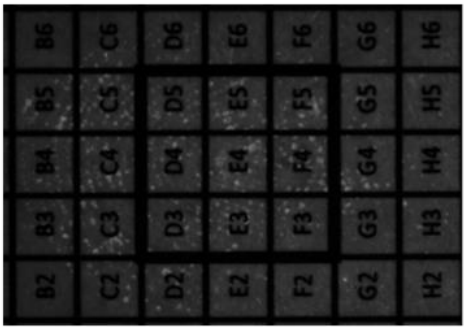
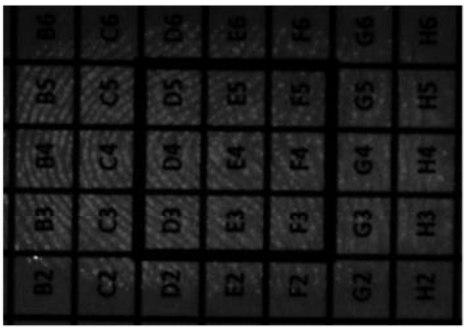
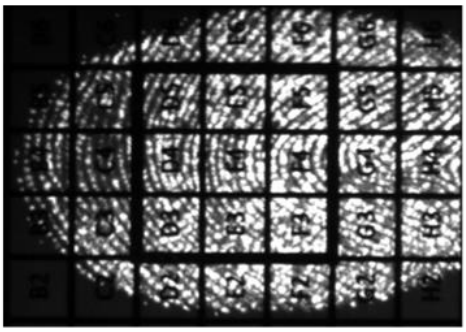
	1	2	3	4	5	6	7
A	A1	A2	A3	A4	A5	A6	A7
B	B1	B2	B3	B4	B5	B6	B7
C	C1	C2	C3	C4	C5	C6	C7
D	D1	D2	D3	D4	D5	D6	D7
E	E1	E2	E3	E4	E5	E6	E7
F	F1	F2	F3	F4	F5	F6	F7
G	G1	G2	G3	G4	G5	G6	G7
H	H1	H2	H3	H4	H5	H6	H7
I	I1	I2	I3	I4	I5	I6	I7
J	J1	J2	J3	J4	J5	J6	J7

Figure 46: Grids printed onto copy paper for deposition of fingermarks (Left: 1:1 scale representation; Right: 2:1 scale representation)

Central region of the grid outlined in thicker lines indicates a 1 cm^2 area where donors were asked to align the central region of their fingermark. This area was subsequently examined microscopically. Letter and number inside each cell is the identifier for the cell and allowed for images from microscopy to be stitched together.

Following examination with the Poliview® system, the central 1 cm^2 region of the grid (Figure 46) of the deposited fingermark was inspected using a Leica fluorescent microscope system (Leica DM4 M microscope with Leica LQ-HXP 120-L-Multipole external light source, Leica Microsystems™ Pty Ltd) and an H3 Leica Mircosystems™ filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm) to determine fluorescent activity. Microscopically observed areas were imaged and the series of images corresponding to the same fingermark were stitched together using Leica software. Cells ranging between 10 – 100 µm were counted [210]. The development and contrast of the fingermark were assessed at each concentration level under the optimal viewing conditions using a modified UNIL scale [212] as shown in Table 39. Samples were not scored in a blinded manner.

Table 39: Modified UNIL scale for assessment of fingermarks treated with DMAB-IND combinations [212]

Score	Description	Representative Image
0	No ridge or fingermark visible	
1	Ridges visible in portions of the mark but poor ridge detail causing difficulty in retrieving level 2 characteristics	
2	Ridges visible in almost the entire mark, with level 2 characteristics retrievable but quality affected by low contrast	
3	Ridges visible and very well defined over the entire mark with level 2 characteristics easily retrievable due to optimal contrast	

Following the determination of the concentrations that offered the best visualisation of the fingermark and cellular material, the formulation was then prepared again per the respective concentration in Table 38 with Solstice® PF replacing HFE-7100. Two donors each rubbed their hands together to homogenise their secretions before ten fingermarks from each of the donors were deposited onto the copy paper. Five of the fingermarks from each donor were then treated with the Solstice® PF formulation, while the remaining fingermarks were treated with the HFE-7100 preparation at the same concentration. Samples were allowed to dry following treatment, then heat pressed between two sheets of paper towel at 160°C. Samples were subsequently imaged under the Poliview® system before being imaged under fluorescent microscopy. The images were then compared to assess the performance of the HFE-7100 replacement reagent.

6.3.2 Results and Discussion

6.3.2.1 *Fingermark development*

Fingermarks were visible under ambient laboratory lighting conditions following treatment with each of the test concentrations. These fingermarks were characteristic of IND development showing faint Joullié's pink on the ridges. Luminescent fingermarks were also visible the when exposed to different lighting conditions with the Poliview® system (Figure 47 and Figure 48). It was observed that the conditions suggested for IND (505 nm excitation; 555 nm interference bandpass barrier filter) [187] provided superior contrast of the fingermark to the background compared to the conditions suggested for DMAB (470 nm excitation; 530 nm interference bandpass barrier filter) [184] where the fingermark is difficult to distinguish from the background. This suggests that the fingermark secretions are primarily reacting with IND over DMAB, thus luminescence is more visible under the conditions for IND. This account for the presence of Joullié's pink

coloured fingermark ridges when view under ambient lighting conditions, rather than brown ridges resulting from DMAB as described by Fritz *et al*. [184].

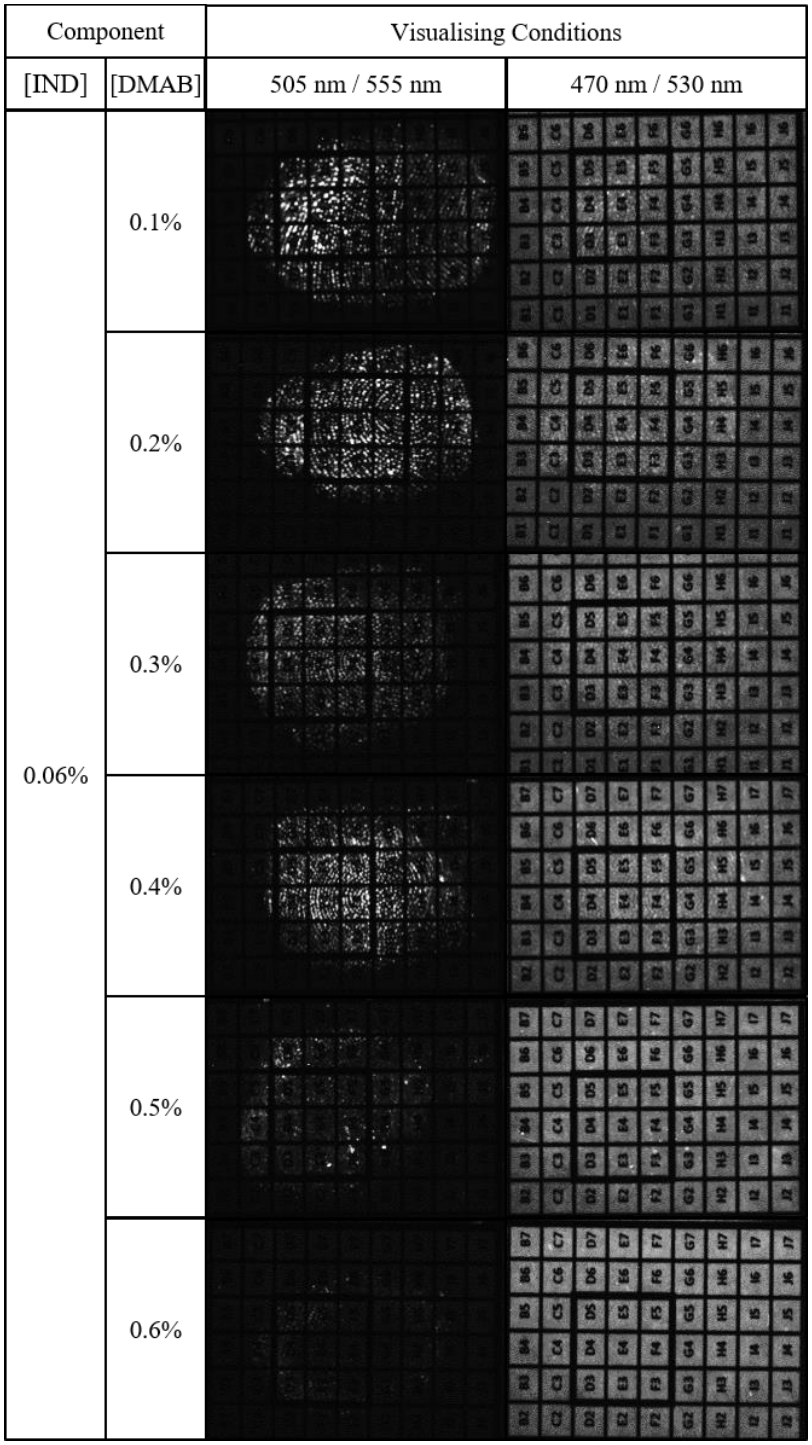


Figure 47: Fingermarks treated with DMAB-IND at various concentrations (IND 0.06% w/v : DMAB 0.1% (1); 0.2% (2) and 0.3% (3) w/v)

Samples viewed with the Poliview® IV System; Visualisation conditions: excitation nm / interference bandpass barrier filter nm.

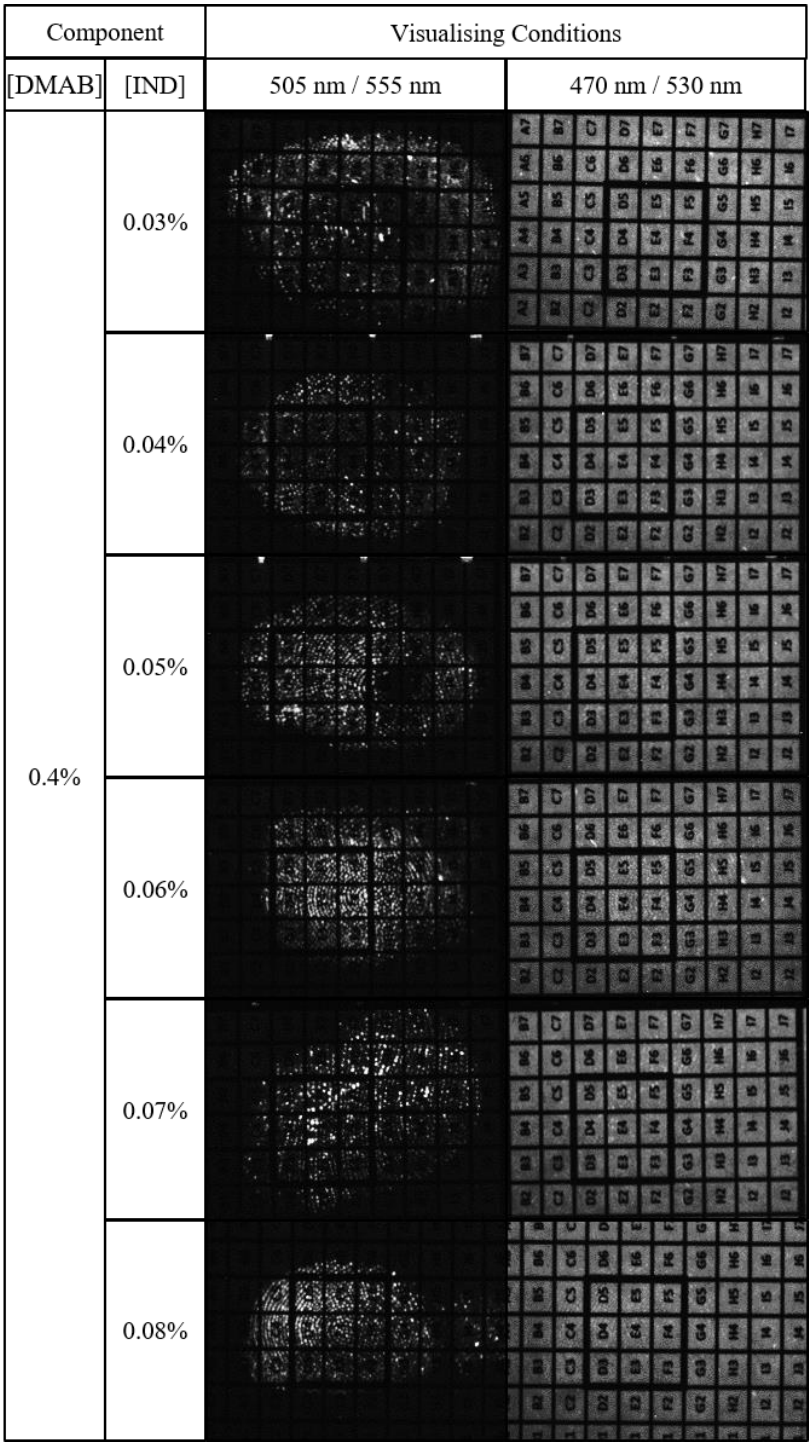


Figure 48: Fingermarks treated with DMAB-IND at various concentrations (DMAB 0.4 % w/v : IND 0.03% (1); 0.04% (2) and 0.05% (3) w/v)

Samples viewed with the Poliview® IV System; Visualisation conditions: excitation nm / interference bandpass barrier filter nm.

From Figure 47, it can be seen that when concentrations of 0.1 – 0.4% w/v DMAB are added to the 0.06% w/v IND reagent, the effect on the fingermarks are the same.

However, when treated with concentrations of 0.5% w/v and 0.6% w/v DMAB, the luminescence of the developed fingermark is less than when compared to DMAB at lower concentrations. This supported by the scores given to the developed fingermarks where the averages over the concentration ranges reflect the visual observation with 0.5% and 0.6% w/v DMAB returning lower scores due to lack of contrast (Figure 49). This suggests that at concentrations of DMAB above 0.4%, there is excess DMAB that may be interfering with the reaction between IND and fingermark secretions, thus resulting in lower luminescence and inferior contrast between the fingermark and background. From these results, the maximum concentration of DMAB was determined to be 0.4% w/v.

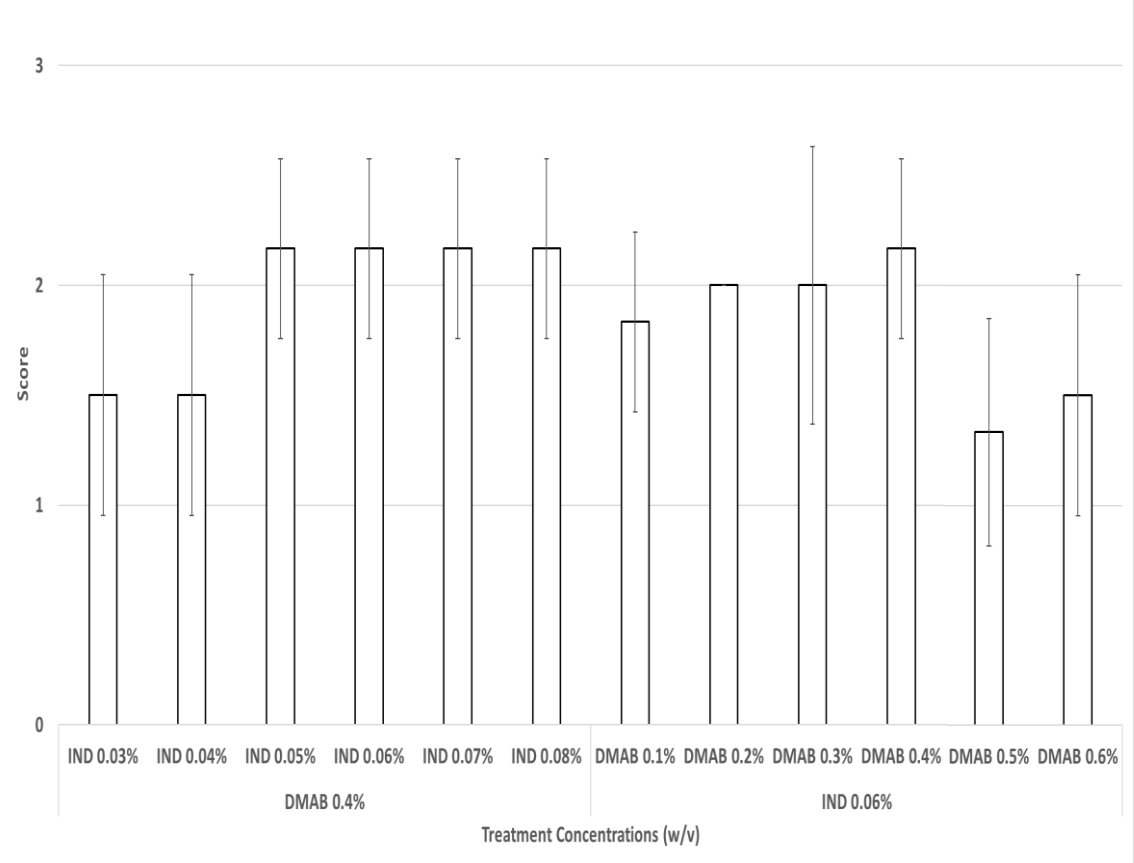


Figure 49: Assessment of fingermarks treated with DMAB-IND concentration combinations under IND visualisation conditions (505 nm excitation; 555 nm interference bandpass barrier filter) Fingermarks were scored per Table 39. Error bars represent the standard deviation of the respective treatment group. NB: DMAB 0.4% : IND 0.06% and IND 0.06% : DMAB 0.4% are the same sample.

Conversely, when the concentration of DMAB was kept at 0.4% w/v, the reagent performed slightly better at that the higher concentrations of IND (0.05% – 0.08% w/v), than the lower concentrations (0.03% – 0.04% w/v) (Figure 48). Scoring of the fingermarks also mirrors this result with lower concentrations of IND achieving lower average scores Figure 49. This would agree with the previously mentioned theory that the fingermark secretions are interacting with IND over DMAB as, when the concentration of IND is low, the luminescence is consequently inferior to that seen with the higher concentrations of IND. However, there appears to be no difference between the luminescence of IND concentrations 0.05% – 0.08% w/v (Figure 48 and Figure 49), thus the minimum level of IND required for this reagent was determined to be 0.05% w/v.

6.3.2.2 *Visualisation of cells*

Cells were visible with each tested concentration of DMAB (0.1 – 0.6% w/v) against 0.06% w/v IND (Figure 50 and Figure 51). Little difference was observed in the contrast of these cells against the background across the DMAB concentration range. Fingermark ridges were also present under these visualisation conditions (Figure 50 and Figure 51) and these became weaker as the concentration of DMAB increased, where at concentration of 0.5% and 0.6% w/v the fingermarks were no longer observed (Figure 51), reflective of that seen when the fingermark was viewed under the Poliview® system (Figure 47).

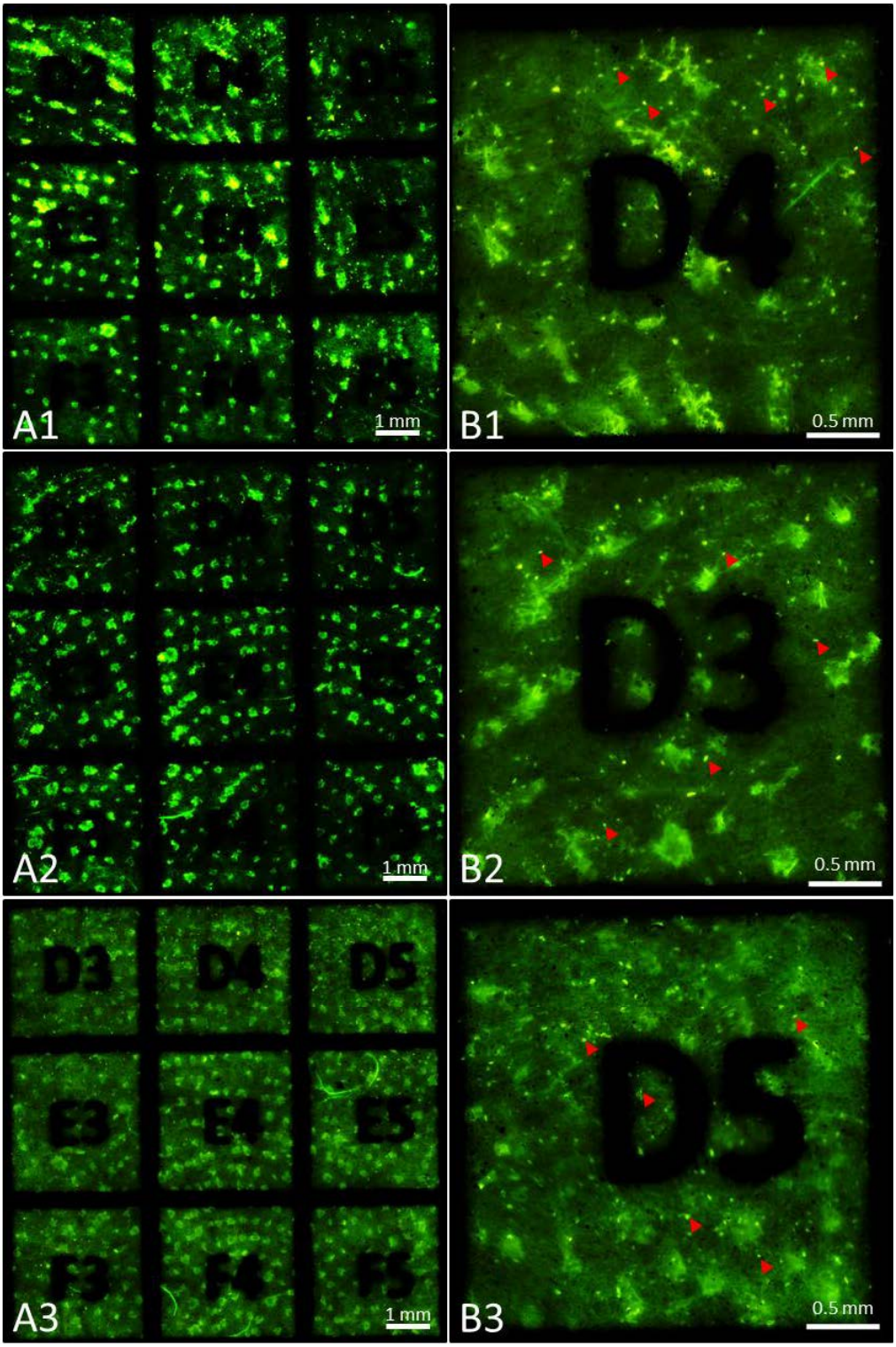


Figure 50: Fingermarks treated with DMAB-IND at various concentrations (IND 0.06% w/v : DMAB 0.1% (1); 0.2% (2) and 0.3% (3) w/v)

Samples viewed under microscopy with H3 filter cube at 50 times (A) and enlarged showing the presence of cells (B). Fingermarks correspond to those in Figure 47. Arrows indicate some of the cells present.

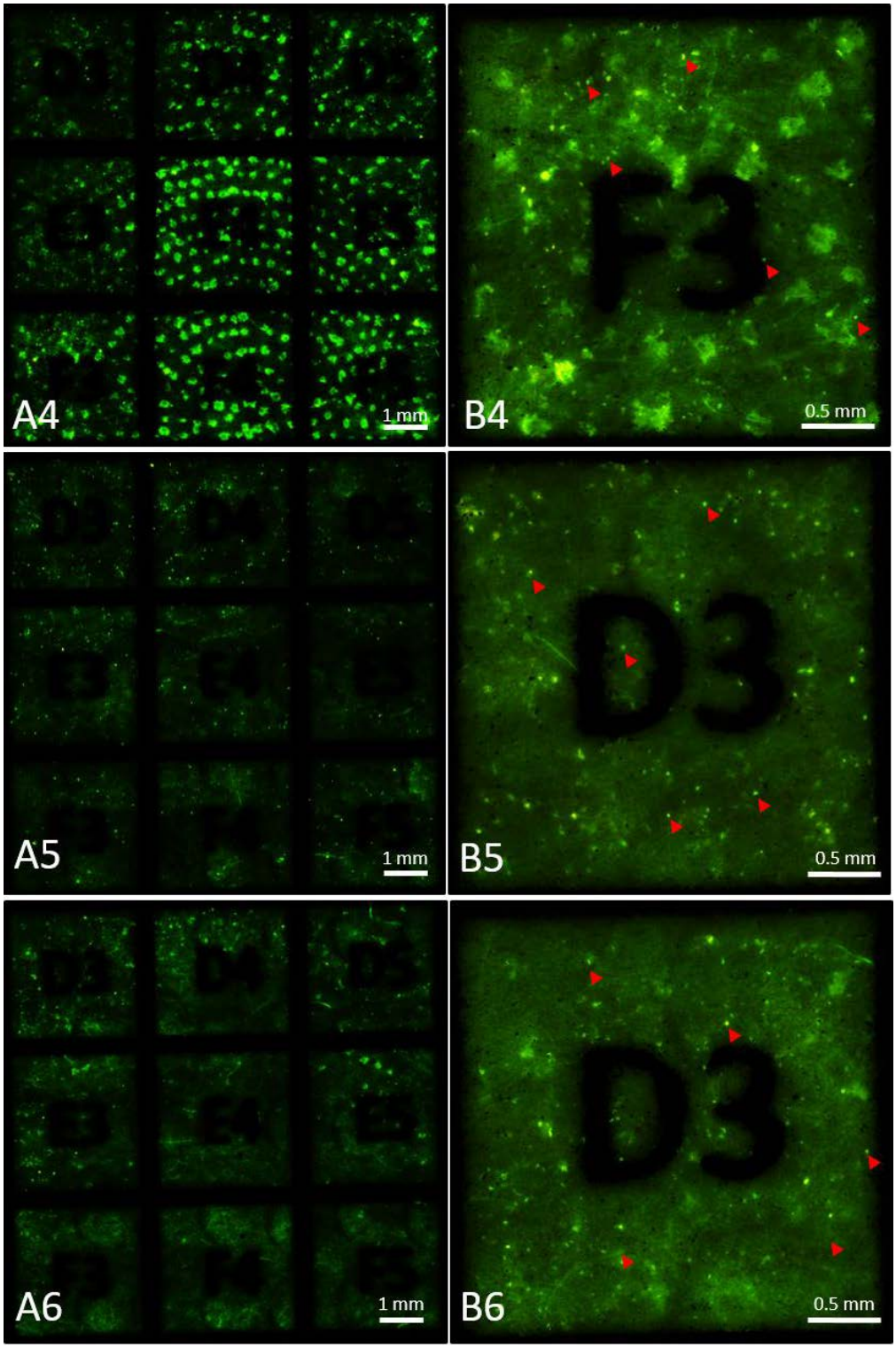


Figure 51: Fingermarks treated with DMAB-IND at various concentrations (IND 0.06% w/v : DMAB 0.4% (4); 0.5% (5) and 0.6% (6) w/v)

Samples viewed under microscopy with H3 filter cube at 50 times (A) and enlarged showing the presence of cells (B). Fingermarks correspond to those in Figure 47. Arrows indicate some of the cells present.

Similar to that seen in Figure 48 using the Poliview® system, when using microscopic conditions, fingermark residue are less apparent at lower concentrations of IND (0.03-0.04% w/v) when DMAB is kept constant (0.4% w/v). Cells, on the other hand, were visible under microscopic visualisation conditions at all concentrations of IND tested (0.03-0.08%w/v). It was noted that at the higher concentrations of IND (0.07% and 0.08% w/v) less cells were visible (Figure 53) and this is also reflected in the cell counts following the respective treatments (Figure 54). This would suggest that IND is reacting with the amino acids present in the cells as well, and at these higher concentrations, the abundance of IND molecules begin to inhibit the interaction of DMAB with cellular amino acids, thus causing less cells to be visible. However, as the contrast of cells at these concentrations similar to those of other IND concentrations, it may be that fingermarks collected for the higher IND concentrations naturally contained fewer cells than those used for other IND concentrations. Alternatively, it could be possible that at the higher IND concentration, certain cells are targeted by excess IND due to their amino acid content which changes as a result of differentiation. A study by Mekkaoui Alaoui and Halamek [213] found IND in the IND-Zn formulation has a higher affinity for L-lysine, L-arginine, L-alanine and β-alanine. As mentioned previously, nitrogen makes up 15% of the dry weight of the cornified layer [208]. Of the amino acids found to have strong interactions with IND [213], lysine and arginine have also been found to be among the most abundant in the cornified layer contributing 3% and 10% of the total nitrogen of the layer, respectively [208]. The interaction of IND with these amino acids could be masking the reaction of DMAB with other amino acids in certain cells thus rendering those cells non-luminescent.

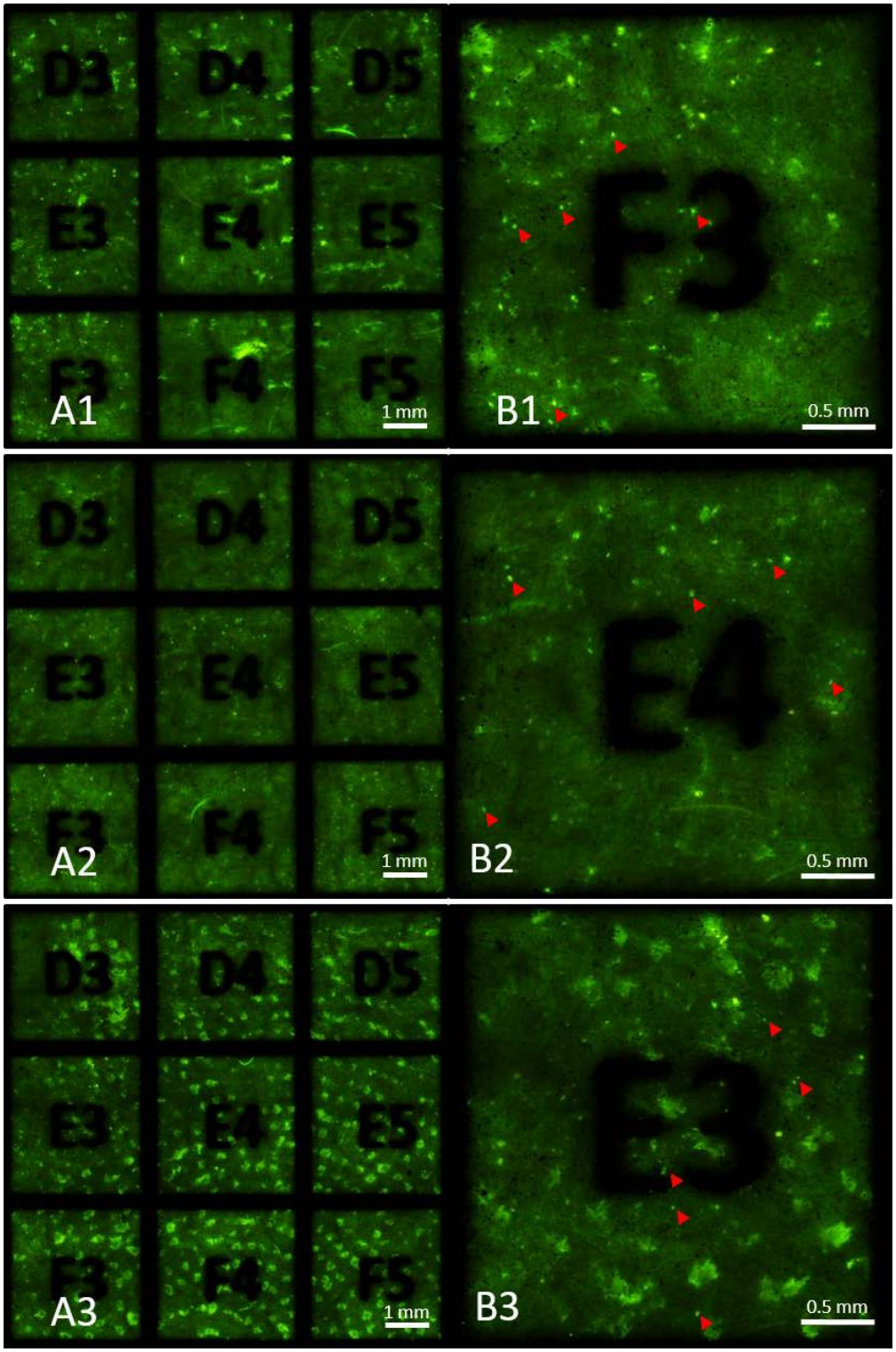


Figure 52: Fingermarks treated with DMAB-IND at various concentrations (DMAB 0.06% w/v : IND 0.03% (1); 0.04% (2) and 0.05% (3) w/v)

Samples viewed under microscopy with H3 filter cube at 50 times (A) and enlarged showing the presence of cells (B). Fingermarks correspond to those in Figure 48. Arrows indicate some of the cells present.

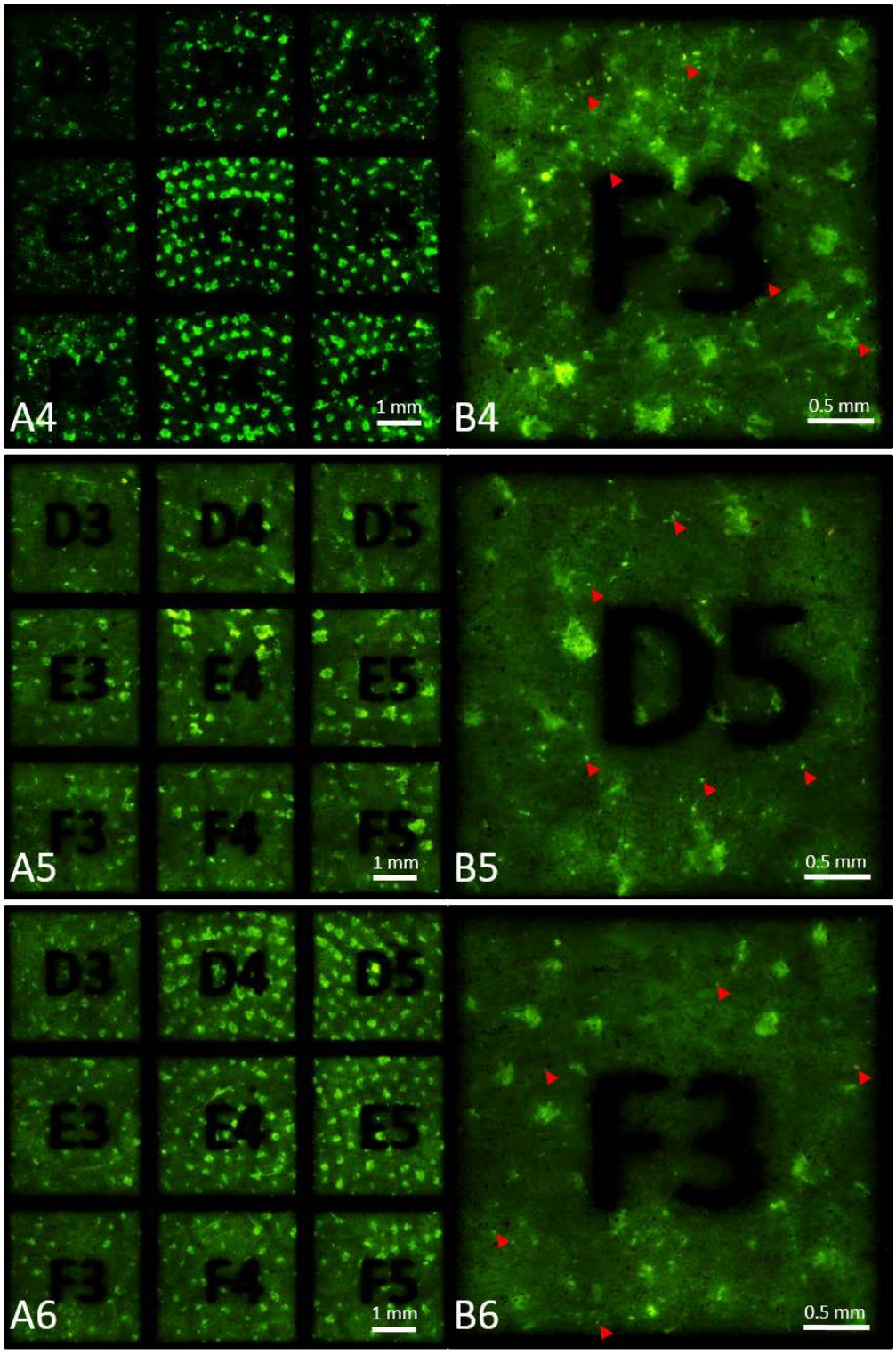


Figure 53: Fingermarks treated with DMAB-IND at various concentrations (DMAB 0.06% w/v : IND 0.06% (4); 0.07% (5) and 0.08% (6) w/v)

Samples viewed under microscopy with H3 filter cube at 50 times (A) and enlarged showing the presence of cells (B). Fingermarks correspond to those in Figure 48. Arrows indicate some of the cells present.

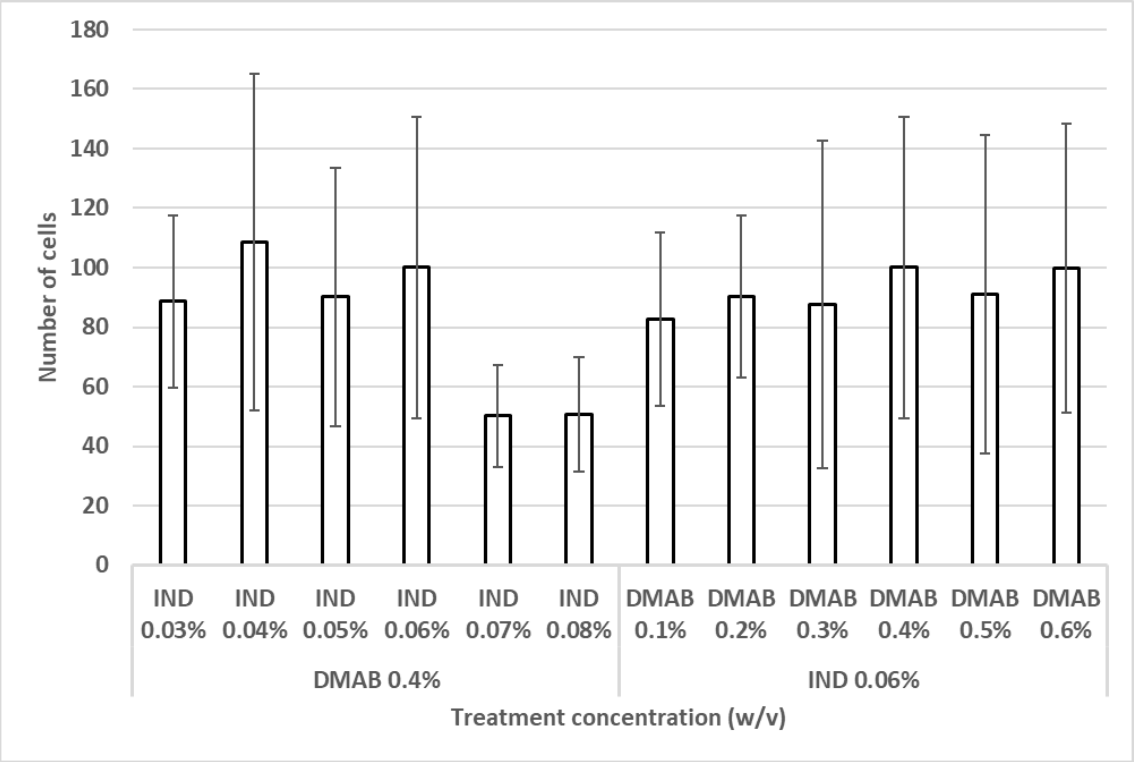


Figure 54: Average number of cells detected following treatment with reagents of different concentrations of DMAB and IND (DMAB 0.4%: IND 0.03 – 0.08% w/v; IND 0.06%: DMAB 0.1% – 0.6% w/v)

Error bars represent the standard deviation of the respective treatment group. NB: DMAB 0.4% : IND 0.06% and IND 0.06% : DMAB 0.4% are the same sample.

6.3.2.3 *DMAB-IND in Solstice® PF*

While HFE-7100 is used in the preparation of the NIN and conventional IND and IND-Zn formulations, it has been recognised to have adverse environmental impacts being a contributor to global warming [176, 214]. As a result, the quantities of HFE produced, imported and exported have been subjected to reporting by the European Union (EU) Regulation since 2014 [176]. In Australia, the process of phasing-down the use of HFE-7100 has begun, hence, there is the need for a replacement solvent for fingermark detection reagents. Olszowska *et al*. [215] have investigated the use of Solstice® PF as a replacement for HFE-7100 in IND-Zn and NIN reagents and found that the results of

treatment were comparable between the two carrier solvents in terms of development quality and detection rates. Olszowska *et al*. [215] also outline the advantages of replacing HFE-7100 with Solstice® PF stating that cost of Solstice® is half that of HFE-7100 and it has superior environmental properties compared to HFE-7100.

With HFE-7100 as the carrier in the DMAB-IND reagent, it was determined that the ideal concentration of DMAB was between 0.1% up to 0.4% w/v while the best concentrations for IND was either 0.05% and 0.06% w/v. As the conventional IND-Zn formulation is prepared to 0.06% w/v of IND it was decided that this concentration was most ideal as it also allows for both the conventional and DMAB-IND reagents to be prepared with the same stock solution. As 0.4% w/v DMAB was used against 0.06% w/v IND and this concentration also provided for both good fingermark and cell enhancement, it was selected as the DMAB concentration for the DMAB-IND reagent.

The reagent was prepared per Table 38 at 0.4% w/v DMAB and 0.06% w/v IND with Solstice® PF replacing HFE-7100. Following treatment of fingermarks deposited on paper, very little difference was observed between the treated samples (Figure 55). Fingermark enhancement was comparable between the two carrier solvents showing the same level of fingermark development and similar contrast when viewed under the same visualisation conditions with the Poliview® system. Cellular luminescence was also observed following both treatments with the contrast of the cells to the background also found to be similar. The change in carrier solvent had no notable negative impact in the reagent. As the active constituents of this formulation are light sensitive, the solution was protected from light. Due to the use of Solstice® PF as the carrier solvent [216], the reagent was also sealed and stored at 4^{o}C to prevent the evaporation of the solvent.

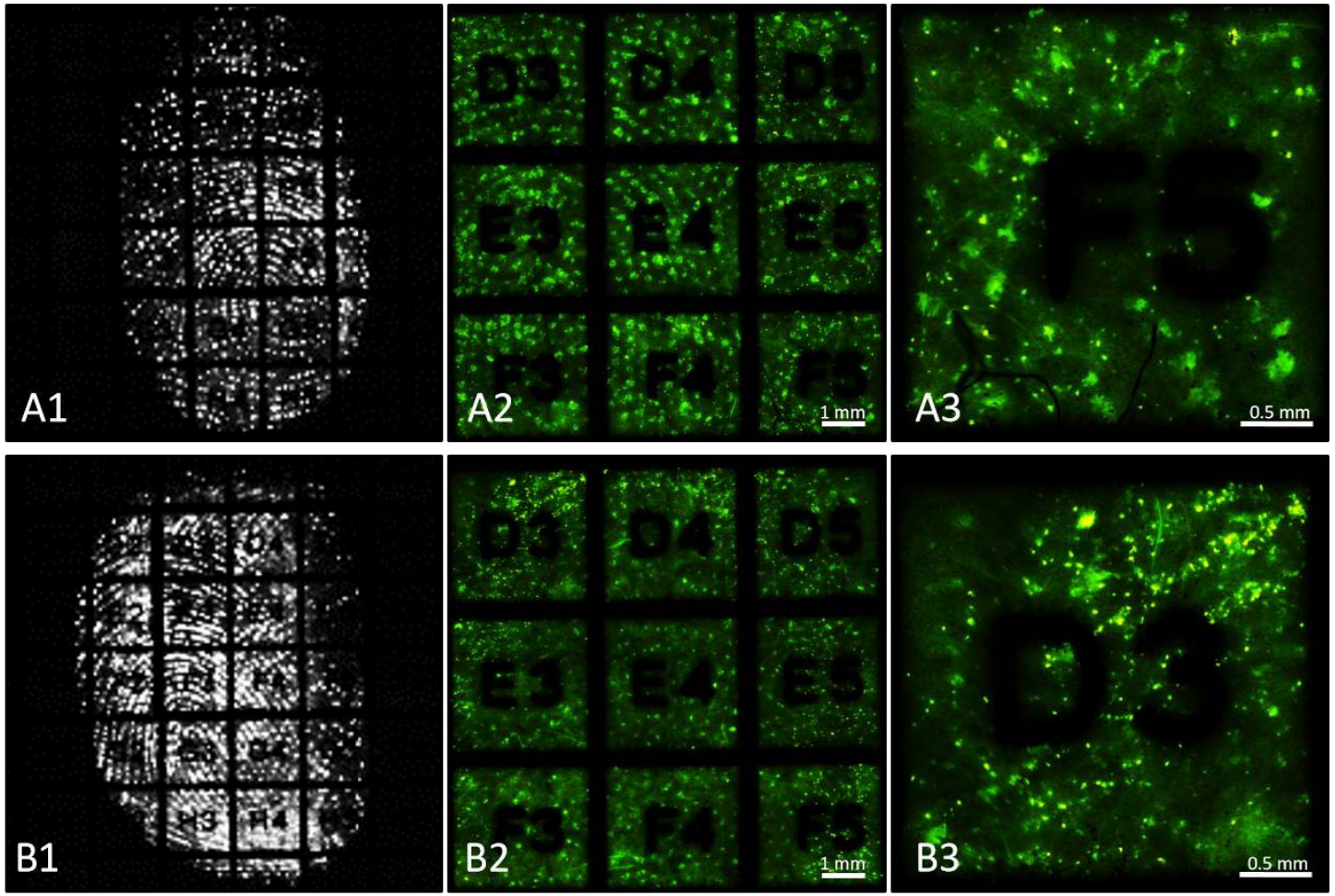


Figure 55: Comparison of DMAB-IND prepared in HFE-7100 (A) and Solstice® PF (B)

Samples viewed with the Poliview® IV System (ex 505; em 555) (1); under fluorescent microscopy (50 times; H3 filter cube) (2) and enlarged following microscopy (3).

6.3.3 Conclusions

When using the DMAB/IND combined reagent, it appears there is a trade-off between cells detection and fingermark enhancement. A reagent with too high a concentration of DMAB allows for good cellular visualisation however the quality of the fingermark development is negatively affected. On the contrary, when the concentration of IND is increased, the ability of the reagent to yield luminescent cellular material is hindered, while allowing fingermark development. Thus in this reagent, the concentrations of 0.4% w/v DMAB and 0.06% w/v IND were selected as these allowed for an ideal compromise between cellular detection and fingermark enhancement. There appeared to be little difference between the use of HFE-7100 and Solstice® PF as the carrier solvent. Consequently, as the use of Solstice® PF is less detrimental to the environment and, as HFE-7100 may be phased out in years to come [176], Solstice® PF was used as the carrier solvent in the preparation of DMAB-IND solutions for the remainder of the study.

6.4 Comparison of Optimised DMAB-IND Reagent with IND-Zn

6.4.1 Methods

6.4.1.1 *General methodology*

The experimental design is summarised in Figure 56. The impact of DMAB-IND solution on the enhancement of fingermarks and DNA was assessed against IND-Zn. Natural fingermarks were deposited onto copy paper and left overnight before being treated with

the respective solution. Fingermarks were then visualised first, before visualisation of cellular material in the central region of the fingermark was performed. Detected cellular material was subsequently recovered and processed for DNA using a direct amplification method. Where cellular material was not visualised on a fingermark, the central regions were excised and processed for DNA. Following the generation of DNA profiles, the quality of profiles was then compared and reported. Additionally, the quality of the DNA following treatment was assessed over time using natural fingermarks and a cell solution on paper per Figure 57.

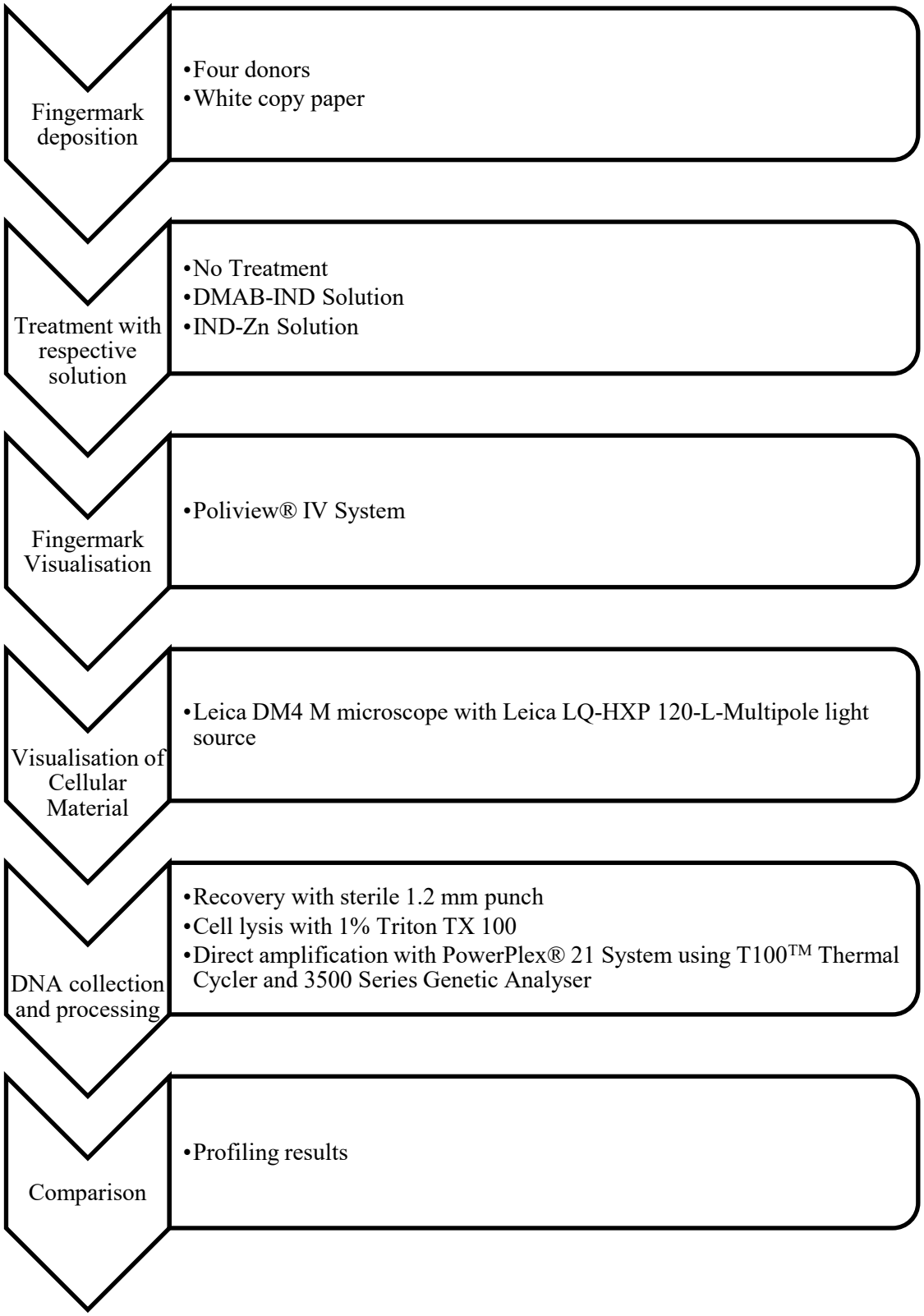


Figure 56: Overall procedure for assessing the impact of DMAB-IND reagent on fingermarks and DNA against IND-Zn

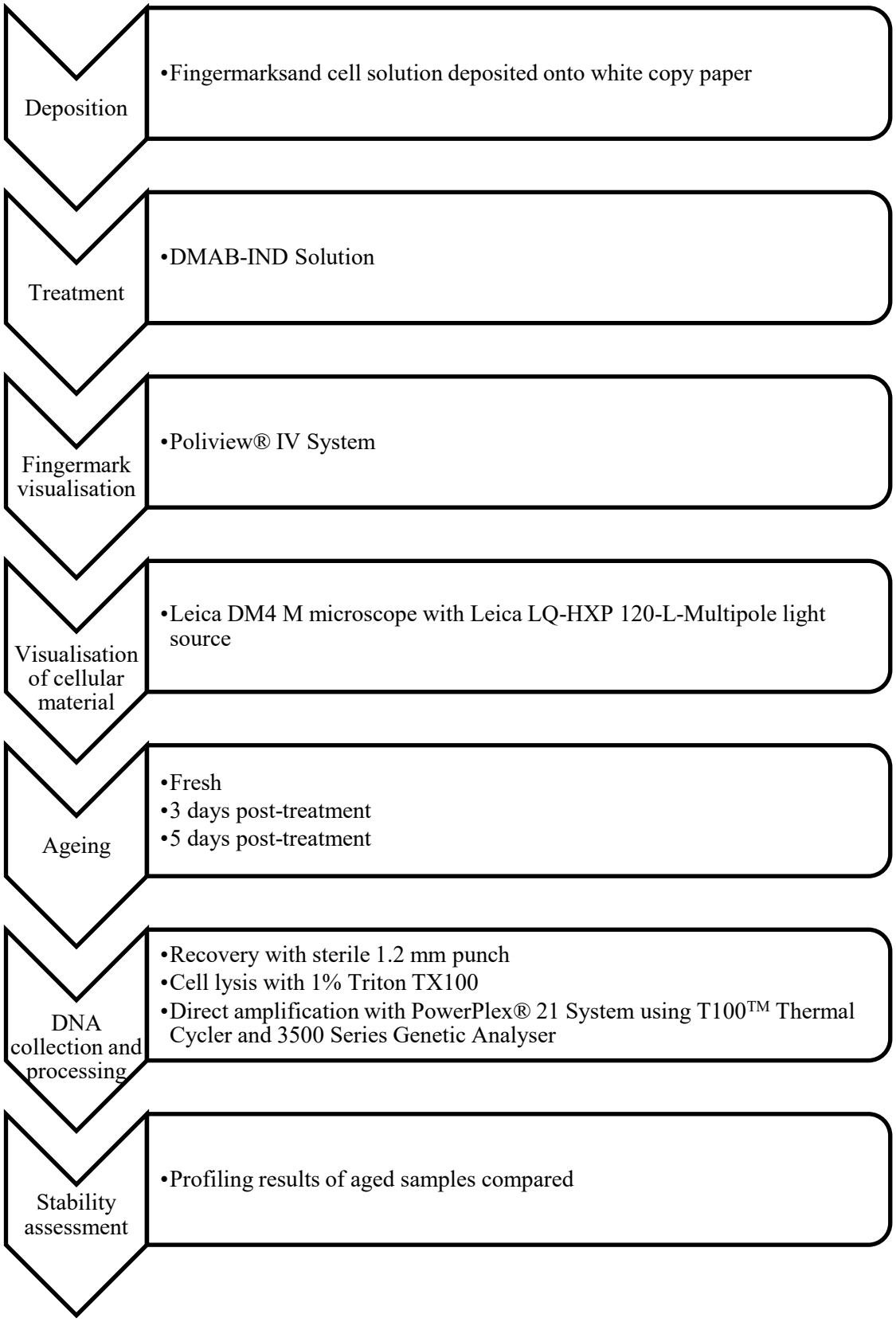


Figure 57: Overall procedure for assessing the stability of following time after treatment with DMAB-IND

6.4.1.2 *Procedure*

Preparation of detection solutions for comparison study

Stock solutions (SS) of IND and $ZnCl_2$ were prepared to the concentrations stated in Table 40. For IND SS, IND was firstly dissolved in ethyl acetate, before acetic acid was added (Table 40). Separately, zinc-chloride was dissolved in ethanol for $ZnCl_2$ SS (Table 40). The two SS were combined with Solstice® PF per Table 41 to produce IND-Zn WS with an IND concentration of 0.06% w/v.

Table 40: Preparation of IND and $ZnCl_2$ Stock Solutions

Component	Concentration	Supplier	Grade
IND Stock Solution			
IND	4.6% w/v	Casali Institute	-
Ethyl Acetate	96% v/v	Chem Supply	AR
Acetic Acid	4% v/v	Chem Supply	AR
$ZnCl_2$ Stock Solution			
$ZnCl_2$	4% w/v	Sigma-Aldrich	Reagent
Ethanol	q.s. 100% v/v	Chem Supply	AR

Table 41: Preparation of IND-Zn working solutions

Stock solution	Concentration	HFE-7100
IND	13% v/v	q.s. 100%
$ZnCl_2$	0.40% v/v	

The DMAB-IND WS was prepared to the concentrations in Table 42. DMAB and IND was firstly dissolved in ethyl acetate, before acetic acid was added to make SS1. Separately, zinc-chloride was dissolved in ethanol for SS2. Finally, the working solution was prepared by combining SS1 and SS2 in Solstice® PF per Table 42. The final concentration of DMAB in the working solution was 0.4% w/v and that of IND was 0.06% w/v.

Table 42: Preparation of DMAB-IND solution

Component	Concentration	Supplier	Grade
Stock Solution 1 (SS1)			
DMAB	3.1% w/v	Fisher Scientific Co.	ACS
IND	4.6% w/v	Casali Institute	-
Ethyl Acetate	96% v/v	Chem Supply	AR
Acetic Acid	4% v/v	Chem Supply	AR
Stock Solution 2 (SS2)			
$ZnCl_2$	4% w/v	Sigma-Aldrich	Reagent
Ethanol	q.s. 100% v/v	Chem Supply	AR
Working Solution			
SS1	13% v/v	-	-
SS2	0.40% v/v	-	-
Solstice® PF	q.s. 100%	Honeywell	-

Fingermark collection

Printed grids on copy paper (Figure 46) were sterilised under UV for 15 minutes prior to collection of fingermarks. The use of printed grids was to allow for microscopic examination and location of cellular material following treatment as well as for stitching the microscopy images to recreate whole image of the area of interest. Four donors were used in this study, three males and one female, ranging from weak to strong fingermark secretors. As a compromise between ideal and realistic conditions, donors did not wash their hands for at least 20 minutes prior to deposition but conducted usual activities up until deposition of fingermarks. Each donor had a separate piece of paper for their fingermarks to minimise instances of DNA contamination. They each deposited five individual fingermarks onto the printed grid area for each treatment (n = 60). Samples were aged for 24 hours in a closed sterile plastic container before being treated with either DMAB-IND or IND-Zn, or left as untreated controls.

Treatments and visualisation

Treatment samples were immersed either into the DMAB-IND solution or the IND-Zn solution until wet. Samples were allowed to dry at ambient laboratory conditions before being heated at 160ºC in a heating oven for 1 minute. An oven was used as opposed to the conventional dry heat press to minimise the risk of contamination and loss of genetic material. All samples, including untreated controls, were stored in the dark, in a wooden box overnight before visualisation with the Poliview® system (Polilight PL500, Peltier Cooled 12 bit CCD camera and lens, with Poli-V®++ software, Poliview® IV System, Rofin Australia) per the conditions listed in Table 43. DMAB-IND samples were visualised using both sets of corresponding conditions, while all samples also underwent white light examination. Following Poliview® visualisation DMAB-IND- and IND-Zn-

treated samples were also microscopically examined (Leica DM4 M microscope with Leica LQ-HXP 120-L-Multipole external light source, Leica Microsystems™ Pty Ltd) for cells using a H3 Leica Mircosystems™ Filter cube (excitation range: violet/blue; excitation filter: bandpass filter 420-490 nm; dichromatic mirror: 510 nm; suppression filter: bandpass filter 515 nm). Only a 1 cm^2 area pertaining to the centre of the grid was processed for DNA in this study.

Table 43: Poliview® IV System visualisation wavelengths for untreated controls sample and samples treated with DMAB-IND and IND-Zn

Visualisation conditions	DMAB-IND	IND-ZN	Control
Excitation (nm)	470[1] 505[2]	505	White light only
Interference bandpass barrier filters (nm)	530[1] 555[2]	555	

Collection of cellular material

After microscopic examination of DMAB-IND samples, all samples were stored in a wooden box and refrigerated at 4ºC overnight prior to DNA analysis. Images of each DMAB-IND-treated sample were merged and assessed for the areas of the highest density of cells, with the three highest being recovered for DNA analysis. A cell was only included in the count if it was fluorescent under the microscopic conditions and approximately 10-50 µm in length [217, 218]. DNA recovery was conducted with a

1.2 mm punch, with three 1.2 mm disks collected from each fingermark, corresponding to areas determined during microscopic examination to have cells, and placed into the same well of a 96 well plate. Three 1.2 mm disks were also collected from within the 1 cm^2 area corresponding to the central region of the fingermark of IND-Zn treated and control samples. Similarly, three 1.2 mm disks from untouched, untreated paper, and untouched paper treated with each of DMAB-IND or IND-Zn were also assessed as negative controls.

Processing of cellular material

Samples were processed for DNA per Figure 58. 15 µL of 1% Triton™ X-100 (TX-100; Sigma-Aldrich) was added to each sample well, along with one extra well as a control (0.1% TX-100 control), and incubated at 70ºC for 30 minutes until dry prior to PCR (PowerPlex® 21 System, Promega Corporation). PCR was performed on all samples according to the PowerPlex® 21 protocol [194] for *Direct Amplification of DNA from Storage Card Punches in a 25 µL Reaction Volume,* with 1 ng of 2800M DNA as a positive control, the PCR amplification mixture prepared per Table 44 and added to an additional well as PCR negative control. PCR was run on a Veriti 96 Well Thermal Cycler (Applied Biosystems).

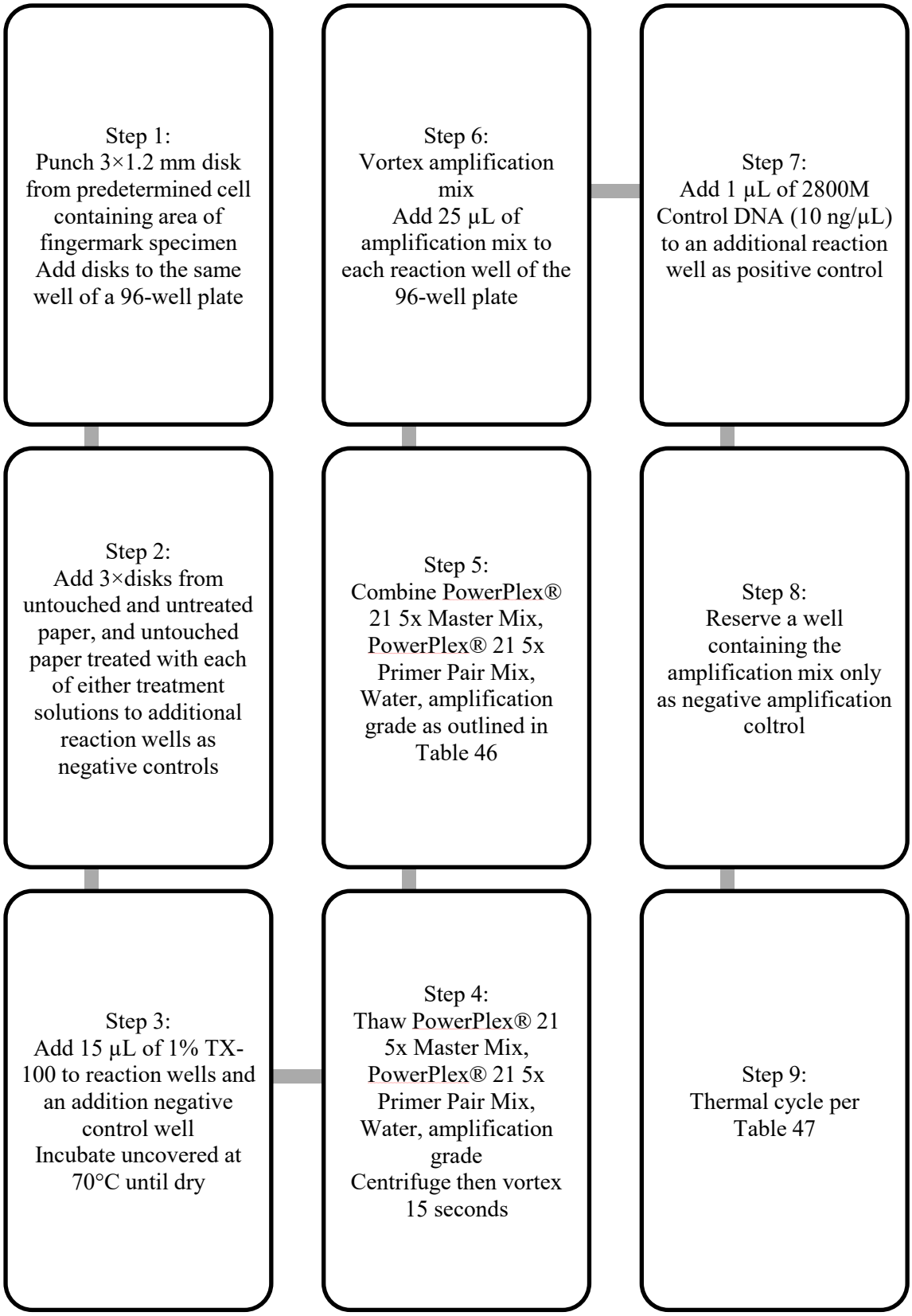


Figure 58: Workflow for direct amplification of DNA using PowerPlex® 21 System

Table 44: Preparation of amplification mixture for direct amplification of DNA with Powerplex® 21 System

Component	Volume required per reaction (µL)
Water, Amplification Grade	15
PlowerPlex21® 21 5x Master Mix	5.0
PlowerPlex21® 21 5x Primer Pair Mix	5.0

Table 45: Thermal cycling protocol for direct amplification of DNA using PowerPlex® 21 System on Veriti 96 Well Thermal Cycler

***adjusted for optimal performance on the samples using the available equipment**

Step	Time	Temperature (°C)	Number of cycles
Initialisation	1 minute	96	1
Denaturation	10 seconds	94	
Annealing	1 minute	59	28*
Extension	30 seconds	72	
Final extension	20 minutes	60	1
Hold	∞	4	-

Detection of amplified fragments was conducted immediately following PCR per the Powerplex® 21 [194] protocol for using the Applied Biosystems® Hitachi 3500 Series Genetic Analyser. WEN ILS 500 and Hi-DI™ formamide were firstly thawed on ice, then a loading cocktail was prepared by combining WEN ILS 500 and Hi-DI™ formamide in amounts corresponding to the number of samples as per Table 46. The cocktail was vortexed before 10 µL was added to each sample well of a 96-well plate (Multiplate™ 96-Well PCR Plates, low profile, unskirted, white, Bio-Rad Laboratories, Inc.). 1 µL of

amplified sample was then added to each sample well, with one well per column having 1 µL of PowerPlex® 21 Allelic Ladder Mix in place of amplified sample and one well on each plate having 1 µL of TE^{-4} Buffer (10 mM Tris-HCl, pH 8; 0.1 mM EDTA) in place of amplified sample as an additional control. The 96 well plate was then covered with a septum and placed centrifuged to remove air bubbles, denatured at 95°C for 3 minutes before being immediately chilled on ice for at least 3 minutes. Following this, plates were placed into a plate retainer and base, loaded onto a genetic analyser for separation and data collection using the 3500 Series Data Collection Software 3 3500 Series Genetic Analyser (Applied Biosystems; Hitachi) and processed with GeneMapper® *ID-X* Software (Applied Biosystems).

Table 46: Preparation of loading cocktail for profiling of DNA with Powerplex® 21 System

Component	Volume required per sample (µL)
WEN ILS 500	0.5
Hi-Di™ Formamide	9.5

Quality of DNA following DMAB-IND treatment

Additional samples treated with DMAB-IND were assessed for the stability of the DNA following treatment. Three time points were assessed: 5 days, 3 days and fresh (processed on the day of treatment). Three single fingermarks from a single donor were deposited onto paper grids (Figure 46) for each time point. As touch DNA is heavily influenced by inter- and intra-donor variability [32], an accurate representation cannot be achieved using fingermarks alone, hence, separate grids were also spiked with a volume of cells equivalent to 10 µL per 3 mm^2. Cells were derived from washed saliva cells, resuspended in PBS. The concentration of this solution was estimated using Plexor™ HY System

[192] on a CFX96 Touch™ Real-Time PCR System. Cell spikes were allowed to dry before being treated with DMAB-IND reagent per the aforementioned procedure outlined in Treatments and visualisation section along with the fingermarks. Following DMAB-IND treatment, samples were aged for either 5 or 3 days or processed for cellular material on the day of treatment per the Collection of cellular material and Processing of cellular material Sections.

6.4.2 Results and Discussion

6.4.2.1 *Visual comparison of DMAB-IND with IND-Zn*

Following treatment with each of the DMAB-IND reagent and the IND-Zn reagent [187], fingermarks were visible under ambient laboratory lighting conditions. Both sets of fingermarks showed the characteristic Joullié's Pink colour of the IND-amino acid reaction [219]. The intensity of that observed in DMAB-IND-treated fingermarks were slightly less than that from IND-Zn-treated samples. This again suggests that DMAB is also interacting with amino acids in the fingermark residue (see Section 6.3.2.1) making it less visible under ambient lighting conditions. Visualisation using the respective lighting conditions (Table 43) showed similar results with IND-Zn-treated fingermarks, again being slightly more intense than those treated with DMAB-IND (Figure 59). While the fingermarks were able to be located and visualised, cellular material was difficult to observe using the Poliview® system due to the size of the trace and unsuitable visualisation conditions (Figure 59; A1, B1).

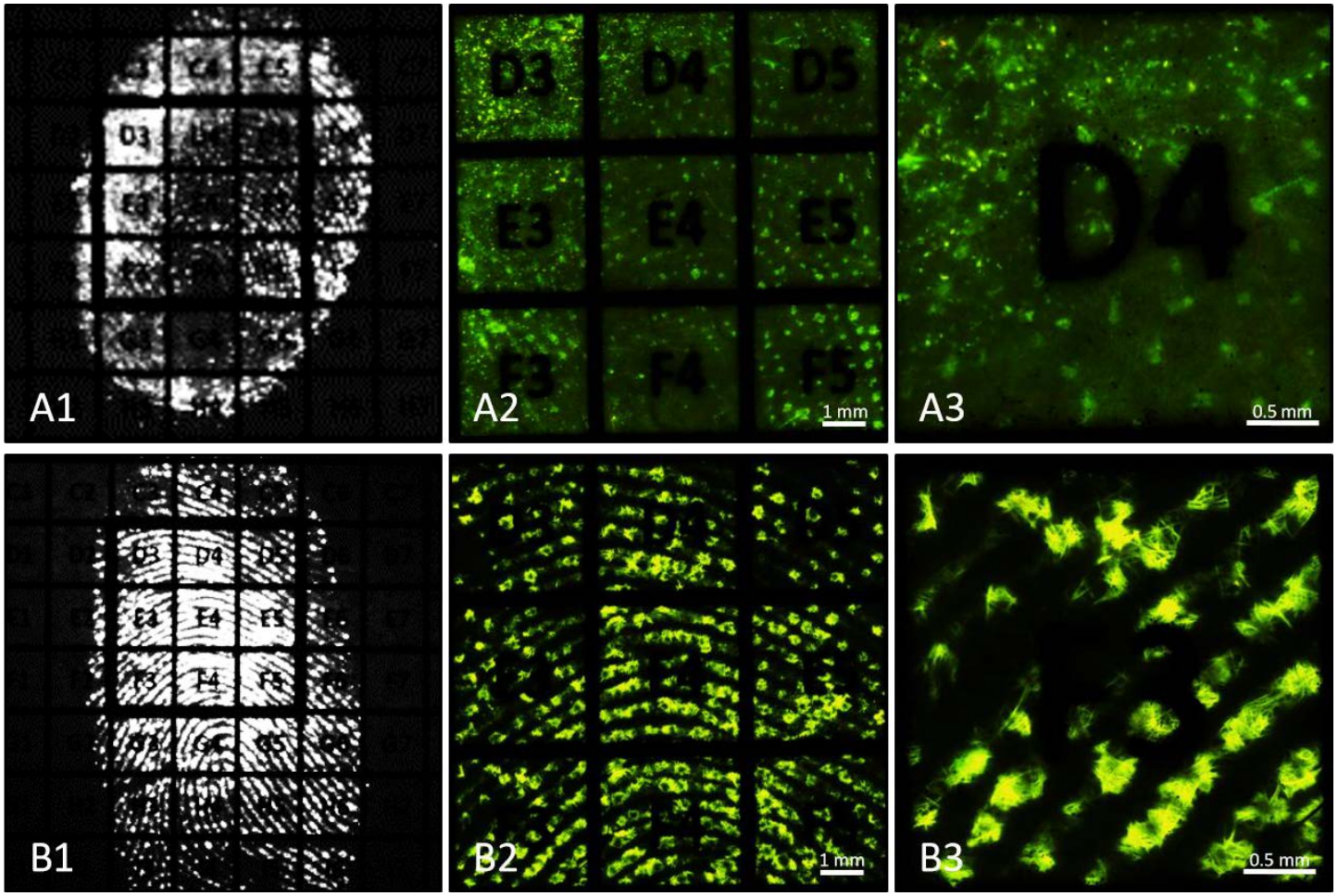


Figure 59: Comparison of DMAB-IND treated fingermarks (A) and IND-Zn treated fingermarks (B)

Samples were viewed with the Poliview® IV System (ex 505; em 555) (1); under fluorescent microscopy (50 times; H3 filter cube) (2); and enlarged following microscopy (3).

During microscopic examinations, cells were visible after treatment with DMAB-IND, however not with IND-Zn treated samples (Figure 59, A2-3, B2-3). The cells from DMAB-IND-treated fingermarks were able to be seen at 50× magnification and found to be more intense than the fingermark residue. This allowed for the differentiation of cells from the fingermark secretions. Interestingly, fingermark secretions were visible following treatment with IND-Zn but much less visible following DMAB-IND treatment when viewed under fluorescent microscopy using a H3 filter cube. This suggests that different reactions may be occurring depending on the presence or absence of DMAB, which are then detected under these visualisation conditions. Although techniques for porous surfaces are typically conducted in the laboratory, the low magnification required suggests that portable magnification device and an ALS could be used to detect cellular material within detected fingermarks if required.

Cells counts were subsequently only conducted on DMAB-IND treated samples as these were the only samples where they could be visualised. From each DMAB-IND treated sample, the three grids returning the highest cells counts were collected for DNA analysis using a 1.2 mm punch. During microscopic visualisation, it was observed that in the majority of DMAB-IND samples the cells tended to be found on the sides of the fingermark but rarely in centre of the fingermark itself. This likely results from the activities conducted by donors prior to deposition, where the centre of the fingerprint skin comes into contact with surfaces more readily and, hence, loses the cellular material on touch [31]. This suggests that sampling the sides or the top of the fingermarks may increase the number of alleles called or even generate filler DNA profiles when a reagent to detect fingermarks alone has been used.

6.4.2.2 *Comparison of profiles*

Prior to PCR amplification, 1% TX-100 was added to each fingermark sample as previous studies have shown that the use of detergents increase the yield of DNA [220] or success of DNA profiling [221, 222]. It is thought that is occurs due to the increased lysis of cells and subsequent release of more DNA [223]. The amount of DNA recovered could not be assessed in this study due to the use of a direct amplification method, which bypasses the quantification step. However, it can be seen in Figure 60 that there is a difference in the performance of these techniques in terms of the quality of the profile recovered.

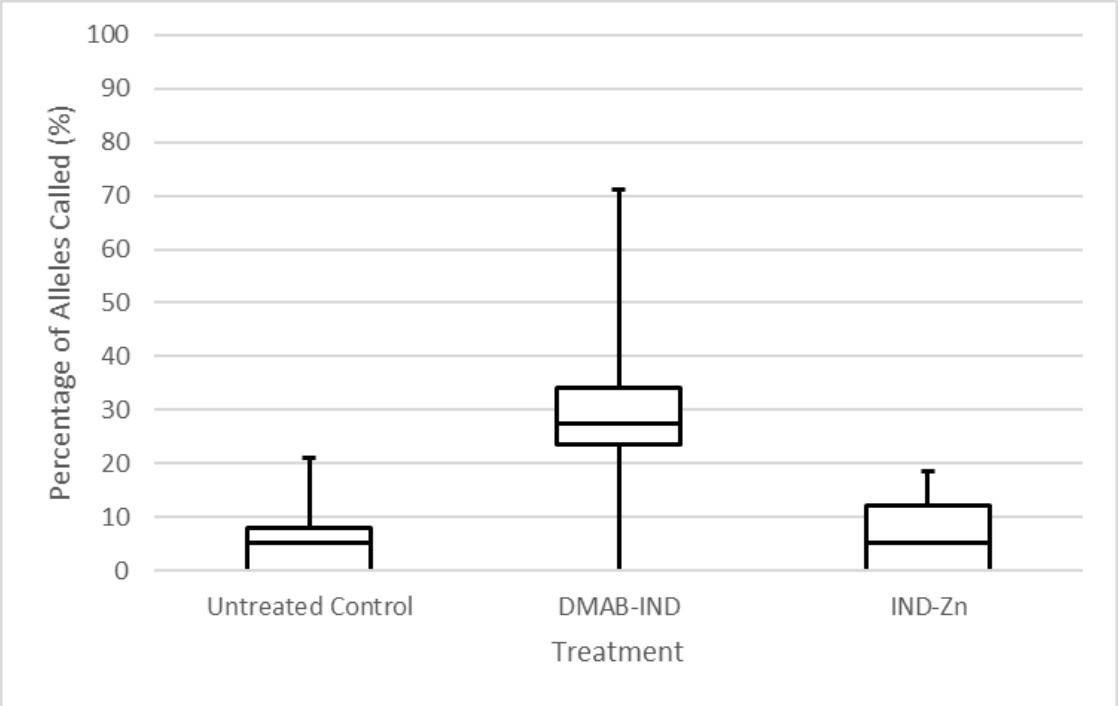


Figure 60: Box plot comparison of percentage of alleles called after either no treatment (Control) or treatment with DMAB-IND or IND-Zn

Alleles were identified following STR analysis with PowerPlex21®. Percentages of alleles called was determined by dividing the number of alleles correctly identified by the expected number of alleles and converted into a percentage by multiplying the determined value by 100.

While the majority of samples showed a lack of useful profiles (< 50% of alleles called) following the respective treatments, ANOVA was performed between all treatments with at least one of the means seen to be significantly different from the others ($F(2,57) = 3.15$, $P = 2.8x10^{-9}$). T-Test ($\alpha=0.05$) results showed that the use of DMAB-IND resulted in a greater percentage of alleles called compared to untreated samples ($t(19) = 6.3$, $p = 2.2 \times 10^{-6}$) and samples treated with IND-Zn ($t(19) = 6.3$, $p = 2.4 \times 10^{-6}$). No significant difference was observed between untreated control samples and IND-Zn-treated samples (($t(19) = 0.64$, $p = 0.26$). This suggests the targeted recovery of cells assists in improving the quality of the profile obtained, hence the treatment of fingermarks with the DMAB-IND reagent allows for a higher level of alleles to be called than when cells could not be visualised. This is an advantage over conventional methods such as swabbing where cells and DNA is collected from a larger area, although the majority of the genetic material may not be released from the swab. Similarly, a smaller surface area of the exhibit may be collected to minimise damage to it; this also allows for less substrate to enter the DNA processing reactions. Additionally, with further optimisation, this reagent could not only be used to reveal sources of touch DNA but used to visualise its transfer, persistence and prevalence in studies.

While a large number of cells were visualised, no full profiles were recovered from fingermarks following DMAB-IND treatment (Figure 60). This could be due to insufficient sampling, where only the three squares from each sample gird with the highest number of cells visualised were collected and thus may not have been enough genetic material. As only partial profiles were detected, this may also suggest that the formulation is likely indicating all cells, not just those that are viable for DNA analysis; hence cells with no useable DNA may also have been collected for analysis. Alternatively, it could be an effect of degradation caused by the treatment. Previous research indicates

that DNA degrades with time following IND-Zn [76, 81] treatment. As the reagent contains most of the components of the IND-Zn formulation [187] it is likely that it will exhibit similar effects. Additionally, the impact of the carrier solvent used in this formulation, Solstice® PF, on DNA is yet to be reported on in the literature. Another factor that may contribute to the lack of full DNA profiles is DMAB itself. A more rapid rate of DNA degradation was also noted in touch DNA following fingermark treatment with PolyCyano UV, a one-step luminescent CA that contains DMAB as the staining agent [188], hence the stain itself could be contributing to the decrease in the usefulness of the profiles with time following treatment.

6.4.2.3 *Quality of DNA following DMAB-IND treatment*

As the DNA may be impacted by DMAB-IND treatment, the quality of DNA from DMAB-IND-treated samples was assessed. Fingermarks or washed cells were deposited on paper and treated with DMAB-IND solution, then aged for up to five days following the treatment. It was observed that there was a rapid decrease in the percentage of alleles called over the five day period (Figure 61).

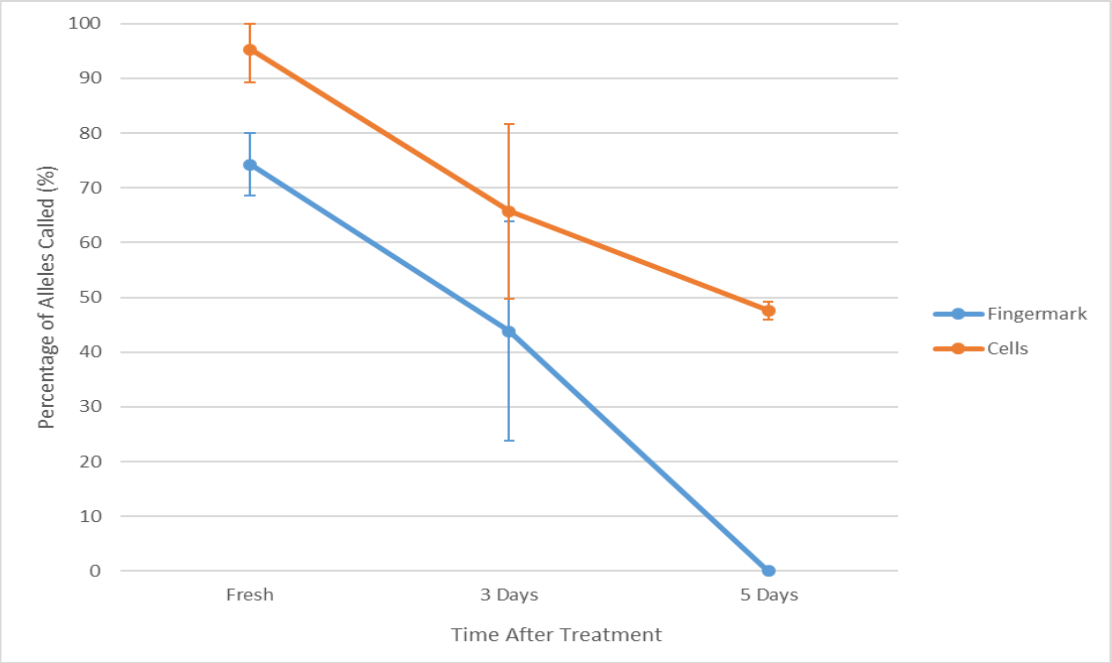


Figure 61: Percentage of alleles called from fingermark and cells samples over time following DMAB-IND

Alleles were identified following STR analysis with PowerPlex21®. Percentages of alleles called was determined by dividing the number of alleles correctly identified by the expected number of alleles and converted into a percentage by multiplying the determined value by 100. Error bars represent the standard deviation between samples at the respective time after treatment.

Where DNA from samples were processed following visualisation on the day of treatment, useful profiles (≥ 50% alleles called) were observed from both washed cell ($\bar{x}$ = 95%) and fingermarks ($\bar{x}$ = 74%) samples (Figure 61). However, it was still observed that fingermark samples did not achieve full profiles (maximum 80%). A loss in the quality of the profile was observed when DNA from samples were processed 3 days following treatment, while alleles were not detected from touch DNA samples after 5 days of ageing. This appears to be in line with that described in previous research [76, 81] where the fullness of profiles from fingermarks treated with IND-Zn dropped dramatically when processed for DNA more than 24-hours after treatment and could not be detected 6 days post treatment.

In addition to the loss of quality of the samples over time, Figure 61 also indicates a slower degradation rate when the starting amount of DNA is high. After 5 days of aging, just under 50% of alleles were still called in washed cell samples. Raymond *et al.* [67] also reported a decrease in alleles called over time in untreated DNA samples. While controlled amounts of DNA were used in the study by Raymond *et al*. [67], the observations suggest that both natural and environmental degradation have an impact on DNA. It can be seen from both this study, with touch DNA from fingermarks, and previous research [188] that the small amount of DNA present in the fingermark residue is more prone to rapid degradation. Hence, while the DMAB-IND reagent does enable the visualisation of both fingermarks and of cellular material increasing the chance of obtaining useful information from the trace, the analysis of touch DNA must be conducted on the day of treatment in order for the results to be meaningful.

6.4.3 Limitations

While this proof-of-concept study introduces a successful technique for the visualisation of cellular material and demonstrates an increase in the fullness of DNA profiles with the targeted recovery of DNA, it is not without its limitations. This study consisted of a small donor pool and the use of a single porous substrate. It must be noted that while the trends appear clear in this study, differences may be observed with increased donor numbers. As it is also difficult to control for touch DNA due to inter- and intra-donor variations, future studies should also use cells spikes to assess the true impact of the DMAB-IND solution especially in regards to degradation effects. However, the use of realistic conditions should continue be assessed alongside controlled conditions to provide representative results to practitioners as the nature of touch DNA is quite unpredictable. Further work should also be conducted on other porous substrates to observe if similar trends are seen

as well as using increased number of punches per reaction to determine if the rate of success is increased. Additionally, a validation of the method would need to be performed prior to this technique being adopted into routine use.

6.4.4 Conclusions

This proof-of-concept study demonstrated the successful use of a reagent containing DMAB and IND to visualise both fingermarks and cellular material. The results of this study support the idea that a targeted approach to DNA collection is required in order to maximise the usefulness of the trace. In addition, the use of a dual detection reagent has been shown to be advantageous in fully exploiting fingermark deposits, where both the fingermark and associated cellular material can be visualised in a streamlined approach. This reagent could also be employed to investigate the transfer, persistence and prevalence of DNA. However, this reagent is limited by the degradation effect it appears to have on the DNA over time, indicating that more meaningful results can be achieved when touch DNA from a sample is processed rapidly following treatment with the reagent. Future work may be conducted to further optimise the solution to allow more time between detection and processing as well as to evaluate the trends with increased samples number and across different porous surfaces.

Chapter 7. GENERAL CONCLUSIONS

7.1 The Challenge of Detection

Fingermarks and touch DNA have the potential to provide identifying information about the source and act as a vector of information, providing a link between cases. The detection of both of these traces is crucial to their exploitation, despite conventional methods of detection favouring one trace over the other. This is a significant problem as the detection of one trace may not be of sufficient quality to provide any useful information, hence the alternate trace may be used, or they may even be used in combination.

When detecting fingermarks, DNA analysis is possible following use of most reagents, but DNA may become contaminated or lost in the process. While there is no routine method for the detection of DNA used in current casework, DNA is generally collected by swabbing or tape-lifting areas that are suspected to contain DNA. This largely results in the destruction of the fingermark patterns and additionally does not always result in a useful DNA profile.

This project aimed to find techniques to detect both latent fingermarks and DNA on non-porous and porous surface types. In this project potential ways to dually detect both latent fingermarks and touch DNA were investigated. Various staining methods for fingermarks and DNA were investigated along with MDA, a DNA amplification method, to assess if these could be used in a dual detection technique. It was found that DNA was too small

of a target, being recorded on a nano-level, and hence required strong magnification to visualise. Additionally, as DNA is typically encapsulated within the cell, methods to penetrate or lyse the cell were required. Methods that specifically detected DNA were found to be costly and in the instance of MDA, required lengthy incubation periods to amplify DNA signals. Thus, targeting the cell was found to be a more visible and cost-effective alternative to provide an indication of the presence of DNA.

DMAB was found to show good potential for use in a dual detection technique. This molecule is generally used as in histology as an indicator of amines in cells. New applications for DMAB include its use as a staining agent in some one-step luminescent CAs (PolyCyano UV and PECA products) that can be used to detect fingermarks on non-porous surfaces, and as a fingermark detection reagent to detect fingermarks on porous surfaces. This indicated that the cell detection molecule showed compatibility with fingermarks.

7.2 Dual Detection

Further investigations showed cells in fingermarks treated with one-step luminescent CAs were able to be differentiated from the fingermark secretions when viewed using a fluorescent microscope. The impact of one-step luminescent CAs on DNA analysis was subsequently determined. It was found that, while fingermarks and touch DNA were able to be located, it is likely that DNA either remains trapped in the CA following extraction or slight degradation occurs due to the staining agents used in the one-step luminescent CAs. However, the main factor contributing to the degradation of DNA was found to be time of exposure to the environment. This indicates the time between deposition and

detection and processing of touch DNA samples needs to be minimised. This also points towards future work being conducted to determine if DNA can be detected, and its quality preserved, with the application of one-step luminescent CAs to assess the impact of exposure to these techniques.

When DMAB was combined with the well-known and widely used IND-Zn reagent for fingermark detection on porous surfaces, the DMAB-IND reagent was found to detect both cells and fingermarks. DMAB was thought to detect the cells, while IND revealed the fingermark secretions. While alternative light sources and microscopy equipment were required to visualise and differentiate the cells and fingermark secretions, the application of this method has proved effective. From a few localised cells originating from a fingermark deposit on copy paper, DNA profiles were recoverable and superior to that treated with IND-Zn alone or that left untreated, where, in both of the latter cases, the cells could not be detected. Similar to the IND-Zn reagent, for the DMAB-IND reagent, time after treatment was observed to be a contributor to the degradation of DNA. This indicates that while touch DNA can be detected, it must also be processed rapidly to avoid loss of information.

Hence, this research suggests that future work could be conducted to optimise to this formulation allowing for improved results and for an increased timeframe between treatment and DNA analysis. This could start at by examining the effect each individual excipient has on DNA and investigating suitable replacements for the most degradative components, while also considering at how this in turn impacts the fingermark trace. Further studies also need to be conducted using increased sample sizes, and the effectiveness of this reagent should be evaluated on a wider range of porous surfaces.

Although this reagent allows for both fingermarks and DNA to be detected, it must be stressed that care still needs to be taken with DNA interpretation. While DNA profiles showing strong similarities to that of the fingermark donor may add weight to that conclusion that the source of the fingermark and the touch DNA are the same, DNA transfer events can present complications in interpretation of profiles that show varied semblance to that of the fingermark donor. Investigation into this particular challenge is clearly beyond the scope of the present research work.

7.3 Final Remarks

The results of this project are in agreement with a number of studies that have called for a targeted approach to DNA profiling [35, 36, 39-41, 44]. It has been shown that DNA profiling is more successful, that is, more alleles are called, when cells are able to be located compared to when they cannot be. Hence the use of DMAB containing one-step luminescent CAs and the DMAB-IND reagent can not only reveal fingermarks and allow for that trace to be assessed, they can also assist in improving outcomes when it comes to DNA profiling. This in effect streamlines the investigative process, saving time, money and resources. The quality of the fingermark trace or the quantity of the touch DNA trace can quickly be determined and priority can be assigned accordingly.

APPENDIX. ELECTROPHEROGRAMS

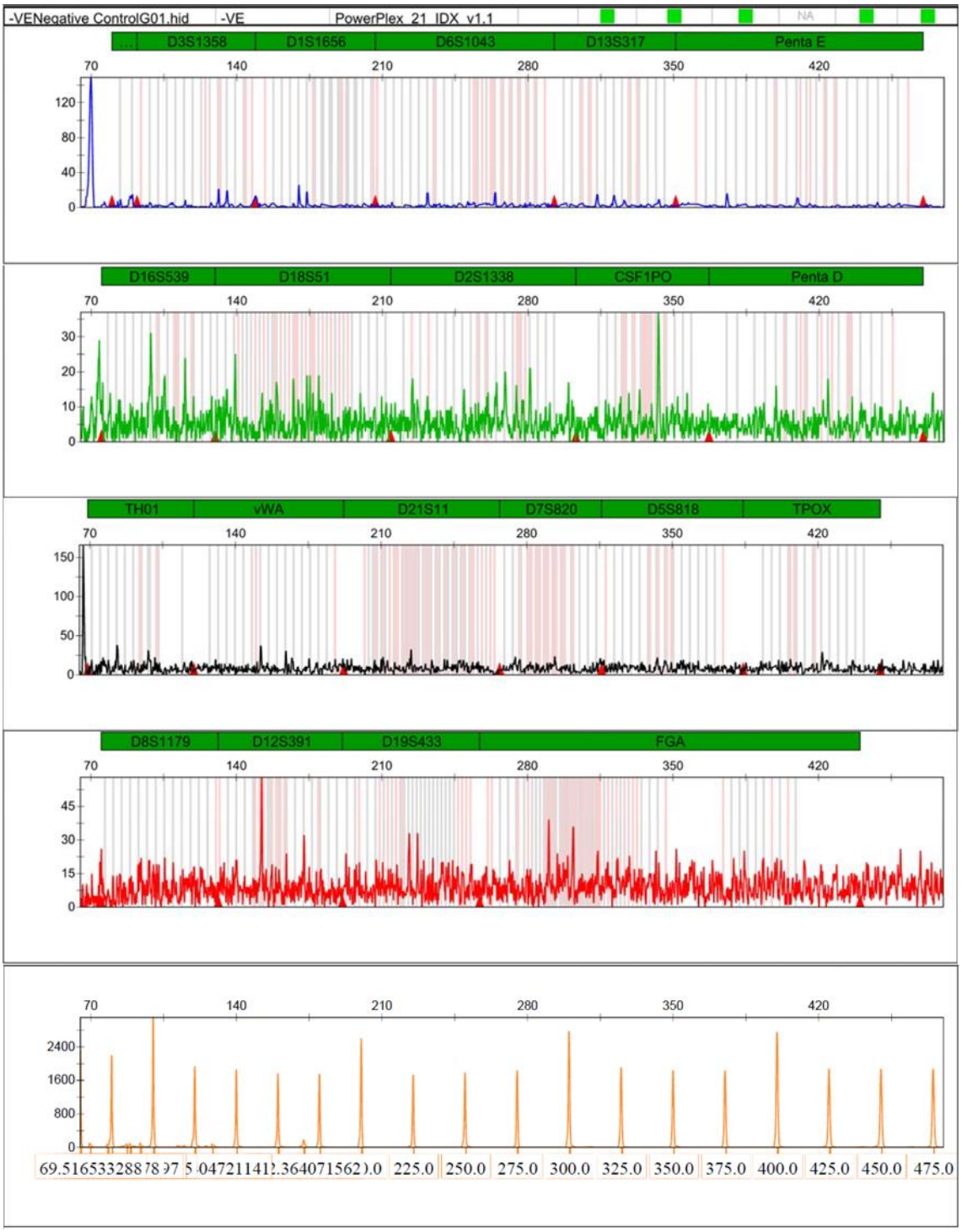


Figure 62: Electropherogram for negative control sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

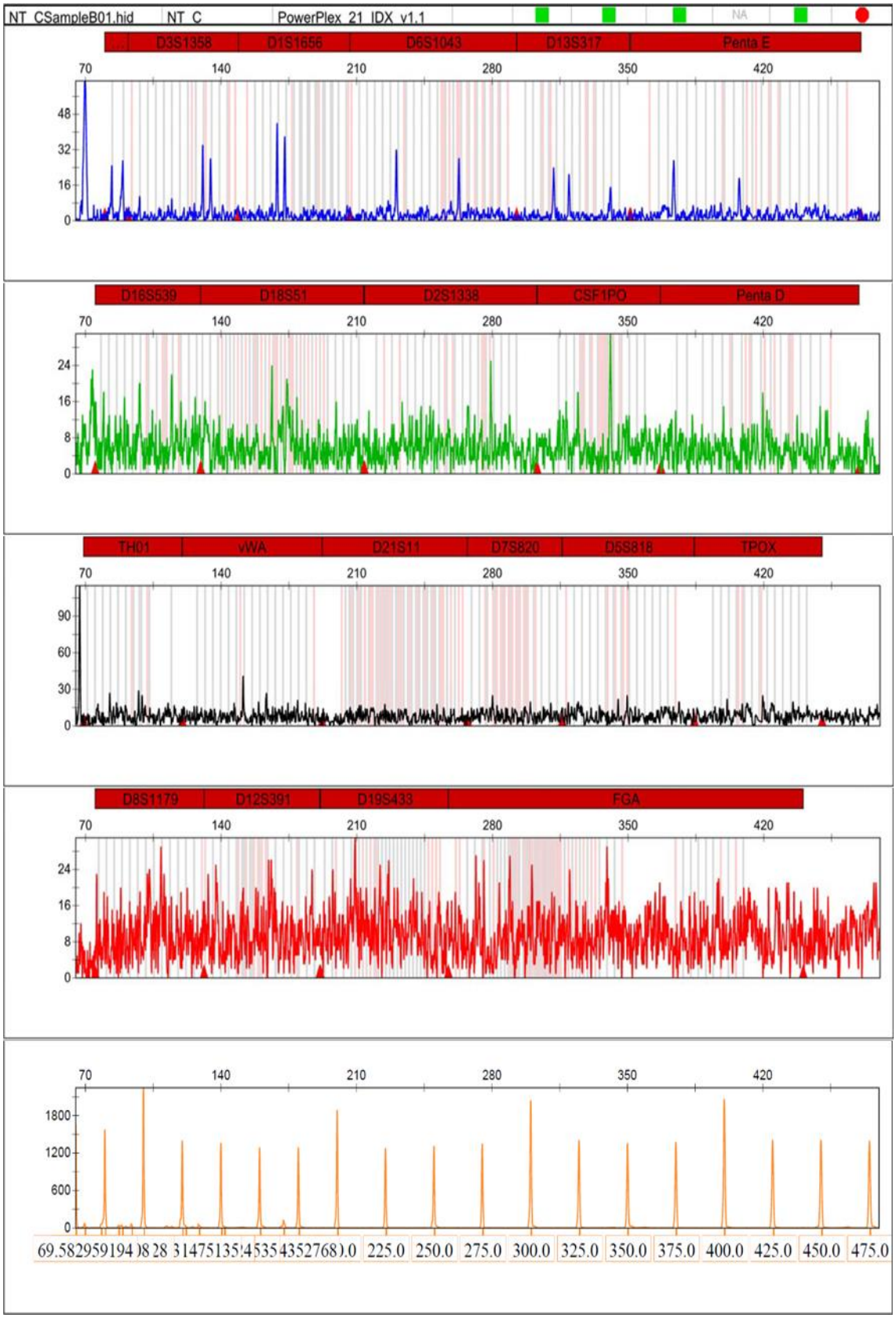


Figure 63: Electropherogram for non-fumed control sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

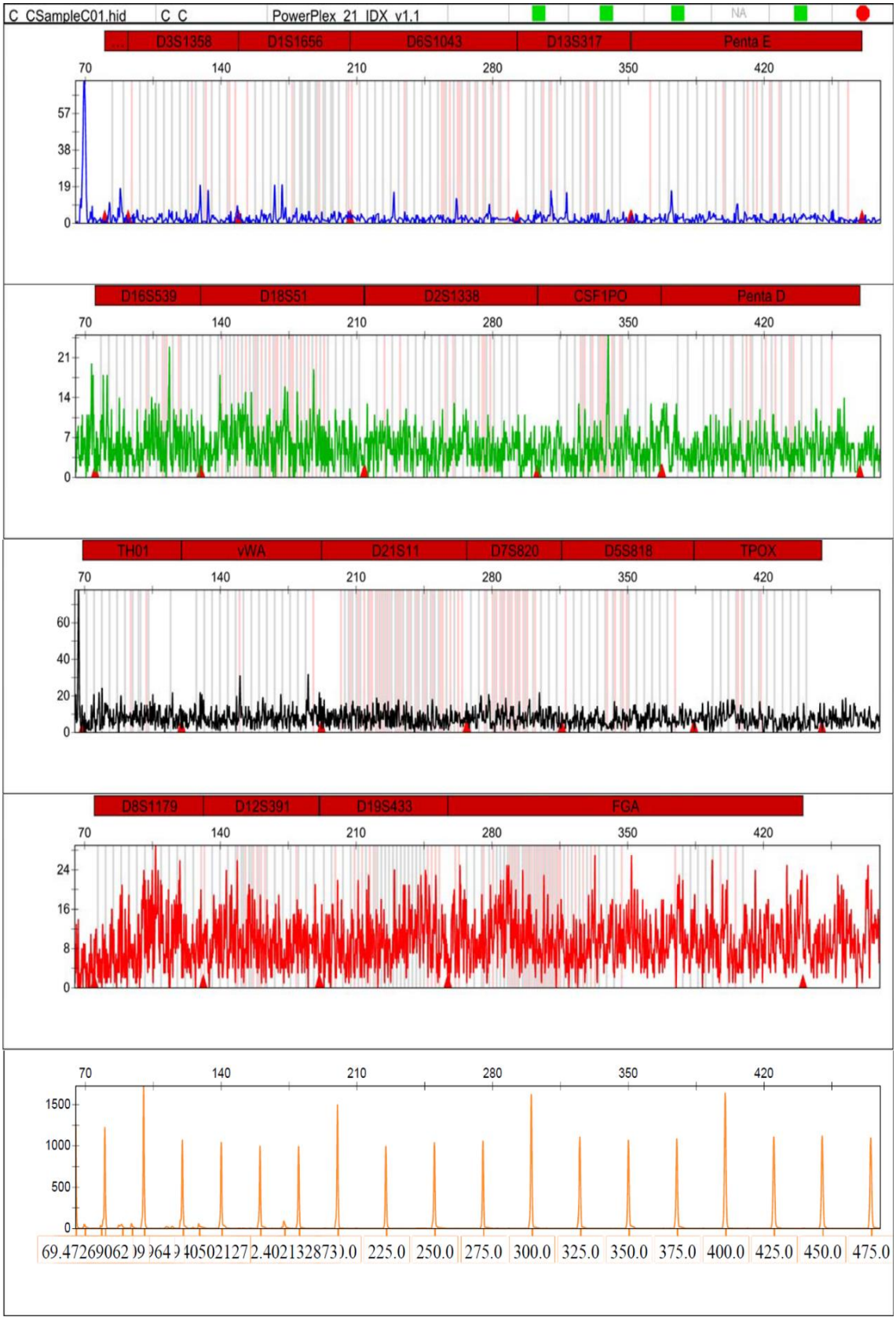


Figure 64: Electropherogram for Cyanobloom fumed control sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

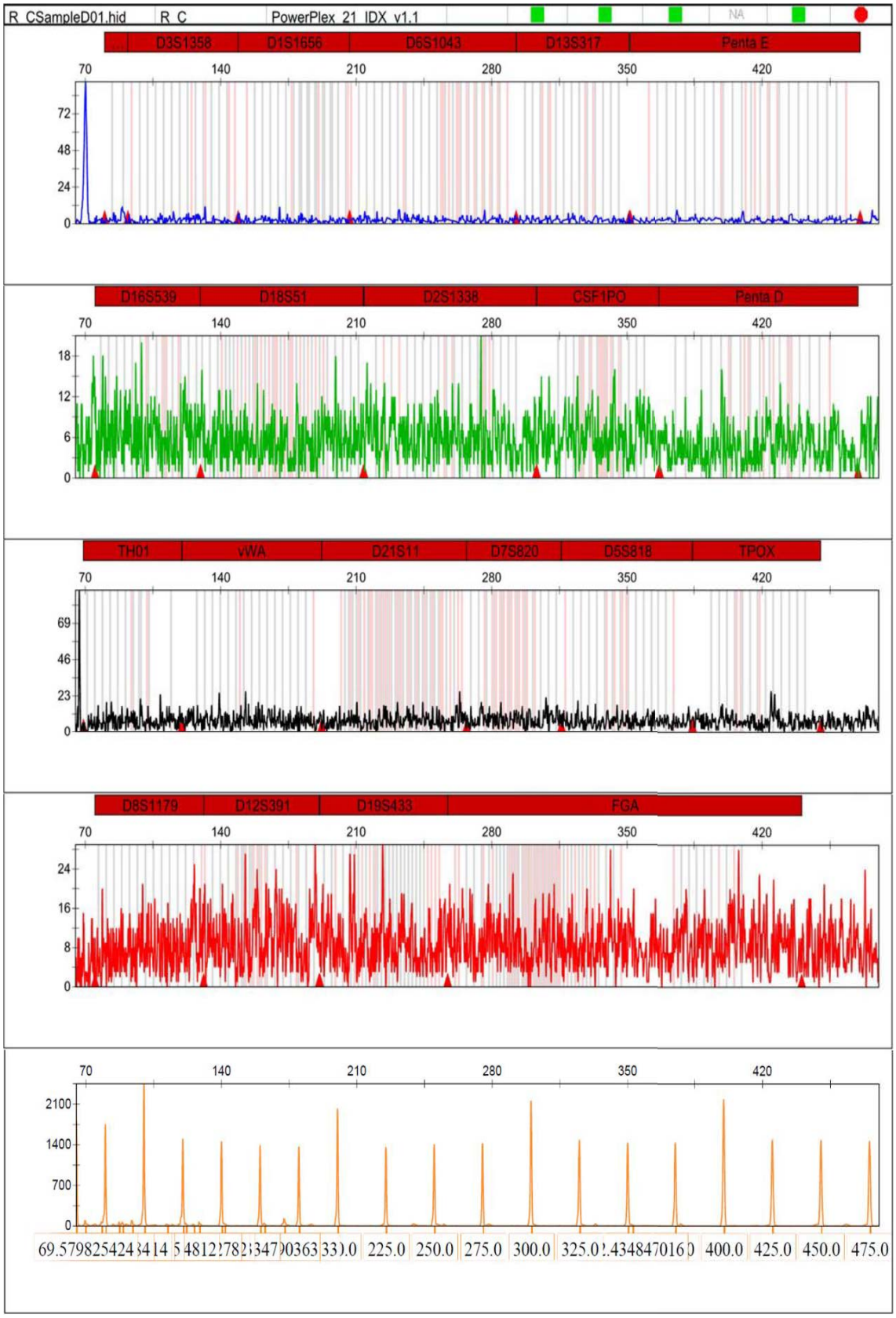


Figure 65: Electropherogram for Cyanobloom fumed control sample post treated with R6G

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

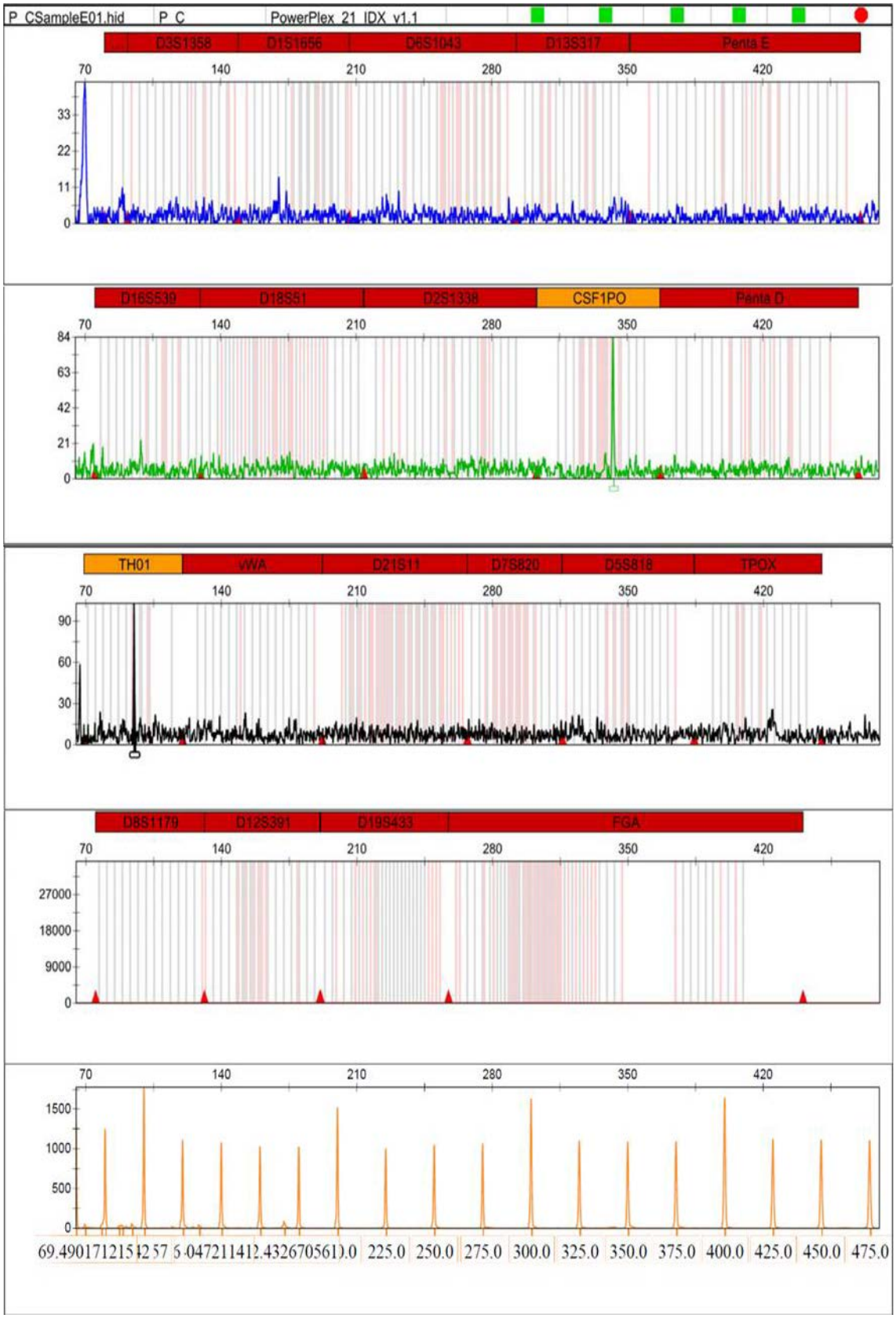


Figure 66: Electropherogram for PolyCyano UV fumed control sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

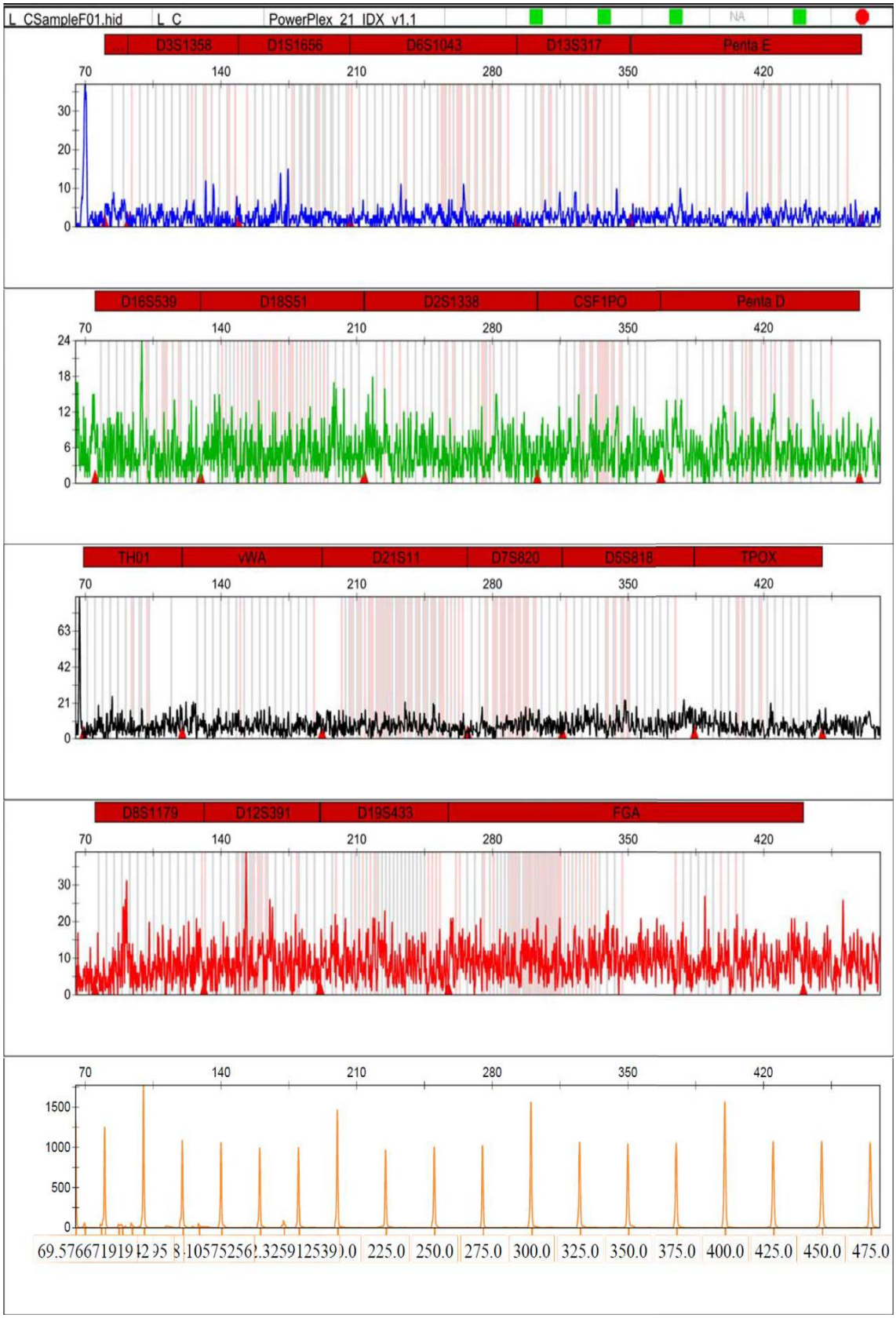


Figure 67: Electropherogram for Lumikit™ fumed control sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

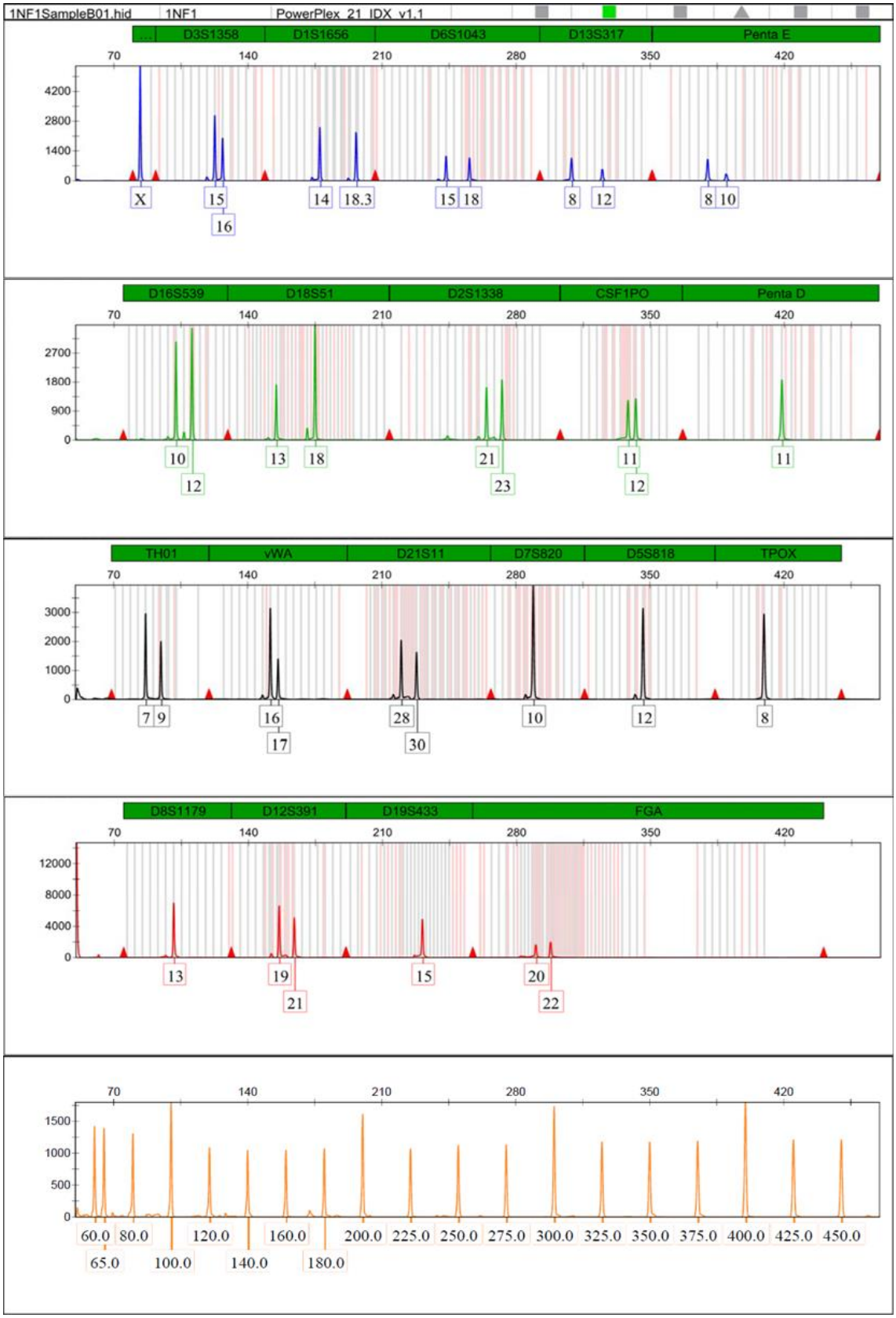


Figure 68: Representative electropherogram for spiked non-fumed sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

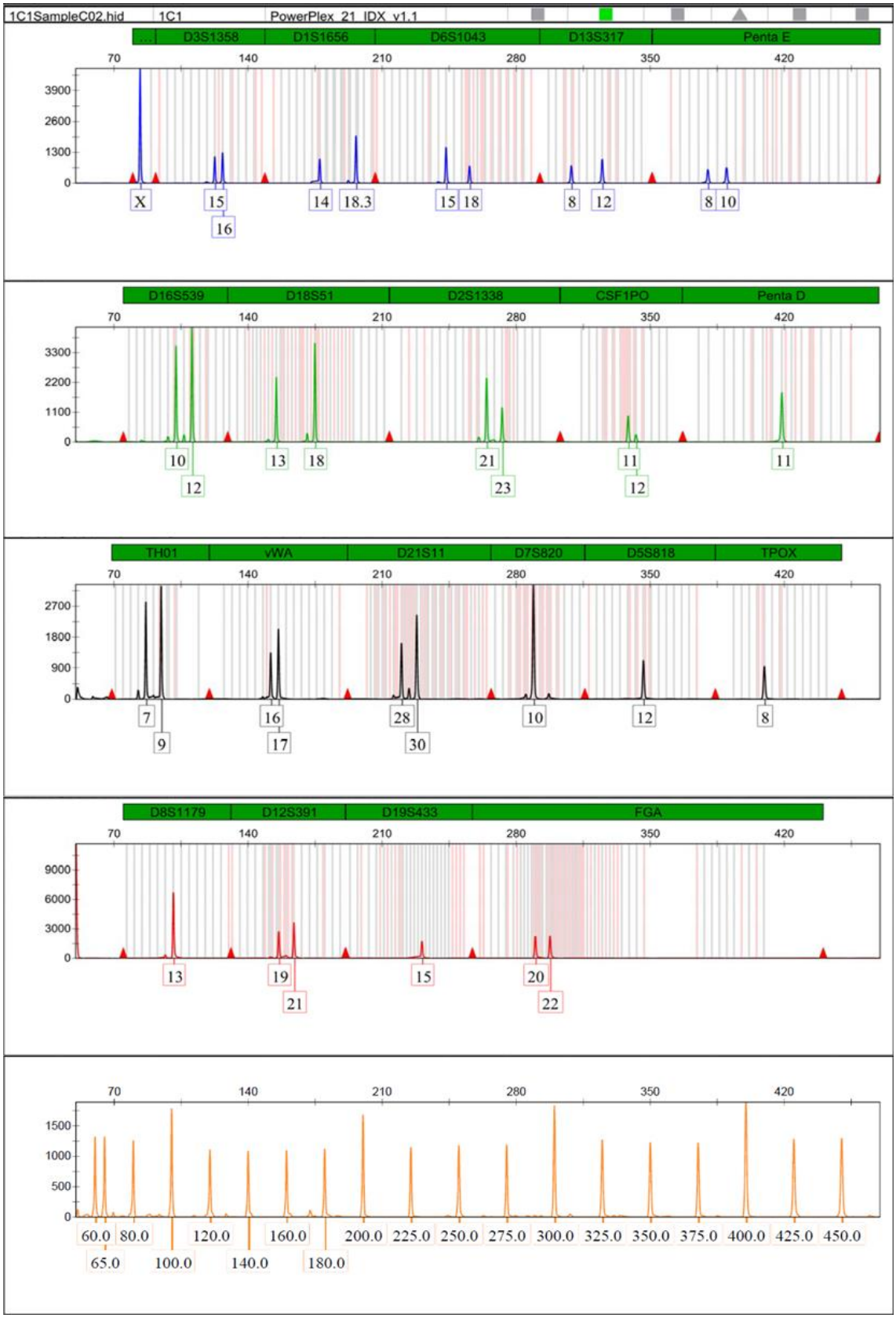


Figure 69: Representative electropherogram for spiked Cyanobloom fumed sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

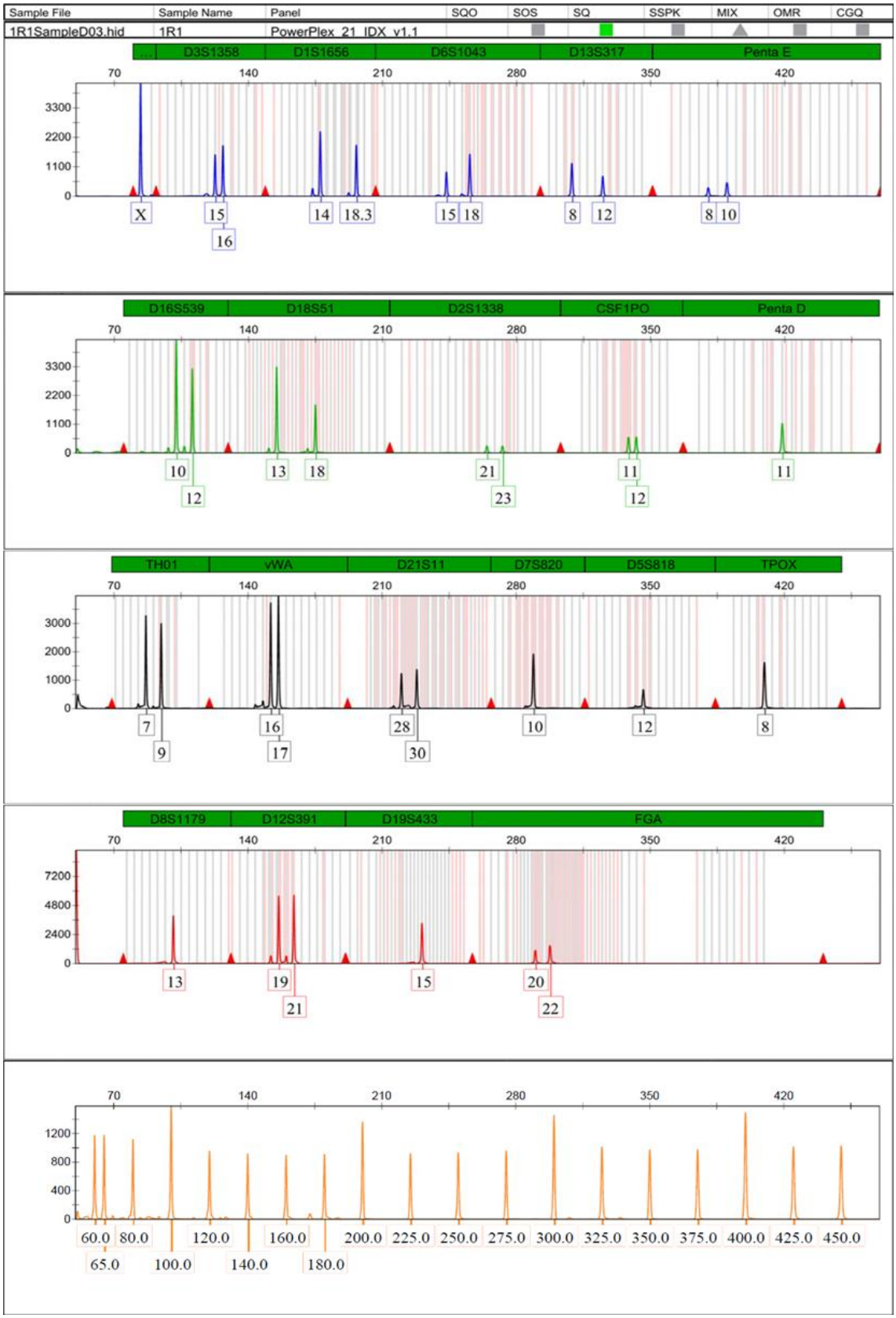


Figure 70: Representative electropherogram for spiked Cyanobloom fumed and R6G stained sample Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

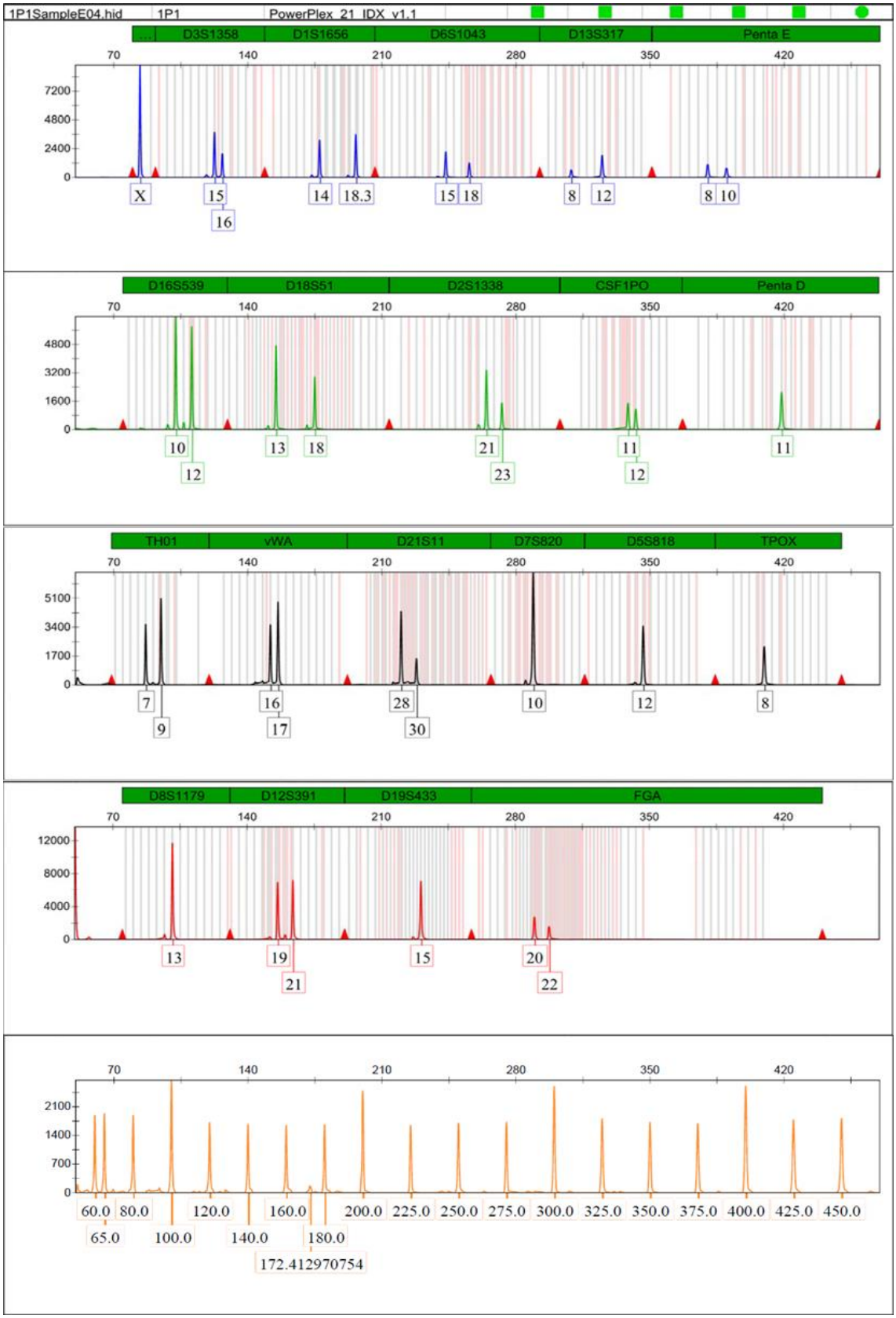


Figure 71: Representative electropherogram for spiked PolyCyano UV fumed sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

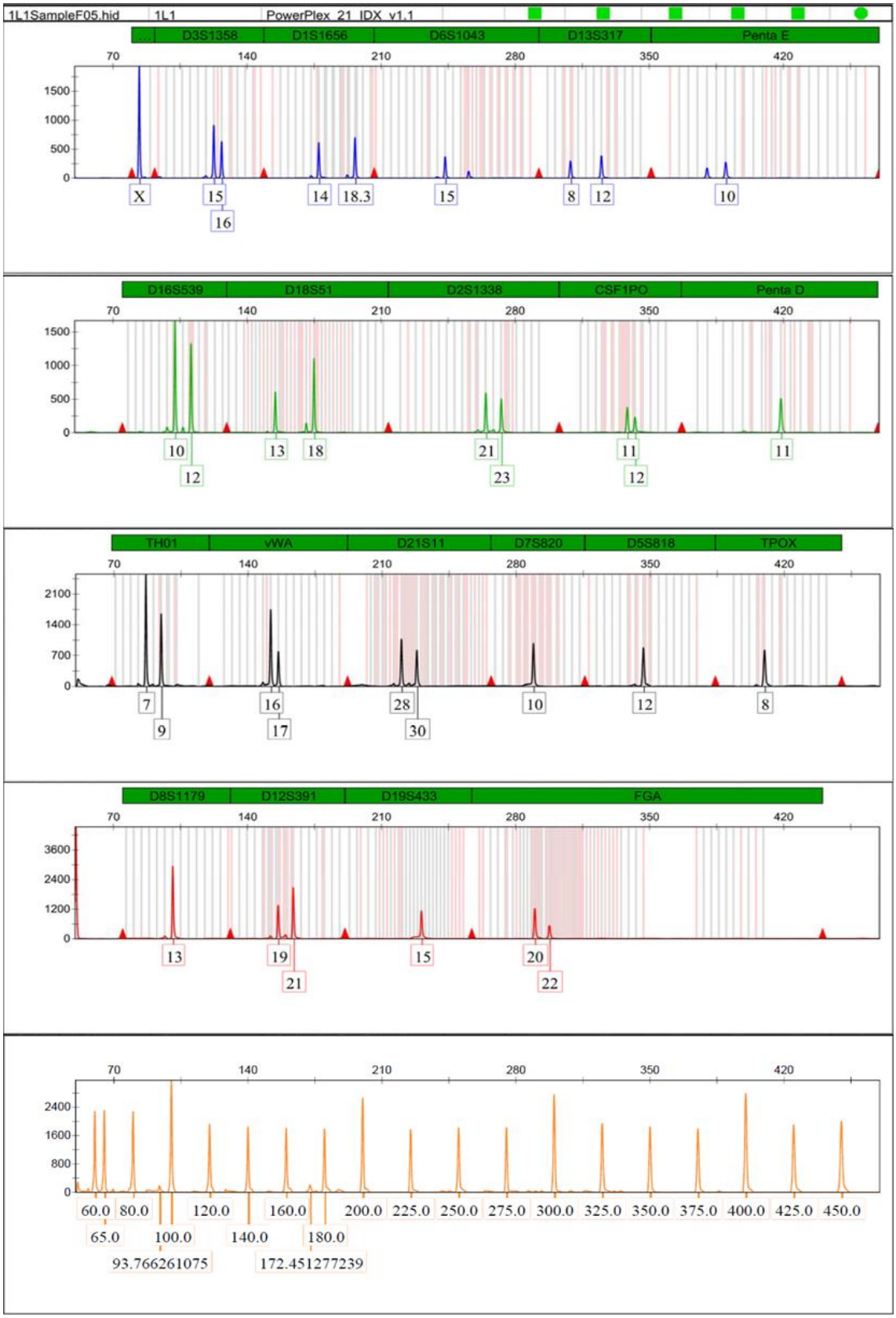


Figure 72: Representative electropherogram for spiked Lumikit™ fumed sample

Sample was amplified using the PowerPlex® 21 System and analysed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.4

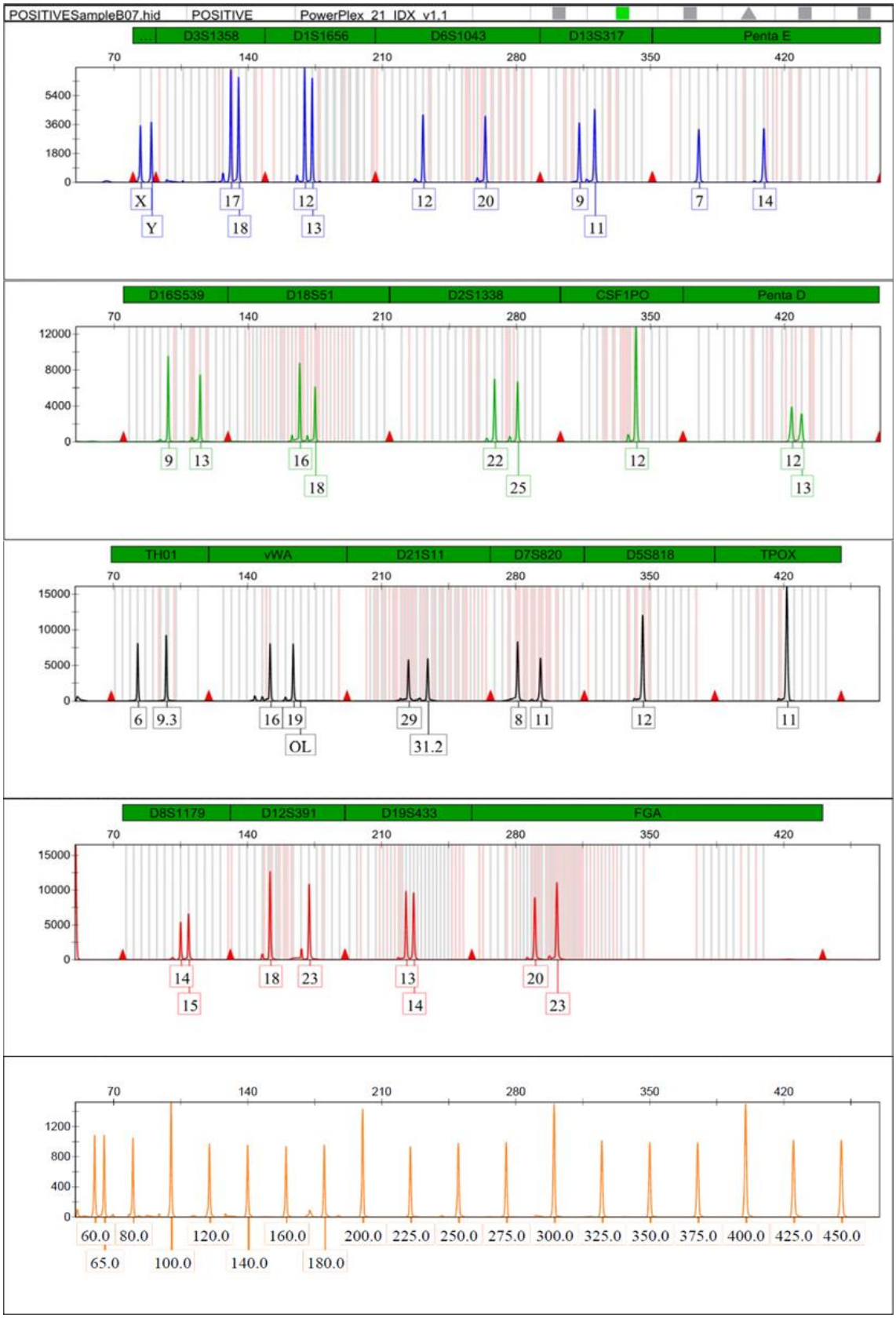


Figure 73: Electropherogram for positive control 2800M DNA

Sample was amplified using the PowerPlex® 21 System and analyzed with an Applied Biosystems 3130 Genetic Analyzer. Results were analysed using GeneMapper® ID software-X, version 1.

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