Enhancement of Trace DNA: Role of Direct PCR in Forensic Practise

by

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THESIS ABSTRACT

DNA profiling success from trace genetic material, such as that deposited within a fingermark, is unpredictable and often low. In a forensic casework setting it may be highly advantageous to obtain genetic data from exhibitory samples to aid in investigations, prosecution, or acquittal. As touched items comprise a large portion of the samples submitted for analysis it is imperative to examine how improvements can be made to the production of DNA profile data.

Standard methods of DNA analyses involve sample collection, DNA extraction, PCR by means of a validated commercial STR kit, CE separation, and profile analysis. The portion of this workflow that can cause the largest reduction in profile information is the template DNA loss during an extraction. An alteration to this workflow, termed 'direct to PCR', involves the collected sample being incubated, but not extracted, with the lysate used in a subsequent PCR. This approach offers inhibitor dilution and no loss of template, however not all template is placed into a single PCR. In a further workflow alteration, termed 'direct PCR', the collected sample can be placed directly into a PCR with no pre-treatment. Direct PCR uses the entire sample in the PCR with maximum template provision, does not require extraction or incubation reagents, but does not facilitate the removal or dilution of any inhibitors present.

The initial part of this thesis looks at optimising direct PCR by analysing six commercially available kits using direct PCR to analyse touch DNA. The work provides informative data and assessments to operational and research-based laboratories of how each of these kits performed. This leads on to investigations into factors that affect touch DNA analysis in a number of ways. Examination of substrate types not previously studied was conducted using direct PCR to determine whether any substrates were more or less suitable for this workflow choice. To better understand how touch DNA acts for genetic amplification, cellular threshold requirements following swabbing and tapelifting for both 'model' cell types and corneocytes were examined through extraction-based and direct PCR workflows. The role of cell-free DNA on profiling success was also observed. This encouraged the assessment of other workflow possibilities for touch DNA analysis with rapid DNA technology being analysed for its suitability.

A crucial issue not previously addressed was whether replicate analysis of a sample could be performed using direct PCR. Here, the analysis of a novel tapelift method showed the ability to produce multiple concordant direct PCR profiles from a single sample. The method also allowed performing both a direct PCR amplification and extraction from a single sample. This provides a solution to a major limiting factor for the implementation of direct PCR to casework samples.

The final two chapters look at sub-optimal sample types or those submitted for examination following extreme conditions; improvised explosive device components post-detonation, small calibre fired bullet casings, and used matchsticks. These studies utilise the optimisation techniques developed through the previous chapters. The data presented within this work demonstrates that STR profiles can be obtained from sample types previously not possible.

Direct PCR is becoming a popular method for forensic science research for touch DNA analysis, due to advantages in cost and time reduction while increasing the probability of obtaining STR profiling data. However, it has not been taken up by operational forensic facilities. Prior to this thesis, the application framework of direct PCR for forensic analysis was not well defined. There was little consistency in STR kit choice, the nature of touch DNA template deposition, and a limited range of substrate types analysed. The outcomes of this work and original contribution to touch DNA analysis and direct PCR knowledge involve an extensive examination of STR amplification kit performance and substrate types, a brief consideration of PCR additive effects, the inclusion of cellular staining for experimental monitoring, determination of cellular template input requirements for PCR, and the examination of extreme conditions on touch DNA template viability.

DECLARATION

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

Signed. .....

Belinda Martin

Date.....

ACKNOWLEDGEMENTS

Firstly, I would like to thank Professor Adrian Linacre, my principal supervisor, for his incredible support throughout my PhD studies. His enthusiasm for this research and wealth of knowledge has been an incredible asset to my professional development and PhD experience. Thank you for facilitating my study as well as for your patience, support, and guidance.

Secondly, I would like to thank my co-supervisor Dr Duncan Taylor, from Forensic Science South Australia, for his invaluable input, support, and advice on both my research and manuscripts. Thank you for your willingness to take time out of your busy days to answer questions and read drafts of everything I sent you. I have really appreciated the time you have invested in me to facilitate the successful completion of this PhD.

I would like to thank the Flinders University forensic research group for providing a supportive work environment. In particular, I would like to thank Renee Blackie, Piyamas Kanokwongnuwut, and Jennifer Young for their personal and professional support throughout my PhD studies. Each of you have played a significant role in maintaining my personal drive to complete this work and provided substantial support to the research itself. To the others in the research group over the years, thank you for your feedback in group presentations and your willingness to share your research with me to allow broader learning.

I would also like to thank my family for the unbelievable level of emotional and physical support they have provided. As well as support with research and writing, maintaining personal support is incredibly important through the PhD years and this is what was provided by my beautiful family. Thank you to my Mum for providing an ear when required and justifying a level of procrastination or relaxation when it was really needed, to my Dad for helping me maintain extra-curricular hobbies to ensure mental stability, and to my brothers for their companionship, interest in my work, and willingness to attempt draft reading. To my beautiful son Eli who came into my life at the one-year milestone of my candidature, although you presented some incredible challenges throughout your little life you have inspired me to complete my PhD with more determination than I previously had. Finally, to my husband Ben who has been incredible throughout these years, thank you. Your understanding and ability to sacrifice a lot to give me the time and room I required at times to do what I needed has been fantastic.

Finally, thank you to the reader. Thank you for making it your task to read this thesis and for your determination to reach the end!

LIST OF ABBREVIATIONS

µL	Microlitre
µM	Micromolar
° C	Decree Celsius
ADIFF	Advanced DNA, Identification and Forensic Facility
AFP	Australian Federal Police
AI	Artificial Intelligence
ANZFSS	Australian and New Zealand Forensic Science Society
ATF	Alcohol, Tobacco, Firearms and Explosives Laboratory
AUS	Australia
bp	Base Pair
CE	Capillary Electrophoresis
CODIS	Combined DNA Index System
CPI	Combined Probability of Inclusion
CTTSO	Combating Terrorism Technical Support Office
DBLR	Database Likelihood Ratios software
DD	Diamond™ Nucleic Acid Dye
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleoside triphosphate
DSTG	Defence Science Technology Group
DPST	Development and Promotion of Science and Technology Talent Project
DVI	Disaster Victim Identification
eDNA	environmental DNA
EDTA	Ethylene-Diamine-Tetra-Acetic acid
ESR	Environmental Science and Research Limited
EPG	Electropherogram
FAME	Flinders Accelerator for Microbiome Exploration
FASS	Forensic and Analytical Science Service
FSSA	Forensic Science South Australia
FST	Forensic Statistical Tool
GLM	Generalised Linear Model
GSR	Gunshot Residue
IBD	Identical by Descent
IED	Improvised Explosive Device
INDEL	Insertion or Deletion
INNUL	Insertion/null markers
IRA	Irish Republican Army
KS	Kolmogorov-Smirnov
LCN	Low Copy Number
LHP	Liquid Handling Platform
LR	Likelihood Ratio
min	Minute(s)
mL	Millilitre

mm	Millimetre
mM	Milimolar
MPOP	Micropopule
MPS	Massively Parallel Sequencing
NDNAD	National DNA Database
NCIDD	National Criminal Investigation DNA Database
ng	Nanogram
NGS	Next Generation Sequencing
nm	Nanometre
OL	Off-Ladder
PCAST	President's Council of Advisors on Science and Technology
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
pg	Picogram
RMP	Random Match Probability
qPCR	Real-time PCR (quantitative-PCR)
RFLP	Restriction Fragment Length Polymorphism
RFU	Relative Fluorescent Unit
rpm	rotations per minute
S	Second(s)
SBREC	Social and Behavioural Research Ethics Committee
SNP	Single Nucleotide Polymorphism
SOP	Standard Operating Procedure
STR	Short Tandem Repeat
TE	10 mM Tris, 0.1 mM EDTA, pH 8.0
TERN	Terrestrial Ecosystem Research Network
UK	United Kingdom
USA	United States of America
UV	Ultraviolet
VNTR	Variable Number Tandem Repeat

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ACHIEVEMENTS

Poster and oral presentations:

"The Australian and New Zealand Forensic Science Society" conference, Perth, Australia, 2018. **Poster presentations**: 'DNA Profiling from Matchsticks'; 'DNA Profiles from Touched Samples'; 'Shedding Light on Shedder'.

"Human Identification Solutions" conference, Kobe, Japan, 2019. **Poster presentation:** 'Maximising the DNA Profile from Touch DNA'.

"The International Society for Forensic Genetics" conference, Prague, Czech Republic, 2019. **Poster presentations:** 'Ideal STR Kit for Direct PCR on Touch DNA Samples'; 'Forensic Identification and Intelligence SNP Data from Latent DNA Using Massively Parallel Sequencing'.

"Flinders University Higher Degree by Research Conference", Adelaide, Australia, 2019. **Poster presentation:** 'Maximising the DNA Profile from Touch DNA'.

Guest speaker at the "Ross Vining Memorial Forensic Science SA Awards Ceremony", Adelaide, Australia, 2019.

"International Association of Forensic Sciences" in conjunction with "The Australian and New Zealand Forensic Science Society" conference, Sydney, Australia, 2021. **Oral presentation**: 'DNA doesn't Dodge Detonation' **Poster presentations:** 'Collection of Touch DNA Evidence: Tapelifts vs Swabs from a Range of Items' and 'Recovery of Touch DNA from 12G Shotgun Casings using Direct or Extract-based PCR and GlobalFiler®'. ***NB: these abstracts were accepted; however conference was cancelled.***

"The 9th Crossing Forensic Boarders", Adelaide, Australia, 2021. **Oral presentation:** 'DNA profiling from improvised explosive devices'

"APR Internship Scheme", Adelaide, online, 2021. **Oral presentation:** 'InfoDust: Recovery of dust microbiomes from personal belongings for counterterrorism intelligence'

Scholarships and grants awarded:

1) The "Australian Government Research Training Program Scholarship".
2) The "Flinders University Research Higher Degree Travel Scholarship".
3) The "Australian and New Zealand Forensic Science Society (ANZFSS) Symposium Award" to support registration for the 22nd Triennial Meeting of the International Association of Forensic Sciences in conjunction with the 25th International Symposium on the Forensic Sciences.
4) APR Internship funding, 3 months.

Teaching performed:

I have conducted lectures and workshops, as well as aided in laboratory and tutorial content delivery for BIOL3793 and BIOL 3792 (2016-2021).

Additional training:

Underwent training in specified DNA-free laboratory usage at Forensic Science South Australia (2018).

Underwent a 'certified peer reviewer course' provided by Elsevier (2020) (See Appendix 8.4.1).

Performed peer reviews of manuscripts (2020-2021) (See Appendix 8.4.2).

Underwent an 'R studio workshop' through Flinders University (2020).

Casework involvement:

Case 1 – Allegation of assault (2018) - Read and analysed casefiles, specifically STR profile results, to read and sign a statement prepared by Professor Adrian Linacre.

Case 2 – Requested by NZ Barrister Nigel Cooke for alleged assault case (2018) - One exhibit examined and STR profiles processed by candidate.

Case 3 – Allegation of assault (2019) - Read and analysed casefiles, specifically BPA and STR profile results, to read and sign a statement prepared by Professor Adrian Linacre.

Case 4 – BPA analysis requested by SAPOL (2020) - Candidate performed Bluestar staining of a crime scene (from 2018) and assisted Professor Adrian Linacre with the analysis of blood patterns observed under fluorescence. Candidate took three blood samples for processing, via swabbing.

Case 5 – Detection and collection of latent DNA (2021) - Candidate performed Diamond™ Nucleic Acid Dye staining, latent DNA visualisation, and subsequent collection on unfired bullet casings on behalf of the New South Wales Police, performed at Flinders University.

PEER REVIEWED PUBLICATIONS

Publications awarded during the writing of this thesis, which I have contributed as first or co-author, are listed below:

Reference	Impact Factor	Citations	Year of publication	Chapter in thesis
Kanokwongnuwut, P., **Martin, B.**, Kirkbride, P., Linacre, A. (2018). "*Shedding light on shedders.*" Forensic Science International: Genetics 36: 20-25: 10.1016/j.fsigen.2018.06.004	4.88	91	2018	3
Martin, B., *et al.* (2018). "*DNA profiles generated from a range of touched sample types*." Forensic Science International: Genetics 36: 13-19: 10.1016/j.fsigen.2018.06.002	4.88	39	2018	3
Martin, B., *et al.* (2019). "*DNA profiles from matchsticks*." Australian Journal of Forensic Sciences: 1-5: 10.1080/00450618.2019.1569138	1.08	10	2019	5
Young, J., **Martin, B**., Linacre, A., (2019). "*Evaluation of the QIAGEN 140-SNP forensic identification multiplex from latent DNA using massively parallel sequencing*." Australian Journal of Forensic Sciences: 1-4: 10.1080/00450618.2019.1573923	1.08	7	2019	Appendix
Young, J., **Martin, B**., Kanokwongnuwut, P., Linacre, A (2019) "*Detection of forensic identification and intelligence SNP data from latent DNA using three commercial MPS panels.*" Forensic Science International: Genetics Supplement Series: 10.1016/j.fsigss.2019.11.003	0.98	3	2019	Appendix
Martin, B., et al. (2020). "*Successful STR amplification of post-blast IED samples by fluorescent visualisation and Direct PCR.*" Forensic Science International: Genetics: 10.1016/j.fsigen.2020.102256	4.88	8	2020	6
Martin, B., Linacre, A., (2020) "*Direct PCR: a review of use and limitation.*" Science and Justice: 10.1016/j.scijus.2020.04.003	2.08	4	2020	1
Martin, B., et al. (2020). "*Direct PCR of fired shotgun casings: a South Australian evaluation."* Australian Journal of Forensic Sciences: 10.1080/00450618.2020.1823475	1.08	1	2020	5
Kanokwongnuwut, P., **Martin, B.**, et.al (2021). "*How many cells are required for successful DNA profiling?*" Forensic Science International: Genetics: 10.1016/j.fsigen.2020.102453	4.88	12	2021	3

Martin, B., Taylor, D., Linacre, A. (2022). "*Comparison of six commercially available STR kits for their application to touch DNA using direct PCR",* Forensic Science International: Reports: 10.1016/j.fsir.2021.100243	2.40	0	2022	2
Martin, B., Taylor, D., Linacre, A., (2022). "Exploring tapelifts as a method for dual workflow STR amplification", Forensic Science International: Genetics: 10.1016/j.fsigen.2021.102653	4.88	0	2022	4
Martin, B., Kaesler, T., Taylor, D., Linacre, A. (2022). *"Analysis of RapidHIT application to touch DNA samples.",* Journal of Forensic Sciences: 10.1111/1556-4029.14964	1.83	0	2022	3
Martin, B., Kaesler, T., Kirkbride, P., Linacre, A. (2022) *"The influences of dusty environments on the STR typing success of post-detonation touch DNA samples."* Forensic Science International: Genetics: 10.1016/j.fsigen.2021.102651	4.88	0	2022	6

LIST OF AUTHOR CONTRIBUTIONS

Title: Shedding Light on Shedders, 2018

Author	Participation
Piyamas Kanokwongnuwut	Carried out all staining lab work, staining analysis, fingermark sampling, and co-wrote manuscript
Belinda Martin (Candidate)	Carried out all STR lab work and analysis and co-wrote manuscript
Paul Kirkbride	Helped to edit the manuscript
Adrian Linacre	Co-wrote manuscript

Title: DNA profiles generated from touch DNA on a range of sample types, 2018

Author	Participation
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Duncan Taylor	Helped to edit the manuscript
Adrian Linacre	Helped to edit the manuscript

Title: DNA profiles from matchsticks, 2019

Author	Participation
Belinda Martin (Candidate)	Conceptual/experimental design, carried out all lab work, analysis and wrote the manuscript
Renee Blackie (nee Ottens)	Assisted with experimental design
Adrian Linacre	Helped to edit the manuscript

Title: Evaluation of the QIAGEN 140-SNP forensic identification multiplex from latent DNA using massively parallel sequencing, 2019

Author	Participation
Jennifer Young	Assisted with experimental design, carried out laboratory work, and wrote the manuscript.
Belinda Martin (Candidate)	Assisted with experimental design and carried out laboratory work
Adrian Linacre	Helped edit the manuscript

Title: Detection of forensic identification and intelligence SNP data from latent DNA using three commercial MPS panels, 2019

Author	Participation
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Belinda Martin (Candidate)	Carried out laboratory work and edited the manuscript
Piyamas Kanokwongnuwut	Carried out laboratory work and edited the manuscript
Adrian Linacre	Helped edit the manuscript

Title: Successful STR Amplification of Post-Blast IED Samples by Fluorescent Visualisation and Direct PCR, 2020

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Paul Kirkbride	Aided in experimental design, performed chemical analyses, and helped to edit the manuscript
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Adrian Linacre	Helped to edit the manuscript

Title: Direct PCR: a review of use and limitations, 2020

Author	Participation
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Adrian Linacre	Helped edit the manuscript

Title: Direct PCR of fired shotgun casings: A South Australian evaluation, 2020

Author	Participation
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Adrian Linacre	Edited the manuscript
Julianne Henry	Conceptual/experimental design and wrote the manuscript

Title: How many cells are required for successful DNA profiling, 2021

Author	Participation
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Belinda Martin (Candidate)	Conceptual/experimental design, laboratory work, data analysis and co-wrote the manuscript
Duncan Taylor	Aided in statistical analyses, wrote statistical portions of manuscript, and edited the manuscript
Paul Kirkbride	Aided in experimental design and edited the manuscript
Adrian Linacre	Aided in experimental design and edited the manuscript

Title: Comparison of six commercially available STR kits for their application to touch DNA using direct PCR, 2022

Author	Participation
Belinda Martin (Candidate)	Conceptual/experimental design, carried out all laboratory work, performed data analysis and wrote the manuscript
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Title: Exploring tapelifts as a method for dual workflow STR amplification, 2022

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Belinda Martin (Candidate)	Conceptual/experimental design, carried out all laboratory work, performed data analysis and wrote the manuscript
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Title: Analysis of RapidHIT Application to touch DNA samples, 2022

Author	Participation
Belinda Martin (Candidate)	Conceptual/experimental design, laboratory work, data analysis and wrote the manuscript
Todd Kaesler	Aided in experimental design, laboratory work, aided in data analysis, and edited the manuscript
Adrian Linacre	Aided with experimental design and edited the manuscript

Title: The influences of dusty environments on the STR typing success of post-detonation touch DNA samples, 2022

Author	Participation
Belinda Martin (Candidate)	Conceptual/experimental design, laboratory work, data analysis and wrote the manuscript
Todd Kaesler	Performed laboratory work, aided in data analysis, and edited the manuscript
Paul Kirkbride	Aided with conceptual design and edited the manuscript
Adrian Linacre	Aided with experimental design and edited the manuscript

CHAPTER I:

INTRODUCTION

Manuscripts Enclosed

Martin, B., & Linacre, A. (2020). "*Direct PCR: a review of use and limitation."* Science and Justice

1.1 Preface

The overarching theme in this thesis is 'more from less' with the task of obtaining greater quality, and more meaningful, DNA data from less material than previously possible. More specifically, this thesis aims to improve the process of direct PCR for forensic purposes and apply it to challenging casework samples. Direct PCR has been applied to forensic exhibits for a number of years, however the expansion into 'touch' DNA (DNA left by handling or brief skin contact) is very recent, and as such, little is known about the boundaries of this technique for DNA data generation. The thesis outlines the application of current and evolving DNA profiling methods to novel or traditionally challenging sample types to improve DNA profiling success during the duration of this candidature.

The work in presented in Chapters II-VI of this thesis includes a series of publications in thematic sections rather than how they were chronologically performed and published. The themes explore and describe advancements to the amplification of latent DNA analysis through direct PCR application and provide a clear framework for direct PCR methodology and use in both research and operational settings. It starts by observing how alterations to currently employed DNA profiling methods can optimise latent DNA analysis and follows this with a comprehensive evaluation of different factors that influence the success of DNA profiling from touch DNA as a cellular source; identifying the types of surfaces direct PCR is able to be performed on, how the individual depositing latent DNA affects downstream processing, how many cells are needed for optimal DNA profiling, and how emerging technology performs on this latent cell source was assessed. Following a research-based understanding of direct PCR, issues surrounding the integration of direct PCR into operational casework are discussed and solutions are postulated through novel sampling device use. With optimised methods, this thesis culminates with the technique's application to touch DNA being tested through trials including arson simulation, ammunition firing, and explosive exposure.

Chapter I concludes with a published review of the direct PCR sphere and its application to latent DNA (see manuscript enclosed: Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*)

1.2 DNA Profiling in Forensic Science

Standard workflow for DNA profiling in forensic science involves multiple, discrete phases. These phases are as follows: the collection of a DNA deposit; extraction and purification of the DNA within the sample; quantification of the DNA template; amplification of genomic target regions by

polymerase chain reaction (PCR); separation by capillary electrophoresis (CE); electropherogram (EPG), or profile, interpretation; and finally statistical analysis of the subsequent profile. Each step in the process serves a purpose, but these are also costly and time consuming.

1.2.1 Sample extraction and purification

Following DNA collection, DNA extraction is performed to purify DNA. Many forensic sample types contain PCR inhibitors, which must be removed to allow for efficient PCR [1]. As well as the purification of DNA, the extraction process provides a DNA extract, which may allow many PCR amplifications to be performed and also allows the quantification of DNA. Although the purification of DNA within a sample, and the formation of the extract pool seems entirely beneficial, the process of extraction is thought to play the most significant role in DNA template loss within standard workflows. It is well established that approximately 20-70% [2] of potential DNA template is lost through the extraction steps; Ottens *et al.* (2013) reported this loss may be as much as 83% [3]. These differences in extraction efficiencies can also be dependent on the surface from which the extraction is performed [4].

There are two main DNA extraction chemistries that have been utilised in forensic science: these are chemically driven such as phenol:chloroform organic extractions [5] or Chelex®100 [6]; and solid-phase extraction [7], such as silica column or magnetic bead use. Bright and Petricevic (2004) demonstrated that DNA recovery from the heel and toe of a shoe was higher using phenol:chloroform method compared to Chelex®100 [5]. Although phenol:chloroform extraction results in higher DNA yield and purity than Chelex®100 [5] and silica-based methods [8-10], this approach is not commonly used in operational laboratories as it utilises hazardous substances [10]. Chelex®100 extraction, while quick, does not effectively remove all the PCR inhibitors or allow for automation easily [11]. Due to this, solid phase extractions, using either silica column or magnetic beads, are most prevalent in current forensic practise as these can be easily automated [10].

Silica-based extraction methods, in the form of spin columns, utilise a thin layer of silica that selectively binds nucleic acids in the presence of high concentrations of chaotropic salts. When the pH of solution is less than 7.5, DNA will adsorb to the silica layer with impurities being washed through the matrix. When the pH becomes alkaline, and salt concentrations are low, DNA is released from the silica and the collected eluate is stored as a DNA extract [12]. As multiple pipetting events and tube transfers are involved, there are multiple opportunities for contamination or sample transfer error to occur.

Silica coated magnetic bead extractions works similarly to the silica spin column as the DNA is reversibly bound to the silica coated magnetic bead under low pH and high salt concentration [13]. These beads are pulled toward a magnet, with any impurities remaining in solution to be removed. This DNA is then released from the magnetic bead silica matrix. This system, unlike extraction with spin columns, can be performed in a single tube and can be easily automated [12, 14]. A comparison between five extraction methods determined that DNA IQ™ (silica coated magnetic bead), QIAmp® DNA Investigator Kit (silica column), and QIAsymphony® DNA Investigator® Kit (silica coated magnetic bead) showed greater success in downstream short tandem repeat (STR) typing compared to the QIAamp® DNA Blood (silica column) and Chelex®100 [7].

For more information on the advantages and limitations of extractions see the enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

1.2.2 Quantification of Template

DNA extracts contain both human and non-human DNA. Accurate quantification of the human DNA component within the extract is important to ensure effective amplification of STR loci and allow high quality DNA profiles to be generated. Quantification also allows triaging of samples to ensure resources are not wasted on samples that have no, or very little, amplifiable DNA present within the extract. Some quantification kits also indicate the quality of the DNA within the extract, which allows further triaging in an operational laboratory setting. In operational laboratories, quantification is an important, and sometimes compulsory, part of their analytical workflow. Concentration and degradation index thresholds are often strictly applied to casework samples to reduce the processing backlog of viable samples most likely to obtain an interpretable DNA profile.

Quantification techniques used in forensic DNA analysis workflows often utilise fluorescence as an indicator of DNA presence and quantity. There are a number of methods available with various advantages and disadvantages attributed to each. For more information as to the importance of quantification see enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

Qubit fluorometers utilise a fluorescent dye that binds to DNA molecules, through intercalation, within a sample. By comparing the fluorescent signal detected and recorded by the equipment to a set of standards of known concentration, the concentration of DNA within the sample

can be calculated. They are reasonably sensitive with a detection range of 0.01 to 100 ng/µL [15]. It is an extremely fast and simple method, when compared to real-time PCR (qPCR), however it measures total DNA, not only the human component, and is unable to indicate the quality of the DNA within the extract pool.

Quantification by qPCR is more expensive, time consuming, and requires a higher level of technical skill when compared to Qubit. Advantages of this method, however, are the human specificity of the resultant concentration due to the primers used within the assay, and that degradation index records are produced through the comparison of large and small autosomal amplicon concentrations. This indicates the quality of the DNA present within an extract pool and may advise downstream processes. In addition to these, qPCR allows for the determination of the male component through the amplification of a target sequence present on the Y-chromosome. Because of the increased accuracy and additional data produced through qPCR analysis, this is the preferred method of quantification for forensic laboratories.

1.2.3 Amplification of Genomic Targets

The first application of genomic typing to forensic science involved analyses of variable number tandem repeats (VNTRs) of telomeric minisatellites using a technique known as restriction fragment length polymorphism (RFLP) analysis [16-19] pioneered by Sir Alec Jeffreys. VNTR analysis by RFLP was originally used in 1985 for the resolution of paternity and immigration disputes [18]. In 1987 Colin Pitchfork became the first person to be convicted of a serious crime, being the rape and murder of a 15-year-old female, on the basis of DNA evidence obtained using VNTRs [20]. VNTRs are repetitive sequences found within the genome of variable lengths. Each repeat motif, or repetitive sequence, comprises 16-64 bp [21]. The number of repeat motifs can vary between individuals; therefore, donor identification can be possible by observing multiple VNTR loci along the genome. VNTR analysis by RFLP was time consuming, often taking between days and weeks to complete, required a large quantity (~1 µg) of good quality DNA, which is often not available in casework, and utilized radioactive material making it hazardous to perform. In addition, the interpretation of the resultant autoradiograph could be highly subjective, especially in the cases of mixed profiles [22], and databases were difficult to generate so comparisons to previously obtained analyses were estimated [23], which limits its evidentiary power in a courtroom.

The development of the PCR amplification process, by Dr Kary Mullis in 1985, enabled more sensitive genetic techniques to be applied to forensic science [19]. PCR amplifies a specific target of

genetic interest through the separation of DNA strands, the annealing of forward and reverse primers, and the extension of these primers along the template strand to produce a double-stranded copy of the original template (Figure 1.1). This process continues over many cycles allowing the initial template to be exponentially amplified to a sufficient concentration for separation and detection. In addition to the target region, each amplicon experiences a non-template addition, by the polymerase, through adenylation at the 3' end of the PCR product. This results in an amplicon that is one base-pair longer than the target sequence. The first application to casework was in 1986 with the amplification of the HLA-DQa gene for the comparison of different autopsy samples, confirmed to be from the same individual, in the People vs Pestinikas case [24]. Wider implementation of the technique to forensic DNA analysis followed in the coming years [25-27]. This implementation was accelerated with the introduction of multiplex PCRs. The multiplexing, or mixing, of multiple primer pairs within a single PCR facilitates the amplification of multiple sites from the same sample aliquot, thus reducing the amount of template required. This allowed the analysis of microsatellites and STRs, which were not the standard method for forensic DNA typing for criminal casework.

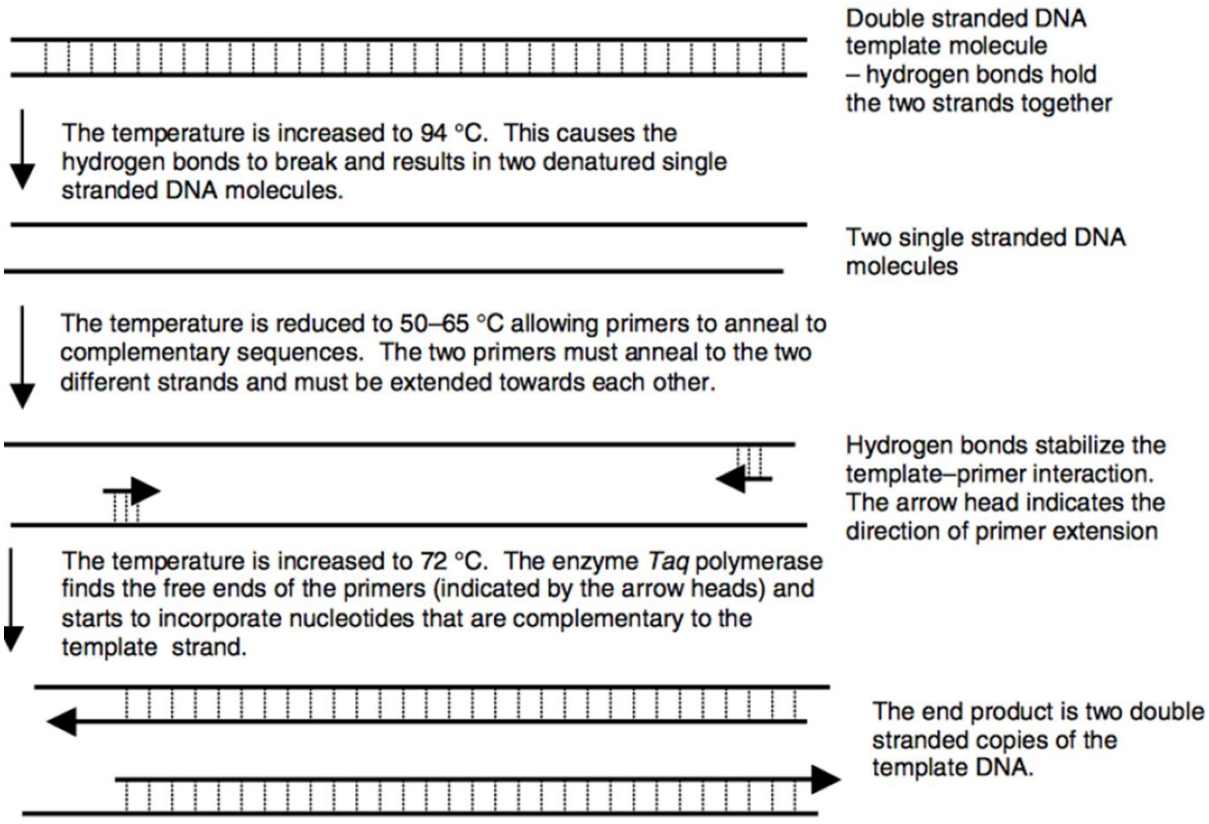


Figure 1.1. Schematic of a PCR cycle utilising a forward and reverse primer to amplify small segments of the template strand. Image sourced from An Introduction to Forensic Genetics by Goodwin, Linacre, and Hadi, 2012 [28].

1.2.4 Short Tandem Repeat Profiling

STRs are segments of repetitive DNA with motifs between 2 (di-) and 6 (hexa-) nucleotides in length [29], occurring approximately once every 15,000 bp across the human genome [30]. For forensic purposes the flanking region of these repeat units must be highly conserved, to allow specificity in primer annealing, while the number of repeat motifs possible within that segment must be variable to increase the ability to differentiate between individuals. There are three STR categories: simple, compound, and complex. Simple STRs consist of one repeat unit only. For example the loci D7S820, located on chromosome 7, which consists of between 5 and 16 repeats of $[GATA]_n$ [31]. Compound STRs are comprised of repeat units with the same length but different motifs, for example vWA, which consists of the motifs $[TCTG]_{3-4}[TCTA]_n$. Complex STRs are comprised of repeat units with variable lengths and motif composition such as D1S1656, which consists of both tetranucleotide and trinucleotide motifs $[TAGA]_n[TGA]_{0-1}[TAGA]_n[TAGG]_{0-1}$ [32].

Each individual carries two copies of DNA, which makes up their genome; one is inherited maternally, the other paternally (Figure 1.2). As a result, two alleles at each locus, should be amplified through PCR. CE analysis of these repeat motifs will indicate the final size, or mass, of the STR sequence. Where the two alleles carry the same number of repeat units a single peak is observed on the EPG and its height will be the collective relative fluorescent units (RFU) value of both single alleles' contributions. The DNA donor is therefore considered homozygous at this locus. Alternatively, each allele carried at a locus can consist of a different number of repeat units; this is termed heterozygous, and two peaks are observed on the EPG.

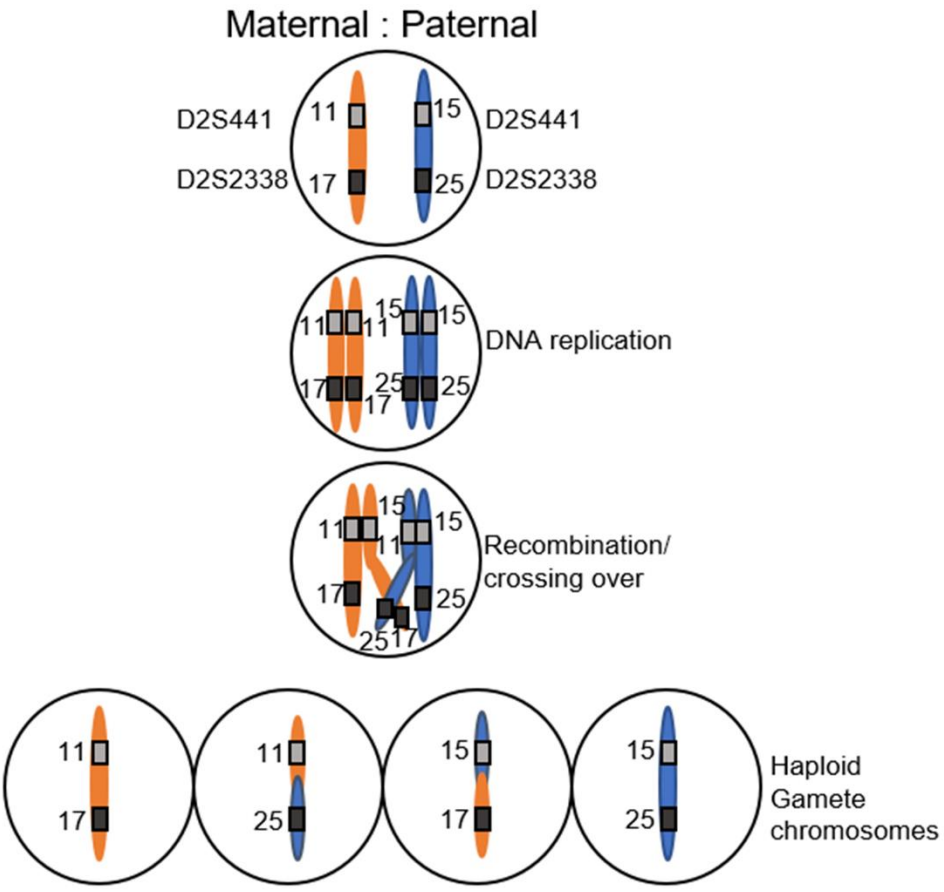


Figure 1.2. Example of allelic inheritance through meiosis. Two loci on chromosome 2, D2S441 and D2S2338, are shown through the replication, recombination, and separation. After zygote production, the fusion of two gamete cells, two copies of each chromosome are present in the diploid cell.

In addition to criminal casework, STRs can be used for kinship testing and familial searching. Paternity testing includes the comparison of the DNA profiles between the parent(s) and child to determine whether paternity is supported based on an understanding of genetic inheritance. Familial testing, described later in 1.2.6.2, can be a useful tool when no match to the profile in question is found in the DNA database [33]. By understanding the relationship between inheritance and shared DNA within family structures, this can aid investigations though the inclusion of individual's whose family members may have been the original DNA donor [34, 35]. Disaster Victim Identification (DVI) casework allows for identification of remains through either direct comparison to the deceased reference profile or through comparison to close relatives [36-38]. An example of this was the 2009 Victorian bushfires, whereby 67 positive identifications were made from samples pertaining to 163 missing people by STR profiling [39].

1.2.5 Commercial STR Kits in Forensic science

The first commercially-available multiplex STR kit was released by Promega Corporation in 1994 and contained three loci - CSF1PO, TPOX, and THO1 - with separation by polyacrylamide gel electrophoresis [10]. Validation for this triplex, commonly known as CTT, was reported by Budowle *et al.* in 1997 with the conclusion that biological samples exposed to a range of environments can yield reliable STR typing results and that this multiplex is suitable for forensic analysis [40]. Through more efficient multiplexing, the incorporation of more loci, and improvement to reaction buffers more discriminatory DNA typing is achieved. The selection and inclusion of various loci influence the price of the STR kit for the consumer, as increased multiplexing complexity increases the number of primers required within a reaction. In addition to this, the transition to newer kits with increased loci data involves internal validation studies, allelic and locus concordance consideration, and potentially database alterations. Because of this, transition to the largest kit available, in terms of locus inclusion, is not always financially or practically viable. An example of autosomal locus typing between five commonly used STR kits is shown below in Table 1.1. In addition to these autosomal targets amelogenin is amplified by all kits, and Y-chromosomal targets are included in GlobalFiler™, VeriFiler™ Plus, and Investigator® 24plex QS.

The incorporation of fluorescent dyes into the multiplexed primer systems further increases the number of loci able to be targeted in a single reaction. Current STR amplification kits utilise four to five fluorescent dye lanes, in addition to the size standard, which allows an increased proportion of smaller amplicons to be amplified from the same DNA source as amplicon mass overlap is distinguishable. As DNA degradation restricts the availability of larger DNA fragments for PCR template, increasing the number of smaller target regions increases the potential of obtaining successful amplification from degraded samples [41]. An example of this is the comparison between SGM Plus® and VeriFiler™ Plus when amplifying control DNA 007 (Figure 1.3). Including amelogenin, SGM Plus®, with 3 primer dyes, amplifies a total of 11 loci (90-360 bp) while VeriFiler™ Plus, with 5 primer dyes, is capable of amplifying 25 (80-430 bp); within the same base-pair range as SGM Plus® (80-360 bp) VeriFiler™ Plus is capable of amplifying 20 loci.

Table 1.1: Autosomal loci amplified by five commonly used commercially available STR kits. VeriFiler™ Plus, Identifiler™ Plus, and GlobalFiler™ Plus are produced by ThermoFisher Scientific; Investigator® 24Plex QS is produced by QIAGEN; PowerPlex® 21 System is produced by Promega Corporation.

KIT/STR LOCI	VERIFILER PLUS	IDENTIFILER PLUS	GLOBALFILER	INVESTIFATOR 24PLEX QS	POWERPLEX 21
D3					
CSF					
D1					
D10					
D12					
D13					
D16					
D18					
D19					
D21					
D22					
D2S1338					
D2S441					
D5					
D6					
D7					
D8					
FGA					
Penta D					
Penta E					
SE33					
THO1					
TPOX					
vWA					
Total	23	15	21	21	20

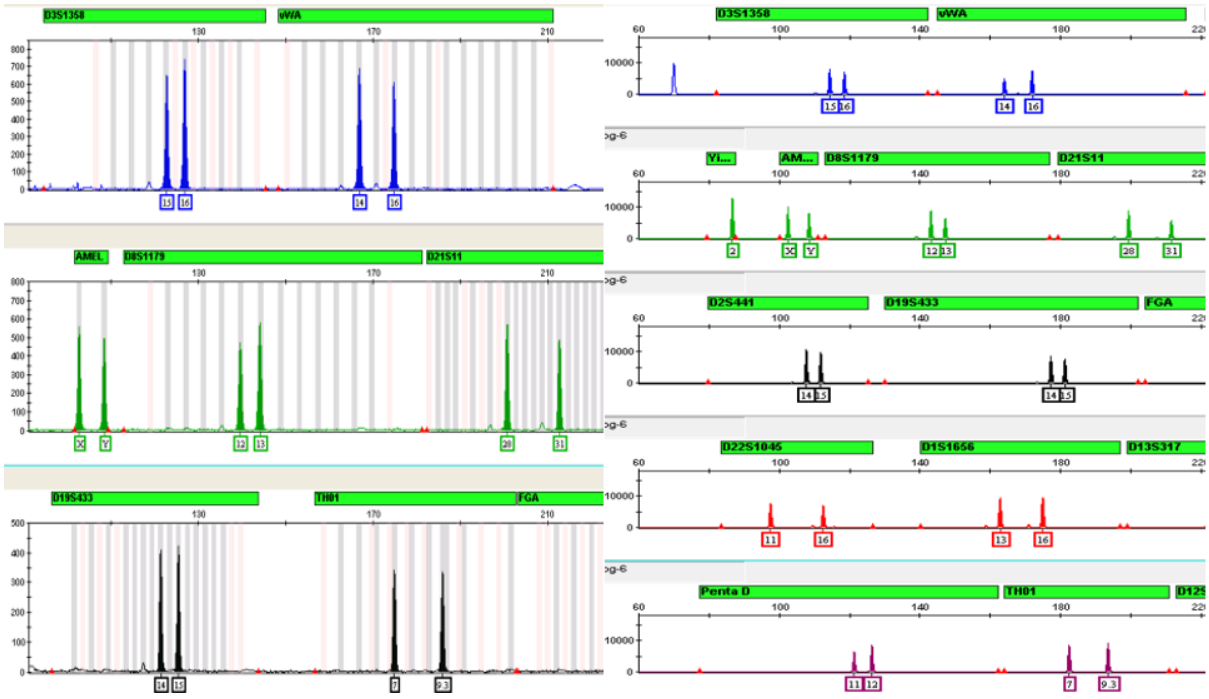


Figure 1.3. 80-220 bp portion of control DNA 007 STR profile amplified with SGM Plus® (left) and VeriFiler™ Plus (right). SGM Plus® contains three dye lanes while VeriFiler™ Plus contains five. Profiles sourced from respective user guides [42, 43].

1.2.6 Separation of Amplification Products using Capillary Electrophoresis

Following multiplexed PCR, products are separated using CE (Figure 1.4). CE offers greater sensitivity and separation resolution than the polyacrylamide gel electrophoresis previously utilised in forensic DNA analyses [44], can be easily automated [45, 46], and eliminates the use of mutagenic or radioactive substances [44]. Electrophoresis causes the migration and separation of molecules, through electroosmotic influence, based on charge. In STR DNA analysis, however, the charge-to-size ratio of amplicons of different lengths is too similar; therefore gels, in the form of a flowable polymer, are incorporated into STR CE to facilitate the separation of DNA fragments based on size [44, 46]. As DNA is negatively charged, movement of the DNA molecules into the capillary is performed through application of a positive voltage across the capillary. For the purpose of DNA separation the internal wall of the capillaries are modified to be inert to DNA thus supressing the electroosmotic effect of the capillary against the DNA molecules [45]. As separation is not based upon charge interaction with the capillary wall, the polymer within the capillary acts as a molecular sieve for size-based separation.

Larger DNA molecules interact with the polymer matrix more frequently than smaller molecules retarding their movement more in comparison [44, 46, 47].

Polymer characteristics have changed over the evolution of CE technology to allow multi-capillary devices to be effectively and simply run. Early size-based separation by CE utilised cross-linked polyacrylamide or agarose gels within capillaries [48], however disadvantages such as air-bubble formation, gel shrinkage, and degradation by hydrolysis led to short lifetimes for these capillaries and the movement to alternate capillary media [45, 49]. Entangler polymer solutions have replaced these cross-linked gel systems for STR CE. These entangled polymer solutions, such as un-cross-linked linear polyacrylamide [50], have been shown to give superior separation than other separation media or dilute solutions [45, 51]. Applied Biosystems supply low-viscosity, performance-optimised polymers of varying concentrations depending on the application or resolution required [52]; POP™-4 and POP™-6 are the same polydimethylacrylamide polymer at different concentrations for STR separation or single bp resolution for sequencing, respectively [45].

Collection of data is based on the emission spectra of the excited fluorescence dyes attached to DNA molecules by a laser-detector module [45]. The fluorescent dyes, which are covalently bound to the 5' end of each primer and incorporated during PCR, are excited by a single solid-state diode (e.g. 3500 series) or argon laser (e.g. ABI 310 and 3100) [53]. The multiwavelength detection capability a currently employed detector module allows increased multiplexing capacities for STR marker analysis; utilising multiple dyes allows overlapping allelic size ranges to be simultaneously analysed. Specific dyes are attached to specific primer sets and each one emits fluorescence at different wavelengths, which is subsequently detected by a camera [45, 53]. This signal is deconvoluted by internal software through comparison to a spectral matrix of the same dye systems [53]. The amount of template passing the detector is measured in RFU. In general, the greater the number of amplicon copies the greater the fluorescence observed by the instrument; therefore, it can give a rough indication as to DNA quantity within the original DNA template. The RFU measurement is taken continuously over a defined timeframe to record influxes of fluorescence from amplicons with specific fluorescent tags. As well as attaching fluorescent dyes to primers for detection and deconvolution of multiple loci's alleles, internal lane standards are injected along with the DNA sample to calibrate the size migration of the alleles present within the sample. Following the deconvolution of the dye's spectra and application of the internal size standard, to indicate fragment mass, the DNA profile is compared against an allelic ladder reference, run in a separate well within the same injection. This

allelic ladder includes all known alleles at each locus, therefore comparison of a sample profile against this offers allelic designation data [53].

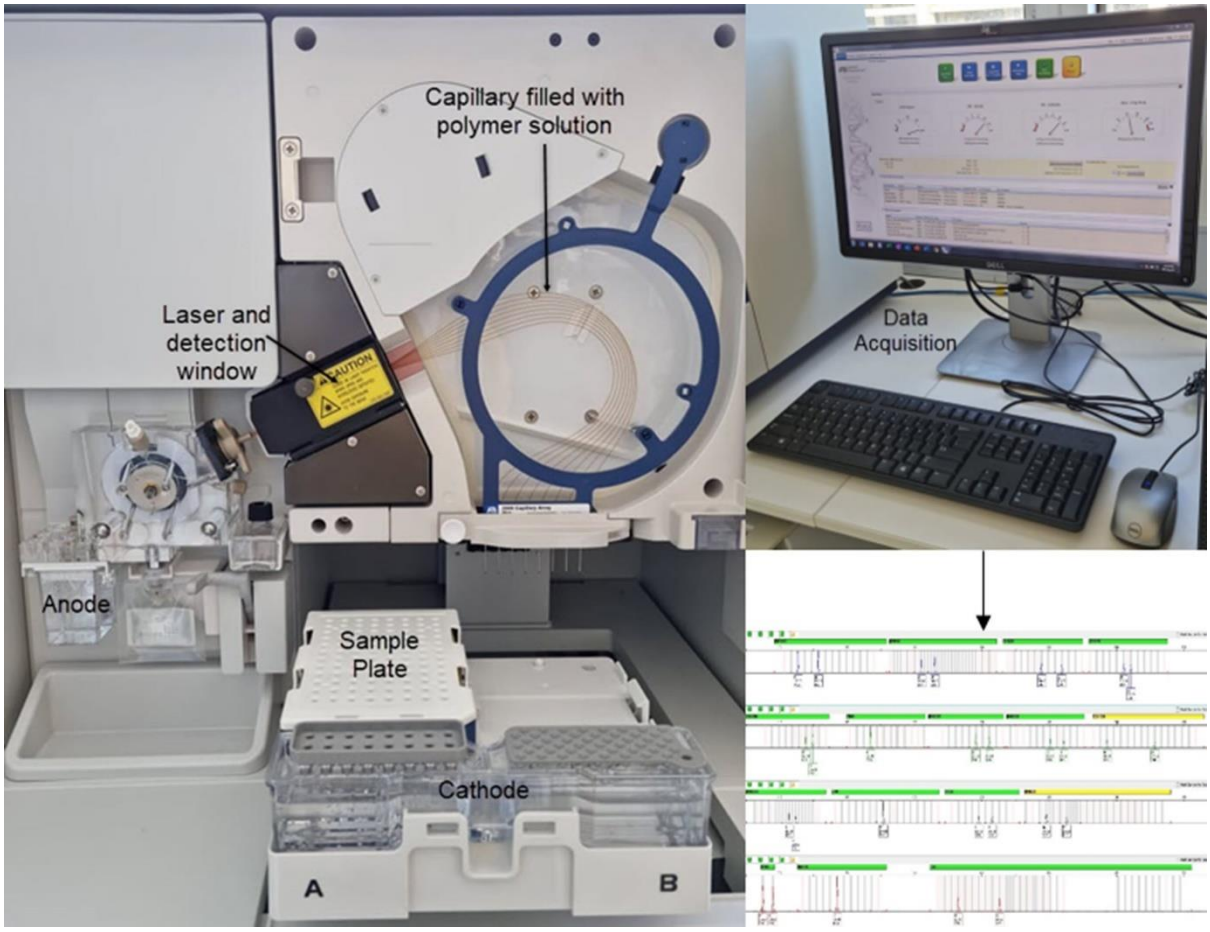


Figure 1.4. Schematic of a basic CE system. Voltage is applied between the buffer reservoirs, cathode and anode, to inject DNA molecules into the capillary, which is filled with a viscous polymer solution that acts as a molecular sieve. As migration occurs the laser/detector unit records the presence of fluorescently tagged primers. Software allows for the development of a DNA profile from this raw data.

The workflow discussed describes individual steps for extraction, PCR, and electrophoresis; all of which require manual preparation and tube changes. Recently, 'rapid DNA technology' has emerged to streamline these combined processes. This hinges on a sample, in the form of a buccal swab, being placed directly into a cartridge that contains specific fluidics and reagents to allow for extraction, PCR, and CE within a closed system without manual handling requirements. A recent validation study

between three laboratories, using the RapidHIT 200, ANDE 6C System and RapidHIT ID, found that 80% of buccal samples analysed produced full profiles with 95% of all heterozygous alleles exhibiting >59% balance [54]. Presently in the USA, under the Rapid DNA Act of 2017, the technology is only permitted for the processing of single-sources reference samples in a forensic capacity [55]. The rapid DNA technology has been applied to bone samples [56], blood samples [57], and tissue samples [58] successfully; latent DNA applications have not been as successful. A study by Hinton *et al.* (2021) describes that only 15.9% of processed latent DNA samples exhibited enough genetic data to be considered comparable to a database [59].

With improvements to rapid DNA technology, in-field applications of the user-friendly systems, which require very little training or technical expertise, will allow for a more efficient profiling workflow in DVI campaigns for repatriation of remains, and this has been shown in practice [60], and for intelligence gathering of bomb-makers in warzone campaigns with widespread use of IEDs in asymmetric warfare [59]. This system is known to perform more poorly than the highly specialised and optimised standard manual workflow [54, 55], however with niche application its value has the potential to be enormous.

1.2.7 Electropherogram Interpretation

1.2.7.1 Electropherogram data interpretation

The analysis of genetic data utilises various expert systems, depending on the type of data to be analysed. STR separation data are analysed on a platform such as GeneMapper™ ID-X, Osiris, FaSTR, or GeneMarker®, which allows for the translation of the raw data output from CE separation into a 'DNA profile' EPG, by the use of specific parameters. Different STR kits examine loci in alternate dye lanes and at different masses across the EPG. Companies supply analytical parameters that are imported into the software, like GeneMapper™ ID-X, to allow accurate analysis of amplified and separated samples. Manual alteration to generated profiles may include the inclusion of peaks that have run off-ladder (OL) or the deletion of peaks representative of artefacts (i.e. stutter, pull-up). Recently, there has been suggestion that movement to an artificial intelligence (AI) profile reader will offer more accurate interpretation of evidence profiles; this would allow increased automation within workflows also [61]. Following the analysis of an STR profile, whether generated from a reference or exhibit sample, comparison to a database that includes previously collected STR profiles ensues.

1.2.7.2 Genetic databases

Construction, maintenance, and access to a database are incredibly important concepts for the collection and use of high-quality information for assistance in criminal activity investigation. Databases can consist of any information that can be compared to other articles of the same type; for example shoe imprints, fingerprints, DNA, paint fragments, glass evidence, and ballistic samples [62].

The purpose of the database must first be established; what kind of evidence is being catalogued for subsequent comparison? An efficient way of collecting, storing, and sorting information uploaded to the database must follow, with the ability to cross-reference information between categories within a single database or between different databases; for example, DNA profiles from 'evidence' may be compared with 'reference' or 'elimination' databases. Determination of database access and sharing must also be considered [63]. There are often separate databases set up within individual forensic institutions whereby cross-referencing of DNA profiles uploaded to one database may not occur with databases elsewhere in the country; special requests are sometimes required for this to occur.

Especially true for DNA databases, concordance among reference points is required within and between databases. In 1997, the national Combined DNA Index System (CODIS) was first established in the USA, following the National DNA Database (NDNAD) becoming operational in the UK in 1995 [64]. In 1999, 13 loci were selected from the available STR kits at the time for inclusion within CODIS [10, 65]; this has since been extended to 20 loci [66]. This CODIS database allows comparisons to be made with reference or casework samples processed with STR kits from different manufacturers, providing the kit contains these concordant loci, from laboratories across the USA as well as internationally [10]. Similarly, the National Criminal Investigation DNA Database (NCIDD) was set-up in Australia to allow interstate comparability of STR data. This database outlines 18 core loci that overlap with the past and current kits utilised in casework. Both local and national databases are crucial in the management of DNA profiles and in the facilitation of cross-referencing to other profiles in the forensic sphere. The ability to search and compare an uploaded exhibit profile against previous exhibits from alternate cases often allows for a connection to be made between two crime scenes that may implicate the same DNA donor [67], sometimes years after the initial sample was analysed; sometimes comparison of exhibit or reference samples against external databases can also be useful to overcome issues of cross-jurisdictional criminal activity [68].

1.2.7.3 Profile to database matching

In a case setting the most informative outcome in a no-suspect case is when the evidentiary profile, believed to be from the offender, can be matched to an individual on a database. Evidence profiles can also be informative if they are compared and matched to other evidence profiles, both within a case or externally. If neither of these come to fruition the STR data carries no donor-identifiable features to be used in a different capacity. In the case where the profile of interest contains the same alleles as a previously analysed profile and a match is reported, specific thresholds are placed on these matches depending on the proportion of similarity between profiles the analyst desires. For investigating a supported profile match between a profile of interest and a reference sample, or previously stored profiles, a high similarity is required to reduce the number of false matches provided by the databasing software. The number of alleles required for a profile to be considered up-loadable to a database for comparison is dependent on the individual laboratory's guidelines [69, 70]. These guidelines are chosen to ensure only profiles with a high statistical weighting potential are compared against the current database. Other considerations into the number of contributors and the RFU thresholds placed within interpretational guidelines helps streamline the analytical process. When using expert systems for statistical calculations, with respect to a DNA match, the processing power of the computer system on which the analysis is being performed is often the limiting factor as to whether a sample profile should be analysed or not. Highly complex profiles, including those with greater than four contributors, can be performed using these platforms, however current technology is not able to run these analyses efficiently; for example, in Australian laboratories it is common that only profiles with four or fewer contributors are analysed against a database for match-probability calculations.

Following a DNA profile 'match', the strength of that match needs to be determined. When only part of a DNA profile can be 'interpreted' as a single source component within a mixed DNA profile or when analysing a partial profile, the likelihood of obtaining the exhibit profile if the 'matched' reference profile donor is the true donor will be lower than when analysing a full profile. For example, if a DNA profile has the potential of amplifying 10 alleles and only 5 are generated in a sample profile, information is missing for the other 5 alleles to increase the power of the sample profile to distinguish between the true donor and non-donors of the DNA. An example whereby two individuals 'match' the sample DNA profile is shown below in Table .1 (exhibit 1); both reference 1 and reference 2 are included. With this example both individuals are equally likely to have been the DNA

donor. If additional alleles are present in the sample profile (exhibit 2) more discrimination between the potential donors ensue (Table .1); reference 1 is excluded while reference 2 is included.

Table 1.2: Comparison of two reference profiles against a partial profile containing 5 alleles obtained from an exhibit (left) and the same profiles compared against an exhibit profile containing 8 alleles (right). All alleles that are present in both the sample and the donor are highlighted. Where only one allele was amplified, the second is noted as '?'; when no alleles were amplified at a locus, 'no result' (NR) is noted.

	Exhibit 1						Exhibit 2					
DNA Locus	Reference 1		Sample		Reference 2		Reference 1		Sample		Reference 2	
1	11	14	11	?	11	15	11	14	11	?	11	15
2	5	9	9	?	9	9	5	9	9	?	9	9
3	14	27	14	27	14	27	14	27	14	27	14	27
4	16	19	NR	NR	17	21	16	19	17	21	17	21
5	17	17	17	?	17	19	17	17	17	19	17	19

As non-coding regions of the genome are targeted by the standard PCR kits used to generate STR profiles, no phenotypic information about the DNA donor is able to be extrapolated from the DNA profile; this is in contrast to single nucleotide polymorphisms (SNP) analysis whereby phenotypic and ancestral information is available where no direct database match is performed. This means single STR profiles carry no informative power, they require comparison to other profiles. If no direct matches can be found to an existing profile within a database, there may be the need for a familial test to be run. Familial searching results in a large number of reported 'matches' as the similarity threshold between the profile of interest and other profiles is lowered. Depending on the relationship between individuals, they will share a specific proportion of their DNA, therefore by comparing the proportion of shared DNA between profiles potential relatives of the sample donor may be identified [71, 72].

1.2.8 Statistical Analysis of DNA Profiles

1.2.8.1 Factors influencing 'match' strength

As well as the number of alleles that match between an exhibit's DNA profile and a reference sample, the type, or repeat motif, of these alleles are also important to consider when calculating the likelihood of obtaining a profile given the suspected donor. Mendelian principles outline the way

meiosis processes randomly separate the two chromosome pairs within a diploid cell, followed by independent assortment, the crossing over of portions of the genetic sequences between chromosomal pairs, and finally meiotic separation into two gametes (Figure 1.2) [73, 74]. The chromosomes within these gametes are not of the same sequence as the chromosomes in the parental diploid cell, however the same nucleotides are present when collectively viewed.

Hardy-Weinberg equilibrium has specific assumptions in terms of the expression and assortment of allele sequence regions within a population, however it is not observed perfectly in real populations. The five principles required to meet true Hardy-Weinberg equilibrium are: random mating, infinite population size, no gene flow, no natural selection of genes, and no mutation [75]. In observed populations, however, these principles (or assumptions) are not met [75]. Random mating does not occur within human societies based on personal preferences, societal pressures upon individuals, and stratification constructs [76]. Also opposed to Hardy-Weinberg's principles, human population sizes are not infinite; this causes a level of inbreeding to occur, even if the degree of relatedness over the entire population is not high [77]. Random genetic drift is known to occur within populations, whereby allele frequencies observed within a population between two generations change by chance alone [78]. Genetic drift, inbreeding, and stratification all cause the reduction in heterozygosity within the population; this is especially true for small populations [79, 80]. Colonisation of Pitcairn Islands, whereby mutiny and conflict resulted in the settlement of 11 male and 6 females contributing to the genetic pool of successive generations, and subsequent procreation through generations is a severe example of loss of genetic diversity due to inbreeding [81]. The people of Pitcairn Island were relocated to Norfolk Island in 1846 [82], however the current Pitcairn Islander population is heavily homozygous and, even considering more recent genetic mixture by individuals who were not an original part of the population, approximately one-third of the Islanders' genomes are derived from the original island's settlers. One individual phenotyped was related to all six of the original Pitcairn Island settlers [81].

Acting against the loss of genetic diversity is random mutation whereby a new allele, or one previously lost, can be reintroduced into the population due to a mutation event [83, 84]. Allele fixation occurs over generations within small populations, especially when viable genomic mutation rates are not high. Therefore, the likelihood of observing specific alleles at each locus within a population varies depending on the factors previously listed. Population studies can be performed to determine the frequency, or occurrence, of each allele at each locus within populations [85, 86]. This

allele frequency data allows the calculation of genotype frequencies that can then be used in various statistical models to assist with evidence evaluation. These statistical tools include the likelihood ratio (LR), which is becoming the predominantly utilised tool worldwide and is described further in section 1.2.8.2, random match probability (RMP) and the combined probability of inclusion (CPI), widely used in the USA for many years. In addition to individual allele frequencies, which differ between populations and should be assigned as such, inbreeding can also be accounted for to make more conservative estimates of a match probability, and in turn the LR. This is done by applying a value, known as theta (θ, or Fst [87], or a co-ancestry coefficient), to populations based on understanding the population size and population breeding patterns to explain the potential for two randomly chosen alleles to be identical by descent (IBD) [88]. Theta is small for large populations and higher for smaller populations with increased inbreeding potential; an example of this is the Caucasian population where θ=0.01 is applied and the Native American population where θ=0.03 is applied [85].

1.2.8.2 Likelihood ratio

The LR, in its simplest form, is the evaluation of evidence given two competing hypotheses; these are to be exhaustive and mutually exclusive. When applied to a DNA match these are often annotated as

- Hp: the suspect is the DNA donor, and
- Hd: someone other than the suspect is the DNA donor.

The discrimination power in the DNA results comes from the fact that in order for Hd to be true the DNA profile of someone else in the population matches the suspected donor by chance.

When considering the laws of probability, basic equations for the probability of homozygous and heterozygous allelic observation are applied. When considering homozygous occurrence, the second law of probability is applied whereby event A and B are independent; the probability of event A and B occurring is equal to the probability of event A multiplied by the probability of event B (Pr(12,12) = Pr(parental allele 12) x Pr(parental allele 12)). When considering heterozygous occurrence, the second and third laws of probability are applied whereby events are different and distinct (mutually exclusive) from one another; the probability that either event A and B or, that event B and A will i.e. Pr(12,13) = Pr(parental allele 12)xPr(parental allele 13) + Pr(parental allele 13)xPr(parental allele 12). From these equations, and considering that allele frequencies within a population at a specific locus can differ, the following are applied to each allele when assessing a DNA

profile 'match': homozygous alleles Pr(12,12) = p (12) ^{2}; heterozygous alleles Pr(12,13) = 2p(12)xp(13). An example of calculating the probability of a homozygous and heterozygous genotype based on population allele frequency data is below (Figure 1.5); given the allele frequencies applied, the probability of observing a 12,12 (Pr = 0.025) at this locus is less than the probability of observing a 12,13 (Pr = 0.078). Considering the understanding that alleles present in the population may be IBD, theta (previously discussed) is applied to these basic equations to give the following [88]:

$$\Pr(homozygote\ 12{,}12) = \frac{2\theta+(1-\theta)p(12)}{1+\theta} \times \frac{3\theta+(1-\theta)p(12)}{1+2\theta};$$

$$\Pr(heterozygote\ 12{,}13) = 2 \times \frac{\theta+(1-\theta)p(12)}{1+\theta} \times \frac{\theta+(1-\theta)p(13)}{1+2\theta}.$$

An example of calculating the probability of a homozygous and heterozygous genotype with the application of θ is shown in Figure 1.5.

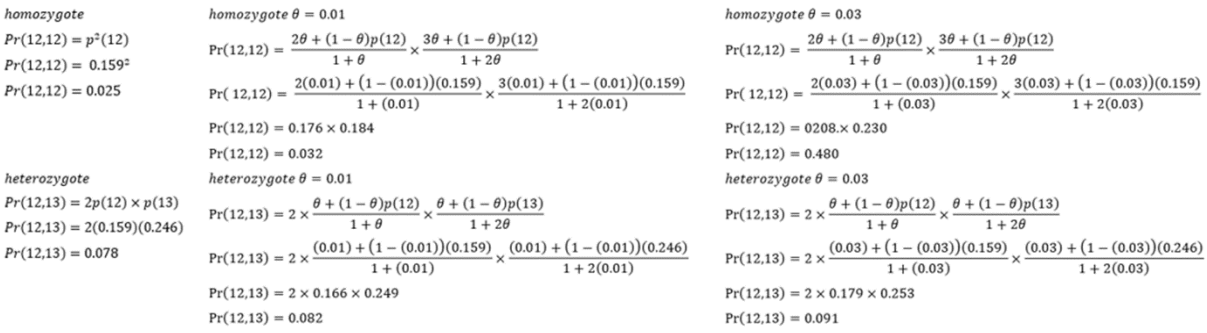


Figure 1.5. Example equations for the genotype probability of homozygote and heterozygote genotypes under (left) no kinship correction, (middle) theta of 0.01, and (right) theta of 0.03. Allele frequencies of 12=0.159 and 13=0.246 are used.

This is the method used to calculate the genotype probability of a locus and must be conducted for every locus examined. This is fairly simple when a single sourced profile is examined (i.e. a profile where there is no indication of more than one contributor), however with additional contributors the equations become more complex as additional genotype frequencies are required to be considered; a defined number of contributors is also applied [89].

The LR and the match probability are closely related. When consideration into the likelihood of obtaining a profile given the two competing hypotheses (Hp:Hd) is made with no additional information beside the evidence profile and the reference profile, the LR is the inverse of the match probability (LR=1/RMP) for complete, single-sourced profiles [89]. If we consider the match probability

of a single locus profile to be 0.032 (from Figure 1.5) homozygote with theta=0.01), the LR$= \frac{\Pr(E|Hp)}{\Pr(E|Hd)} = \frac{1}{0.032}$ = 31.25. This could be reported verbally as "The DNA profile recovered is 31 times more likely to have been obtained if 'suspected match' was the contributor rather than an unknown unrelated individual". In addition to the numerical value, a verbal scale can be attached to the strength of evidence [90]. This would be reported as "There is moderate support that the DNA profile came from 'suspected donor' rather than an unknown unrelated individual".

The benefit the LR has over the match probability is the ability to include additional information to improve the evaluation of profile observation. In addition to this, LRs can be used to analyse mixed DNA profiles and consider other complexities, such as allelic drop-in or relatedness [91-93]. There exists a number of mathematical modelling software options for operational laboratories to utilise to allow LRs to be calculated, with supplementary data generated, considering a large number of additional factors such as those previously listed. 'Case related prior odds' can be built into a Bayesian inference or network to provide more relevant statistical strength to the evidence being presented in court [94]. There are several papers that describe methods, suggested and utilised by forensic statisticians, of LR modelling or ways of constructing these networks [94-99], however this will not be covered within this chapter.

1.2.8.3 Mixtures

DNA profiles often contain allelic data from more than one individual. When this is observed, more complex statistical analyses are required to maintain the robustness of the LRs presented in a subsequent statement. As previously stated, expert systems exist to enable efficient and accurate mixture deconvolution and LR calculation.

There are many examples of expert systems utilised for the interpretation of complex DNA profiles for the generation of LRs; these use semi-continuous or continuous models. Semi-continuous probabilistic genotyping software includes the Forensic Statistical Tool (FST) [100], LRmix [101], and Lab Retriever [91]. Fully continuous probabilistic genotyping software includes TrueAllele® [102] and STRmix™ [93]. STRmix™ includes adjustments for the suspected relatedness of contributors [103], kinship factors within the population group, allele RFU, any level of degradation within the sample [104], allelic drop-out, drop-in and stutter ratios [105] for each loci independent of one another within the statistical framework with which mixed DNA profiles are deconvoluted and LRs can be assigned. The software builds conceptual STR profiles and grades these against the evidentiary profile to find the profile combinations that best explain the DNA types present in the profile. Data from

deconvolution includes other useful information such as the likely contributor contribution percentage. The software has been extensively validated for the interpretation of casework samples and an in-depth paper involving multiple laboratories worldwide was published in 2018, following the President's Council of Advisors on Science and Technology (PCAST) report [93]. Database Likelihood Ratios software (DBLR™) works with STRmix™ to calculate LRs based on different hypotheses and allows mixture to mixture analyses to be performed between samples and within/between different databases. In addition to the LR statistic presented with each of the potential 'matches', output data can include common contributors between mixtures, and expected LR ranges for a true contributor compared with a non-contributor, which can be visualised for any contributor within the profile. When the output from STRmix™ or DBLR™ is received by the forensic officer it can be easily placed within a statement for court presentation.

1.3 'Touch' DNA

STR typing is highly successful, returning full DNA profiles for database comparison, for high-quality DNA template, as previously discussed; however, many of the samples encountered in routine casework are from handled items and do not present high-quality template for collection and subsequent analysis. Thankfully, technology has advanced significantly to facilitate the amplification of low-yield DNA sources. In 1997 it was first recognised that genetic data could be produced from the DNA present in fingermarks [106-108], which introduced the potential to obtain genetic data from a new class of evidentiary exhibits termed 'touched items'; these are now commonplace in forensic laboratories [109, 110]. Comparison of a DNA reference profile from a range of cellular sources including blood, saliva, sweat, and 'touch' DNA present on an evidentiary item is possible as nuclear DNA within each human diploid cell is effectively identical, per individual, independent of cell type. Touch DNA is defined as cellular material complexed with oil or sweat residue deposited by touch or contact with the skin [108, 111, 112]. While the origin of DNA deposited via touch is still largely speculation [113] it is believed that cellular material within a touch deposit exists at various stages of degradation and is comprised of keratinocytes, corneocytes and has a cell-free component [112-116]. Keratinocytes are epidermal cells that are responsible for keratin production and the formation of the skins protective layer. Corneocytes, terminally differentiated keratinocytes, lack a cell nucleus and cytoplasm and are not viable sources of genetic material once differentiation is complete [117, 118].

1.3.1 Casework Examples

The use of the term 'touch DNA' in casework is somewhat contentious as contact mechanisms, be it primary, secondary, or tertiary etc, cannot be accurately predicted based on the amplification of genetic data from a sample. Because of this it is postulated that the presence of DNA does not indicate its donor's participation in a crime [119]; to ensure deposition activity is not implied within operational laboratory settings this template source is commonly referred to as 'latent DNA'. Due to little research surrounding the persistence of touch DNA on a range of exhibit types over time as well as the complexity of its transfer mechanisms [107, 120], much speculation over the power placed on DNA results generated from such sample types has been raised [119, 121, 122]. An example of misleading DNA results from the amplification of latent DNA is the 'Phantom of Heilbronn' case, whereby the DNA profile of a female was amplified from samples involved in over 40 cases between 1993 and 2009 spanning a wide variety of criminal activity [123-125]. This profile was not present on exhibits as a result of contact by the DNA donor, instead it was contamination present on the cotton swab collection device by a factory worker. This case outlined the importance of understanding and considering transfer mechanisms of touch DNA, as well as controlling contamination potential to reduce the occurrence of touch or latent DNA evidence presenting misleading results. Although caution is advised, touch DNA analysis has resulted in the arrest of many criminal offenders and has been used to exonerate wrongfully arrested individuals [126-128]. Some examples of high-profile cases involving touch DNA evidence are outlined below.

Investigation into the murder of Peter Falconio in 2001 utilised touch DNA obtained from the tape used to bind the victim and on the gearstick of the victim's campervan. It was analysed by low copy number (LCN) techniques to provide valuable evidence linking Bradley Murdoch to the murder. In 2005 Murdoch was convicted on the murder of Peter Falconio and the abduction and assault of Joanne Lees.

After the Omagh car bombing in 1998, touch DNA was again targeted for amplification by LCN. A DNA match was made between the tape found on the bomb timer and the accused, and Sean Hoey was charged in 2005 based on these results. In 2007 he was acquitted, however, as the evidence was challenged and deemed inadmissible in court [122].

Following the Massereene Real IRA terrorist attack in 2009, touch DNA was recovered from a seatbelt buckle, the interior of a mobile phone, and a matchstick. DNA results obtained from these items implicated two individuals known to have been involved with the Real IRA (R v Duffy and Shivers)

[129]. This evidence was considered admissible in court and was used to support the involvement of these men in the attack. In 2012, Shivers was convicted of two counts of murder, found guilty of all charges, and was sentenced to life in prison. Although the DNA evidence implicating Duffy was presented, there was no additional evidence supporting his involvement in the murders and he was therefore acquitted at a subsequent appeal.

1.4 Exhibits with Low DNA Profiling Success: Touched Items

'Touch' DNA has been notoriously difficult to generate substantial genetic data from using traditional extraction-based workflows. In 2008 the Institute of Environmental Science and Research Limited (ESR) produced a paper outlying the types of DNA exhibits examined within their facility over approximately six months, 908 exhibits in total, and the profiling success experienced in the analysis of each one [106]. Of these 908 samples amplified with AmpFlSTR SGM Plus™, 259 were designated as 'handled', which included items where touch DNA was the suspected template source, with 5% of these giving a full profile and 69% resulting in no result. This evidentiary group type was the least successful analysed [106]. Other operational laboratories have described poor amplification success from touch or latent DNA samples; Castella *et al.* (2008) noted only 26% of the 1739 latent DNA samples analysed were considered up-loadable to the Swiss DNA database [130], Raymond *et al.* (2009) reported 44% of 252 casework samples failed to produce STR alleles following amplification [131], Dziak *et al.* (2017) reported that handled items returned 10%-33% of profiles considered up-loadable for database comparison [132], the Queensland Police Service recently reported that only 9.5% of their latent DNA samples returned full profiles, however 40% of all suspect identifications were made from this template type [133], and Forensic Science SA (FSSA) reported in 2013 that amplification from drug balloons was most successful, with 56% being considered up-loadable for database comparison, and firearms grip swabs produced the poorest success with 12% generating sufficient results for database comparison [134]. Difficulty in comparing success across laboratories or studies lies in the presentation of results, whether full DNA profiles or up-loadable DNA profiles are reported, differences in database comparison thresholds and in operational methodologies used.

As cartridge cases are one such exhibit where very little or no STR data are obtained with the current post-extraction PCR, much research is being conducted into potential variations to this workflow. Recently, Bille *et al.* (2020) found that through a range of pre-rinses, utilising and retaining the run-off after each rinse, STR profiling success of casework samples was between 27%-56% being considered suitable for database comparison. This is reported to be an improvement on current

procedures employed at the Alcohol, Tobacco, Firearms and Explosives Laboratory (ATF) [135]. Other research groups have tried to amplify a swab taken from fired ammunition by subjecting it to either extraction-based PCR or direct PCR workflows with results showing no significant difference between the methods; 95% confidence intervals of 3.0–7.8 alleles per profile and 3.9–10.3 alleles per profile were obtained following direct PCR and PCR post-extraction respectively [136]. Thanakiatkrai and Rerkamnuaychoke (2019) discuss different results in another paper where they describe an average of 11.1 alleles present within the profile of fired bullets utilizing the direct PCR protocol and 5.6 alleles with amplification post-extraction [137]. It is understood that inhibition from the bullet casings, when fired, poses increased challenges to the successful amplification of STR amplicons [138]. A study conducted in 2008 compared the MiniFiler™ and PowerPlex® 16 BIO kits for their allelic amplification success from shotgun shells with no significant difference observed between the kits [138]. The area on the shotgun shell from which the sample was collected, when separated and treated independently, did show significance in the number of alleles successfully amplified; the hull was shown to be superior to the head in terms of genetic data recovered [138].

Improvised explosive devices (IEDs) are commonly utilised in terrorist attacks or wartime regimes due to their cheap and simple assembly coupled with their devastating damage. These home-made explosive devices, made using improvised materials at hand, are used by insurgent or militia groups as well as by individuals; their design, construction, and use is in contrast to mass-produced commercial explosives (personal communication). Due to the nature of the DNA template, being touch DNA in origin, the extreme conditions to which this DNA is exposed, and the presence of potential PCR inhibitors within the collected sample successful amplification of IED components post-detonation is uncommon [139], although when loaded with DNA template it is not unheard of [140]. Very limited research has been conducted into the application of recent developments, such as DNA binding fluorescent dyes and direct PCR, to the analysis of IED samples [141, 142]; samples from these studies remained undetonated. This, therefore, remains an area for further investigation to facilitate an improvement in the STR results obtained from these sample types.

1.5 Factors Complicating Touch DNA Analysis

As previously mentioned, although technically possible, many exhibits fail to produce adequate DNA profiles [41, 106, 107, 143] due to a number of complexities associated with ‘touch DNA’. The deposition of cellular material onto an exhibit and its persistence is influenced by a number of factors including the shedder status of the DNA donor, activity prior to deposition, the conditions

of the transfer event, and the time since deposition [4, 107, 144-146]. In addition to the technical difficulties surrounding the analysis of touch DNA, issues surrounding a lack of communication between forensic institutes as to best practice handling, analytical protocols, training and research has been highlighted as a limiting factor to its routinely successful use in casework [147].

1.5.1 Factors Affecting Cellular Deposition and Persistence

1.5.1.1 Shedder status of the individual

The rate at which individuals deposit, or shed, cellular material differs between individuals [109, 148-150]. Corneocyte desquamation is carefully regulated to allow for the shedding of single corneocytes [151]. It balances keratinocyte production with subsequent differentiation into corneocytes to ensure the health of the stratum corneum, the outermost layer of skin consisting of keratinocytes [152]. In addition to corneocyte production, keratinocytes can be lost from the stratum corneum to the environment through touch. Literature surrounding shedder status, the rate of keratinocyte and corneocyte deposition by touch, is contradictory with some studies suggesting age [153], gender [154, 155], and hand dominance [150, 156] are influential factors whereas other literature report these do not play a significant role [154, 157]. Several studies have also indicated that the area on the hand with which contact was made will influence the number of cells transferred [158-160], and that different locations on the body have different propensities to deposit DNA [157, 161].

1.5.1.2 Activity prior to deposition

Some literature suggests that activity prior to deposition influences the quantity of cellular deposition during the targeted touch event. Time since handwashing [149, 162, 163] and prior contact with other surfaces [2, 148] have been shown to be influencing factors, however the mechanisms as to why have not been shown definitively. To date, studies observing the effect of activity on DNA deposition have been focussed on previously cleaned, DNA-free, items. Limited work has examined activity on items that belong to the DNA donor, and thus presents the potential to pick up their own cells during contact. The presence and persistence of non-self DNA, DNA from exogenous sources carried by an individual, also varies depending on a number of factors namely surface type [164, 165], and time since deposition or interaction [166, 167]. Because of this, DNA deposited by touch can contain both self and non-self components and the proportion of each can be dependent on activity.

1.5.1.3 Transfer conditions

The surface onto which cellular material is deposited is known to affect the quantity of transferred cells [120]. Typically, transfer of cellular material to non-porous surfaces will be less than

that transferred to rough or porous surfaces The highest DNA transfer between substrates has been previously determined to be from non-porous to porous [168], as a secondary transfer mechanism. Surface type can also influence the efficiency of DNA extraction methods, with one study reporting a significantly higher DNA yield from flannelette compared to plastic [168] and another obtaining greater DNA profiling success from porous substrates than non-porous [109, 169, 170].

Although counterintuitive, it is documented that the handling time does not significantly alter the deposition of DNA onto an object [107, 108, 171]. It has been shown that the length of contact time may not play a role in DNA deposition [108], with the greatest transfer of cells occurring in the initial moments of transfer. Rather than the time associated with the contact, other transfer conditions such as pressure [145, 172], friction [145], and moisture [145] play a greater role. This was outlined in a study performed in 2007, which investigated the potential for obtaining DNA profiles from the burnt edges of street drug packages [173]. Two sealing styles were investigated, deemed 'square' and 'amorphic', with vast differences in the STR profiles observed. It was found that DNA profiles were only obtained from samples exhibiting the 'amorphic' sealing style, where 16 of 20 samples exhibited alleles, with all 19 'square' sealed samples failing to produce a DNA profile. The authors concluded that the handling differences with each sealing style influenced the deposition of DNA onto the plastic surface.

1.5.1.4 Time post-deposition

Persistence of DNA over time has been a difficult area to research in ways that are applicable to casework samples. The persistence of DNA is not only dependant on time, but the environment to which it is exposed. Conditions such as pH, humidity, UV exposure, temperature, micro-organism presence, and rain can influence the rate at which DNA degrades, is lost, or becomes inviable as template for PCR amplification [107, 174-176]. As a result, the persistence of DNA on a given item in a specific environment cannot be accurately determined; however, trends and estimations can give some guidance [109, 145, 177] as well as mock casework studies[178]. Porous surfaces have been shown to retain more DNA than their non-porous counterpart [169]. Touch DNA has decreased persistence compared to DNA within biological fluids or protected within matrices, such as bones and teeth, due to the quality of the original DNA template and mechanisms which may act to protect DNA [174, 175, 179], such as the acrosome in spermatozoa [180, 181]. Counterintuitively, a recent study found that when submerged in cold water touch DNA deposited onto pig skin was retained more effectively for DNA profiling purposes than blood not dried before submersion, [176]; when

submerged in warm water DNA within blood samples was more stable than touch deposits. A 2010 study performed by Goray *et al.*, discussed that the transfer rates between substrates differed based on the 'freshness', or time since deposition, of the cellular source for biological fluids, such as blood and saliva, while the 'freshness' of a touch deposit did not alter transfer rates [169].

After the transfer and deposition of touch DNA to an exhibit, the time between this/these event(s) and collection, as well as the environment the exhibit was exposed to can cause loss of template through degradation [131, 182]. Increased time between initial DNA deposition and seizure as evidence presents increased opportunity for the item to be handled by a second donor; this will also influence the persistence of the DNA originally present. Moreover, the final handler is not always the major contributor in the resultant DNA profile [164, 183, 184]. Loss of DNA from an exhibit can also occur in the collection, packaging, and transportation of an exhibit prior to analysis occurring [185, 186]. In addition to loss, additional exogenous DNA can be transferred to an exhibit through the handling, packaging, and examination stages of an exhibits analysis [186]

An increase in temperature and humidity has been shown to increase the susceptibility of DNA to hydrolytic cleavage which causes hydrolysis of phosphodiester linkages over time [176, 187, 188]. Fragmentation of pyrimidine and purine ring structures can be caused through oxidative damage when exposed to specific environmental factors over time [189], however it has been suggested that the time of exposure has less effect than the temperature at which the samples are stored, or left [188]. In addition to these, irradiation with UV can cause DNA to become inviable for PCR through the cross-linking of thymine nucleotides, creating thymine dimers, when adjacent in sequence [190].

1.6 Laboratory-Based Considerations for Touch DNA Analysis

As well as factors surrounding cellular deposition and loss, laboratory-based factors influence the STR profiling success of touched items. Currently, touch items are processed following the standard DNA profiling process used for other biological sample types where DNA template is more abundant. This process includes DNA collection from the substrate, using a swab or tapelift, DNA extraction and purification, PCR amplification, separation by CE and subsequent analysis (Figure 1.6). The amount of cellular material deposited by touch, including corneocyte, keratinocyte, cell-free and exogenous sources, required for successful STR amplification is currently unknown [111, 191]. In addition to this, DNA loss through inefficient laboratory processes results in a high DNA profiling failure rate being associated with touched items.

The failure to generate genetic data from latent DNA deposited by touch is influenced by the efficiency of collection from an exhibit due to surface type [192-196] or sampling device [197-200] and amplification workflow choice [3, 201, 202]. Interpretation of resulting profiles, when generated from touch DNA, can also pose increased difficulty when compared to pristine cellular sources.

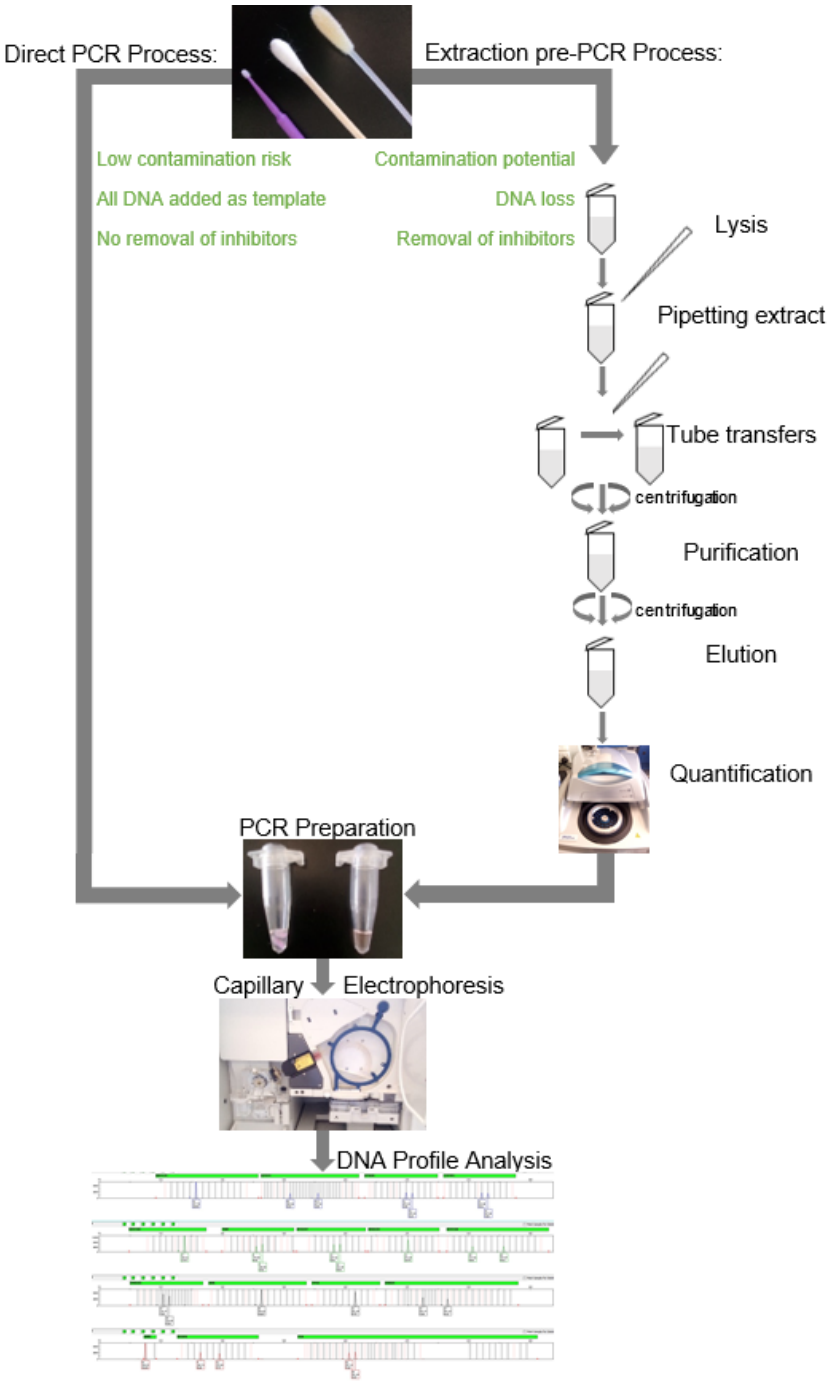


Figure 1.6. Schematic representation of direct PCR workflow (left) and standard PCR post-extraction (right). Image sourced from "Direct PCR: a review of use and limitations" by Martin and Linacre 2020.

1.6.1 Collection

DNA collection by swabbing or tapelifting may not allow for complete collection of cellular material from the exhibit or release from the sampling device for downstream processing [171, 197, 198]. The choice between swabbing or tapelifting is important as optimal DNA recovery will depend on the substrate being analysed [5, 147, 198, 200, 203]. The general rule of thumb is that DNA from porous substrates is collected with a tapelift, while DNA from non-porous substrates is collected using a swabbing technique [200, 204], while some research suggests porosity does not affect tapelifting results [133]. The swab chosen [205] and swabbing technique employed [203, 206] can also influence the recovery rate of DNA from an item. Different swab composition and fibre weave are understood to affect the collection of DNA from surfaces or the release of that DNA into solution for efficient extraction [205]. Double-swabbing compared to single-swabbing is understood to increase DNA recovery [147, 206-208]. Often the first swab is moistened to facilitate increased transfer with the second swab being dry to collect any remaining cells [207].

A study performed by Wood *et al.* (2017), examined the difference in collection and DNA extraction efficiencies between nylon-flocked swabs and cotton swabs on a range of non-porous surfaces [209]. It was determined that a greater proportion of DNA is able to be extracted through the use of the nylon-flocked swab (~85%) than cotton (~55%) when seeded with DNA. DNA yield was significantly lower from the non-porous surface and the performance of each swab was not consistent with the seeded trials. Cotton swabs resulted in greater DNA yield post-extraction for four of the five tested substrates [209]. Although there are studies that have compared collection methods from various substrates utilising the extraction pathway, the is little research that compares methods with respect to the direct PCR pathway. This needs to be addressed if the direct PCR technique is to be effectively integrated into casework analysis.

1.6.2 Amplification

As success in the analysis of touch, or latent, DNA samples through standard workflows is poor [106, 131, 210], there are alternate processing methods that have been explored to enable genetic material to be amplified at increased rates compared with standard STR PCR conditions. These include the use of mini-STRs [211-218], which designs primer positions closer to the targeted repeat motif than standard kits, or increasing the number of PCR cycles such as in LCN PCR [219-222].

The use of mini-STR systems offers the forensic community the potential to generate increased genetic material from samples with low quantity or quality DNA compared with the core

STR systems currently used [223]. This involves the re-engineering of primers to reduce the length of the flanking regions around the repeat units within an amplicon to amplify smaller target regions across the genome [223]. Although this has been shown to increase the alleles generated within resultant STR profiles from low-level DNA samples [218], the requirement of specialised kits for certain sample types makes incorporation into current casework-based forensic laboratory workflows difficult. The currently employed STR kits have altered their primer sequences to allow highly informative loci to be amplified as smaller amplicons and loci with less informative strength to be larger; if samples are degraded or of low template, amplification of highly informative markers is preferential over larger amplicons of the less informative markers.

An alternative to altering the STR kit utilised is to increase the number of cycles performed within a PCR amplification protocol. This is conducted in a technique known as LCN and allows for increased amplification of amplicons of target regions that may be present within a sample in low quantities, which would otherwise be unable to be amplified to a point in which subsequent CE can detect its presence and record a peak [224]. In addition to this, concentrating the eluted template DNA prior to performing PCR can increase the success of amplification. This can increase the genetic data obtained through analysis, when compared to amplification and separation of the standard eluate, however sample volume loss ensues [225, 226]. Increasing CE injection times or voltage can also increase the genetic data obtained through separation by increasing the number of molecules of each amplicon present in the capillary for detection [41, 227].

For more information on LCN amplification see enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

1.6.3 Profile Interpretation

The process of PCR as well as CE separation can cause increased stochastic effects or artefacts in resulting STR profiles from touch DNA. These can cause complications in profile analysis through the incorporation of false peaks or masking of true alleles.

1.6.3.1 Sample-related complications

Many of the anomalies or difficulties experienced with DNA profile interpretation can be caused by multiple factors. Sub-optimal DNA input, due to PCR inhibitory factors co-extracting with DNA template, DNA degradation, or low-template concentration can influence the quality of the resulting STR profile or exacerbate issues observed due to PCR or CE separation.

Degradation of DNA is not uniform across the genome. Because of this, heterozygous alleles from the same locus can experience different levels of degradation and subsequent PCR may result in peak imbalance; when this is severe, drop-out can occur. Degradation is often identified where as a 'ski-slope' is observed in the STR profile. The smaller amplicons' amplification is less likely to be impacted by degradation than larger amplicons' [218]. Therefore, the alleles of loci with a larger target sequences, producing amplicons of higher mass, often experience little or no amplification while those with smaller target sequences amplify successfully. In addition to drop-out and imbalance, degraded and low-template DNA sources experience elevated stutter ratios [228].

Inhibition to PCR is observed in a similar way to degradation, where inhibition to amplification is not complete. Drop-out, the 'ski-slope' effect, and peak imbalance can all be observed in STR profiles of DNA samples with PCR inhibitors present. These are explained below.

Microvariants are alleles that are not present in much of the genotyped population and exists as sequence variations between STR alleles commonly encountered. These rare microvariants can be in the form of insertions, deletions, or nucleotide variations [229]. They can appear on the EPG as OL alleles, and can easily be identified in heterozygous loci where one allele is aligned with the allelic ladder while the other is not [229]. In addition to this, the allele will continue to run OL at the same location if subsequent reruns of the sample are performed.

1.6.3.2 PCR-related complications

Artefacts are deformation of the observed DNA profile through the addition, removal, or modification of peaks in a way that means the EPG becomes less representative of the DNA present in the sample. Artefacts of PCR include incomplete adenylation, stutter, allelic drop-out, heterozygous imbalance, and null alleles.

During the amplification process the polymerase adds a non-template base to the 3' end of the amplicon. This is often an adenosine and is therefore termed adenylation in STR amplification [229]. When excessive template is present, or the efficacy of the polymerase is reduced, incomplete adenylation may occur. This will result in two peaks, or split peaks, being observed in the EPG where only one allele exists: the +A and -A forms [230]. This can become a complication when the profile contains multiple contributors or where microvariants are known to exist within a locus as the peak may be incorrectly determined to be a true allele. Where split peaks are observed and easily identifiable as such, the allele designation should be taken as the +A form (peak to the right).

Stutter is one of the most commonly experienced artefacts resulting from the PCR process [231]. Strand slippage by the polymerase during amplification can cause the amplification of an amplicon one repeat unit in excess of the true allele, while when the template forms a hairpin structure the polymerase skips one motif causing a deficit in amplicon length [229, 231-233]. 'Back' stutter is the most common form, whereby the resultant amplicon is one repeat unit smaller than the true allele; the alternative being 'forward' stutter [234]. This artefact due to a template hairpin formation or strand slippage occurs on both pristine and degraded template, although stutter ratios will generally be higher with degraded template amplification [235]. Both commercial companies and internal laboratory validations inspect stutter ratios, as the ratio comparing the RFU of stutter peak to the true allele, for each locus. As a general rule, a peak can be determined to be stutter if it is less than 15% of the parent peak [229, 231] (Figure 1.7), however each locus has its own stutter ratio constraints.

Allelic drop-out is commonly observed in STR profiles due to low-template concentrations, degraded DNA template, or PCR inhibition [229, 236]. It can be experienced as either a missing peak or a peak of RFU below the analysed threshold. Complications from allelic drop-out include incorrect homozygosity classification, loss of differentiation between profiles, and increased estimates of inbreeding [236, 237]. Loss of genetic data through allelic drop-out also reduces the LRs applied to matches performed against databases. Allelic drop-in can be described as extraneous alleles present in the profile that remain unexplained by the DNA template present [238, 239]. Both allelic drop-out and drop-in probabilities can be estimated [239], and allowances for these phenomena can be made under probabilistic modelling [91, 238].

'Null allele' is the term used to describe the failed amplification of a present allele by the primer sets present within the PCR multiplex system; it is a form of allelic drop-out. When there is a mutation within the primer binding site on the template strand, often at or close to the 3' end, the allele may fail to amplify due to the primer's inability to bind [229, 230]. This can cause complications whereby a heterozygous locus may be incorrectly assigned as a homozygous or no locus data is obtained. Large concordance studies between different kits for the same DNA samples can identify the presence of null alleles. Alteration to primer sequences and incorporation of degenerate primers into multiplex systems can reduce the occurrence of null alleles [229].

Where mutations, or sequence polymorphisms, exist within the primer binding sites of template DNA but they do not completely restrict the primer annealing to the template, inefficient

binding may occur whereby primer annealing occurs some but not all of the time. When this happens on one allele at a heterozygous locus, the allele with complete primer-template concordance will amplify at a far greater rate than the target region whereby primer-template concordance is not complete. This can cause peak imbalance, where one peak at a heterozygous locus exhibits an RFU value much lower than the other [229]. PCR inhibition, DNA degradation, and low-template sources are also common causes of peak imbalance [10, 230]. Guidelines for imbalance varies between laboratories, however a general guideline has been suggested as a heterozygous peak height ratios of <70% being considered imbalanced [10, 230]; as the second peak's presence may also indicate a second contributor, with at least on donor being homozygous, the quality of the profile as a whole must be determined (see Figure 1.7). Modern DNA profiling evaluation systems, such as those detailed in 1.2.7.3, contain models for these artefacts and treat them probabilistically. This removes the need for homozygosity consideration and heterozygous peak balance thresholds.

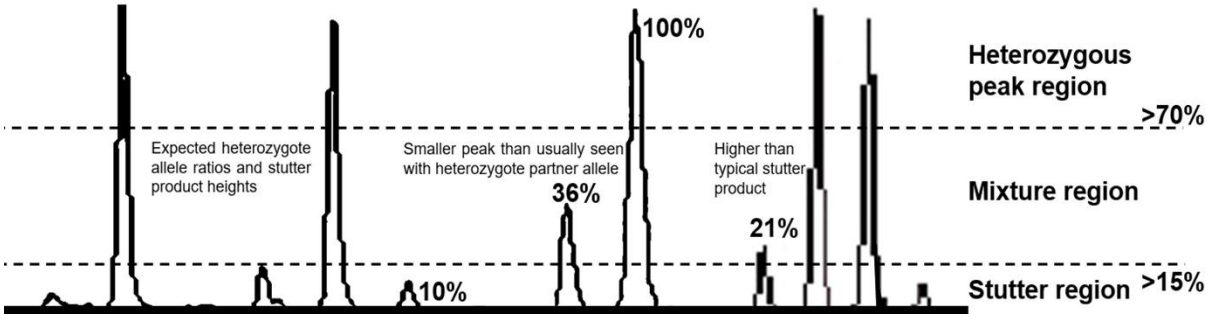


Figure 1.7. Illustration of stutter, mixture, and imbalance designation guidelines for (a) single sourced profile, (b) mixed DNA profile, and (c) a single sourced profile experiencing elevated stutter. X-axis indicates peak mass while y-axis indicates RFU. As perfect amplification of heterozygous alleles would result in identical peak heights (RFUs) and no stutter, these RFU values can be useful in determining the quality of a DNA source or the number of contributors to a profile.

1.6.3.3 Chemistry and instrument related complications

Matrix failure, or pull-up, is often observed when excessive input of the amplified sample occurs [233, 240]. When oversaturation of the detector occurs, the instrument is unable to accurately resolve the fluorescent dye used to label the STR amplicon. As a result, the instrument records the fluorescence at another wavelength, and another colour is 'pulled-up' in the resulting profile. This is identified where a peak is observed at exactly the same mass designation as another of far greater height, often in a dye lane of similar spectral range.

Sharp peaks present at the same location in all dye lanes are typical of voltage spikes [233, 240, 241]. These are an anomaly of the injection, therefore if the sample is reinjected and analysed again these peaks should not be observed. Dye blobs can be observed as broad peaks on the EPG, often at the smaller end of the profile. These are caused when the fluorescent dye molecules become disassociated from their respective primer and migrate through the capillary independently. Contaminants within the injected sample can also cause false peaks to be present within a DNA profile when they possess fluorescent properties at the same wavelengths as the dye matrix utilised. These also often appear as broad peaks, with a similar morphology to dye blobs, and can often be reduced by alternating the extraction protocol conducted [240].

In addition to these, background noise may be increased by a number of factors. Current fluctuations, air bubble presence, urea crystals within the system, and sample contamination can increase the baseline noise in some samples [240]. On occasion these may cause a peak observed on the EPG due to noise to be confused with a real allele. Alternatively, if the baseline is suffering from increased noise, or the sample injected is of poor quality, real alleles may be mistaken for background noise. As true alleles should be reproducible upon re-injection, while the noise profile is not, one method for determining noise vs allele is to re-run the sample [241].

OL peaks can be observed were an interruption in temperature stability occurs [230]. When temperature increases, molecules move through the capillary faster, therefore alleles will be recorded as being smaller than their true mass. If the allelic ladder is compared with samples that have experienced temperature fluctuations, the comparison between mass and allelic designation will not be accurate and a peak may be labelled as OL. Electrophoretic mobility variation of the DNA molecules relative to the internal size standards can cause OL designation. In addition to this, OL peaks can be caused by the presence of microvariant alleles; these are, however, true alleles [229].

1.7 Direct PCR

To overcome the loss of DNA associated with low quantity DNA samples, and thus increase the STR data generation for latent DNA analysis, protocols that circumvent the extraction step prior to PCR, termed direct PCR, have been explored. The concept of direct PCR was not devised for the purpose of forensic examination. The technique, whereby a portion of a sample is placed directly into a PCR was first used in colony PCR research [242-246]. Cultures from various growth mediums, such as bacteria or yeast, would be scraped and placed into a PCR for amplification without carrying out an extraction process. It was found that PCR amplification was successful, and with the elimination of the

extraction protocol the time and financial resources required for these research projects were reduced. The application of direct PCR to reference samples is not uncommon in forensic practice [247-250]. Body fluids such as blood, semen, and saliva have also been successfully amplified directly using both standard STR kits [251-254], specially designed direct PCR STR kits [255] following immersion in a specialised buffer [256, 257]. These direct PCR STR kits utilise an initial submersion into a buffer system designed to aid in the release of DNA and reduce the effects of any inhibitors present to the PCR amplification process [255].

The direct PCR workflow, as the exhibit or sample taken from the exhibit used directly in the PCR, eliminates the DNA loss caused by the extraction step, however any PCR inhibitors also remain. This is different to utilising a direct PCR STR kit available from commercial suppliers, such as Identifiler® Direct, as these methods include wash or mini-extraction steps. Many studies have been performed comparing the direct PCR and post-extraction PCR workflows on a range of casework style samples and have found increased genetic data has been amplified from samples that underwent the direct PCR workflow [143, 193, 258]. The use of an STR kit in which a hot-start enzyme is utilised may aid in the success of subsequent amplification when compared to kits with non-hot start enzymes [254], and the addition of surfactants, that do not post any potential interference with the PCR process, could be investigated to aid in the disruption of the cellular membrane and release of DNA into the PCR solution for amplification.

For more information on the successful amplification of latent DNA samples utilising the direct PCR workflow see enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

The application of direct PCR to casework samples was first demonstrated by Linacre *et al.* (2010), who successfully applied direct PCR to amplify human touch DNA from fibres typical of fabric encountered in forensic examination [259]. Since this initial study, direct PCR has been tested on a wide range of substrates typical of those encountered in forensic investigations including fingernail or toenail segments [260, 261], hair [262, 263], and latent DNA from fabric, plastic, and glass surfaces [3]. Templeton *et al.* showed that through sampling a single fingermark, deposited under controlled deposition conditions, full STR profiles were able to be obtained from glass slides [264], and plastic slides [199, 264] with profiles considered up-loadable to a DNA database, whereby ≥12 autosomal alleles is the guide. Direct PCR has also been trialled on wood, masking tape, unfired nickel and aluminium cartridges but has shown variable success [264]. Since these initial studies, direct PCR has

been applied to amplify latent DNA in a broad spectrum of research [136, 141, 142, 196, 265, 266], including from exhibits where amplification post extraction is notoriously difficult such as with bullet cartridge casings [136] and IED components [141, 267]. Due to the understanding that surface type influences both the persistence and recovery of DNA from an exhibit, increased analysis into alternative surface types are required to establish a body of knowledge that more accurately encompasses exhibits observed through forensic casework.

For more information on the use of direct PCR on forensic exhibits, see the enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

1.7.1 Benefits of Direct PCR

Direct PCR, by which a portion of the exhibit or a sample taken from said exhibit is placed directly into the PCR mix, has shown greater amplification of STR amplicons than post-extraction PCR for many low template DNA source samples [3, 143, 193, 258]. This has enabled template sources of low DNA quantity to be profiled where no, or little, STR data would be obtained when using traditional extraction-based workflows. With increased research into the types of substrates and DNA sources to which this technique is suited, the collection method best suited to these cases, and specific investigation into the relationship between direct PCR and extraction-based PCR workflows for these cases operational laboratories will be better equipped to incorporate direct PCR into their workflows where applicable and advantageous.

1.7.2 Limitations of Direct PCR

A few commonly mentioned limitations of the direct PCR technique within literature are as follows: as no extract pool exists subsequent testing cannot be performed on the sample [204]; quantification data are not available [204, 253]; and due to its increased sensitivity, inferences as to the applicability of results to a case can be challenging to make. Where current accreditation or internal requirements require quantification data to be collected from a sample prior to STR amplification, internal validation of the direct PCR technique under specific sample or case requirements/considerations would need to be performed. This would allow the workflow's integration into practise for the chance to improve profiling results from sub-optimal sample types or template sources.

For more information on the benefits and limitations of the direct PCR technique, see the enclosed manuscript Martin, B., Linacre, A. (2020) Direct PCR: A review of use and limitations. *Science and Justice*.

1.8 Scope of Thesis

This thesis is written as a series of publications published in relevant journals or as additional studies not written in this format when publication was not appropriate. The work is not presented as it was chronologically performed throughout candidature, rather as collections of work in themes. These themes, described below in 1.9, explore and answer different caveats within the literature surrounding direct PCR and its use for trace DNA amplification enhancement. As the works are not presented chronologically there are some instances where methodology and phrasing changes in oscillation rather than in an evolving singular direction; this has been explained where pertinent.

Sampling methodology within this work includes the use of three types of tapelift; DNA-free Lovell, 2.5 mm Sellotape; and Packmate packing tape. The choice of tape within a given study was based on the purpose of the study. DNA-free Lovell tape was utilised where methods were to be directly translated into operation practise as being a certified DNA-free tape this is the choice of many laboratories. Sellotape was utilised when a study was more heavily focussed in a research capacity; it was found to be DNA-free, however it is not certified. Packmate packing tape was used in only one study. This tape presented the least background under nucleic acid staining and microscopy of any tape trialled; the selection of this tape was purely for cellular visualisation aid in the study.

The use of the phrase “touch DNA” within the thesis refers to donor corneocytes deposited by touch. This is used when a known donor touches an item and leaves their DNA within the deposition. It can constitute all or a portion of the latent, or trace, DNA template present on the item, which can comprise additional cells such as donor epithelial cells and non-donor cells. As such, ‘latent DNA’ is more appropriate in the wider forensic sphere as the deposition mechanism is not usually definitively known as primary or by touch; however, for the purpose of the work presented in this thesis, where only donor DNA deposited by touch is considered, ‘touch DNA’ is also appropriate and is interchangeable when the cell-source is known.

Similarly interchangeable language is observed between the phrase ‘informative profile’ and ‘up-loadable profile’. In the truest sense, the use of ‘up-loadable’ is more applicable as a forensic standard in literature as the terminology ‘informative’ can imply that a profile that does meet the

desired threshold, ≥12 autosomal alleles in this body of work, has no informative value. This is not the case as profiles that present fewer alleles than required to be considered up-loadable to a DNA database can present intelligence value. When 'informative profile' is used within this thesis it is in reference to its ability to be up-loaded to a database, rather than any comment as to its value in an intelligence format.

1.9 Aims of Thesis

This thesis aims to build on the current understanding and use of direct PCR for amplifying 'touch' DNA. Within this work, the ability of direct PCR to amplify 'touch' DNA has been explored on a range of exhibit types which previously result in poor DNA profiles success. The main goal of this research is to provide a substantial resource that correlates sample type and collection and processing mechanisms with DNA profiling success using direct PCR analysis to aid operational laboratories in triaging casework exhibits to maximise DNA recovery. To achieve this goal peer reviewed manuscripts were compiled into the following chapters:

Chapter I: Introduction Chapter

This chapter explains relevant concepts and background literature pertaining to the body of the thesis. This chapter includes one published paper written as a review of direct PCR within the field of forensic science.

Chapter II: Optimising Methods for Direct PCR Implementation

This chapter aims to address questions within the current body of knowledge pertaining to the application of direct PCR to the analysis of latent DNA deposited by touch. A comprehensive analysis of commercially available STR kits will provide empirical data to operational laboratories for educated conclusions to be drawn as to the potential for direct PCR to be utilised within their facilities in specific cases. In addition to producing data surrounding STR kit choice and application, the collection of latent DNA will be investigated on a range of forensically relevant samples. It has the following aim:

"To provide empirical data from which laboratory groups can make educated decisions as to the ideal STR kit to utilise within their direct PCR workflows"

This chapter includes: one manuscript draft, and two poster presentations.

Chapter III: Exploring Factors Influencing STR Amplification Success from Touch DNA Deposits

This chapter explores factors that may influence the success of downstream STR profiles pertaining to touch deposits. This includes research into the amplification of DNA utilising direct PCR on a range of forensically relevant substrates, and investigation into the relationship between cellular deposition and DNA profiles. In addition to exploring the relationship between touch DNA analysis with traditional extraction protocols and direct PCR, analysis into emerging rapid DNA will be conducted to determine where this technology sits with respect to DNA data production from latent DNA samples. It has the following aims:

"To investigate further the viability of the direct PCR amplification workflow to a wider range of exhibit surface and sample types"

"To identify whether a relationship exists between the shedder status of an individual and the expected quality of an STR profile once a fingermark is processed"

"To discover the visualised cellular material thresholds at which an informative and full DNA profile are able to be obtained given different cell types and workflow choices"

"To analyse the potential use of the RapidHIT system for the amplification of latent DNA samples"

This chapter includes: four peer reviewed publications, and two poster presentations.

Chapter IV: Reproducibility in Direct PCR

This chapter addresses issues of reproducibility within the direct PCR workflow. This is a major limitation to the uptake of direct PCR within forensic laboratories where accreditation or internal guidelines require multiple tests to be able to be performed. To somewhat resolve this issue tapelifts will be utilised to test their capacity at facilitating sub-sampling for replicate analyses or use in multiple workflows. In addition to this, a comparison between swab and tapelift data acquisition from the same substrate types is performed to address the concern raised in 1.5.1 to provide a reference tool for best practice for operational laboratories. It has the following aims:

"To determine whether the generation of multiple concordant profiles is possible from a single tapelift sample following direct PCR amplification"

"To investigate the potential of obtaining data from both extraction-based PCR and direct PCR from a single tapelift to facilitate a more seamless introduction of direct PCR into laboratories that require a sample to be re-testable"

This chapter contains: one peer reviewed publication.

Chapter V: Application of Direct PCR to Sub-Optimal Sample Types

This chapter extends the application of direct PCR to a range of historically challenging sample types, namely matchsticks and bullet cartridges. The crimes committed with these sample types can be widely devastating, and improvement to the DNA data production possible from these exhibits will translate to a higher possibility of suspect identification. The data presented within this chapter pushes the scope of currently analysed samples wider than previously possible. This chapter has the following aims:

"To determine whether direct PCR is a viable alternative to LCN and extraction-based PCR workflows for the analysis of struck matchsticks"

"To examine the application of direct PCR to the examination of spent ammunition in comparison to other methods"

This chapter includes: two peer reviewed publication, and one poster presentation.

Chapter VI: Analysis of DNA Following Extreme Conditions; Post-Detonation Processing

Following the data presented in Chapter V it follows that the techniques and technical expertise gained through this candidature should be applied to samples with the highest stressed prior to collection and amplification. To fully test the boundaries and capabilities of optimised direct PCR, post-detonation improvised explosive samples will be processed. These data will provide response teams with triaging information and the technical specialists guidance as to potential success or failure rates around specified variables. This chapter presents two major bodies of work and addresses the aims:

"To investigate the potential for direct PCR to provide informative STR data from post-detonation IED samples"

"*To investigate the difference an outdoor, dirty, environment has on direct PCRs capability to provide informative STR data from post-detonation IED samples*"

This chapter includes: two peer reviewed publications.

Chapter VII: Concluding Remarks and Further Applications

Chapter VIII: Appendix

This chapter includes the published and unpublished bodies of work I was involved in during my candidature that are outside the scope of this thesis as well as proof of training and peer review participation. This chapter includes: two peer reviewed publications, one poster presentation, and one internship report.

Chapter IIX: References

1.10 Manuscript: Direct PCR: A review of use and limitations

Manuscript published: *Science and Justice*, 2020

Statement of authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**80**	-	-	**100**	**80**		03.08.21
AL	20	-	-	-	20		05.08.21

1.10.1. Introduction

Direct PCR has been utilized for sample amplification for many years in various biological fields, however it has only recently been applied in forensic science to evidential [259] or reference samples [247, 248]. The concept of direct PCR is simple: any material collected, by whatever means, is placed directly into the amplification reaction without prior treatment and then subjected to PCR. The purpose of direct PCR is to increase the available DNA template for amplification by omitting the extraction step, where it is recorded a loss of DNA up to 83% can occur [2, 3, 201, 259]. A comparison of the workflow involved with each technique is shown in Figure 1.6. Direct PCR as discussed within this review refers to exhibits or samples that are directly added to the PCR amplification; this is different to the workflow followed using 'direct PCR' STR kits, which involves pre-processing prior to amplification.

Since the first application of DNA profiling in forensic science (then termed DNA fingerprinting) for the resolution of an immigration dispute [18], the laboratory procedure for DNA analysis has involved an extraction process to isolate and purify DNA from the other cellular components that might either inhibit the restriction enzyme or, since the 1990s, the DNA polymerase. The composition of cellular material in substrates such as blood, where the haemoglobin binds molecules that carry a negative charge, spermatozoa, which are coated in an acrosome protecting the DNA from enzymatic action, or within hairs, where the DNA is surrounded by proteins such as keratins, have presented inhibition to the PCR process: this presented a requirement for an extraction process to allow these types of substrates to be analysed successfully. The process by which DNA has been analysed has therefore always been initiated by a DNA extraction step to ensure that any subsequent

laboratory-based methods have the best chance of success and are not affected by any potential inhibitors.

The process of DNA extraction is commonly implemented to purify the DNA within a sample taken from cellular material such as saliva, blood, or semen, to a state where any inhibitors to the PCR are no longer present [268-270]. Extraction processes also work to release DNA for PCR template from within more solid biological matrices such as fingernails, hair, bone and teeth [271, 272]. Benefits of automation of DNA extraction methods for high throughput laboratories include consistency in processing, rapid throughput, reduction in staffing requirements and contamination potential, as well as the removal of associated human error. The end result of a DNA extraction is that there is a sample pool enabling multiple PCRs to be performed on the same DNA sample. Although the process of DNA extraction and isolation has advantages with respect to the removal of inhibitors and in quality control, the significant loss of DNA, reported to be up to 83% [2, 3, 201], can reduce the potential for DNA profile generation for samples where initial template may be sparse. Quantification of DNA within a sample, for accuracy of downstream processing, requires an extracted sample pool. Quantification data are advantageous in reducing the financial waste associated with STR profiling of samples with limited or no DNA template, as well as recording the quality and DNA concentration of the extract. As it is a requirement of many laboratories to perform DNA quantification on routine samples, due to internal or accreditation requirements [273, 274], DNA extraction became a necessary part of the sample analysis process.

An amplification method previously used to increase the sensitivity of the PCR process involved increasing the number of PCR cycles [219, 222, 275]. LCN was introduced as an exhibit STR analysis workflow for the purpose of low-level DNA analysis [276].

Increasing the cycle numbers within PCR leads to an increased amount of PCR product such that alleles may be recorded when no results were obtainable with the standard number of cycles. In increasing the amplification of alleles there was also an escalation in stochastic effects within the resultant profiles, such as allelic drop-out and increased stutter, thus the complexity of analysis increased [220, 221]. To counter allelic drop-in and drop-out in particular, LCN was performed in either duplicate or triplicate reactions and only alleles that occurred at least twice were confirmed as true alleles. Although LCN results have been used in a number of cases, and is supported by the Caddy report [277], LCN has not been implemented in many operational forensic laboratories as it is not considered admissible within many legal systems [221, 278-280]. Validation and interpretational

difficulties, as well as the requirement for an ultra-clean laboratory, had been other barriers to this technique's use for casework samples. Maximising the available DNA template and improving collection techniques presents an alternate method for the analysis of low-level DNA without the need for increasing cycle numbers, thereby reducing the stochastic effects observed in LCN [266].

Touch DNA samples, being cellular material in the form of keratinocytes or corneocytes transferred to a substrate by direct contact with skin, are commonly submitted as evidence for forensic examination. Although the touch DNA itself is free from many known inhibitors to PCR, such as haemoglobin, bile, polysaccharides, calcium, phenols and humic compounds [269], the touch deposit may include additives from the environment or additional DNA sources, such as exogenous nucleated cells [113], that contain elements that co-extract with the touch DNA that may hinder subsequent amplification [1]. Touch DNA is therefore a potential template for the amplification process without the requirement for purification, or release from a protein coat, by an extraction process. Although the first application of DNA profiling from touch DNA followed the standard processes of DNA extraction, followed by quantification, and then amplification of STR loci [108], the lack of inhibitor presence within the touch deposit itself was the premise behind the first use of direct PCR from a touched item [259]. The application of direct PCR to the forensic analyses of evidentiary samples, circumventing the DNA extraction process, ensures that the entire mass of DNA within a sample is transferred to the PCR. This increases the probability that a DNA profile can be generated from trace material in such a way that the resulting DNA profile is able to be compared to a reference DNA profile. Direct PCR also decreases the analysis time of a sample and the associated extraction costs such as commercial kit purchasing, equipment upkeep and staffing. Additionally, fewer tube changes within and between stages also reduces the opportunity for contamination or sample confusion [193]. Prior to workflow choice for a given exhibitory sample, consideration must be taken into the quality or type of sample being analysed, whether it is free from most inhibitors, the ability of the buffer to overcome traces of inhibitors and the chosen enzymes activity.

A major factor affecting the strength of the resulting STR profiles from touch DNA analysis is the shedder status of the individual depositing their DNA. Many studies have found that a person's ability to deposit DNA differs between individuals. It has been suggested that factors such as age [153], handedness [155] or gender [281] may play a role in an individual's propensity to deposit, or shed, DNA. Since the introduction of the concept of a good or poor shedder [149], there have been varying results as to the relevance of individual designation into categories [150, 282].

1.10.2. Introduction of direct PCR to forensic science

Direct PCR has been common practice in the analysis of bacterial cultures for decades. Apart from within this field, it had not been implemented in other research work prior to 1993. In 1993 Panaccia *et al.* presented the first description of direct PCR amplification from tissues including liver, brain, testes, ovaries, heart, kidney, skin and skeletal muscle [283]. Here they concluded that their direct PCR protocol has applications in genetic analysis of tissue biopsy samples, origin determination of meat samples and in forensic science. Blood has also been able to be amplified using direct PCR since the 1990s [284].

When considering forensic applications, DNA embedded in FTA™ paper after the deposition of blood or saliva was successfully amplified without the requirement for purification [257, 285, 286]. In two studies [257, 285], DNA profiles generated from blood samples amplified directly showed no locus drop-out and were considered identical to those obtained post-extraction with respect to criteria such as peak balance, artefact presence and preferential amplification. Their methods utilised commercially available primer sets, such as AmpFℓSTR Identifiler®, AmpFℓSTR Yfiler™, or Powerplex®Y, with an alternate buffer mix. Although amplification of loci was successful, the use of alternate buffer mixes can complicate the incorporation of direct PCR methods into laboratory workflow where adherence to accreditation standards is strictly controlled and validation of new techniques for use on forensic samples can be a lengthy process. Kline *et al.* amplified FTA™ card punches with AmpFℓSTR COfiler following the standard manufacturer's protocols for PCR. Amplification following pre-treatment wash steps, to reduce inhibitors within subsequent amplification, was performed with results indicating strong correlations, with respect to standardized average peak area, between amplification post-extraction (Chelex®) and direct amplification post-pre-treatment [286]. Some commercially available STR kits, deemed 'direct PCR' kits, now incorporate an elution or treatment step into their validated protocol to allow amplification of samples without extraction [249, 255, 257, 287, 288], however recent direct PCR protocols have circumvented this step also [143, 259, 260, 262, 264, 266]. Limited literature has compared 'direct PCR' STR kits with their counterpart utilizing the direct PCR workflow, as discussed here (no pre-processing), however Martin *et al.* concluded that Identifiler® Direct did not perform as well as Identifiler® Plus when examining touch DNA samples [289].

As there is no specified requirement for quantification of casework reference samples under some laboratory guidelines, providing the laboratory has a validated system by which to analyse these

samples without prior quantification, direct PCR has been incorporated into some laboratory workflows for reference samples to allow faster [290] and less expensive [196] analysis turn-around for reference samples. In the absence of quantification data there is the potential for excessive DNA template within an exhibit or reference sample to be amplified. This may cause an increase in the occurrence of stochastic effects, pullup, and split peaks [247]. Some of these effects can be reduced by decreasing the time used for the injection of the PCR products into the capillary or by reducing the amount of PCR product loaded for separation [249, 263]. When considering trace or touch DNA, excessive DNA template is unlikely to be acquired from the given exhibit; although this problem of overload was reported when analyzing nails by direct PCR [260]. ABO typing through SNP analysis has also been performed by direct PCR from blood samples and was determined to be an efficient, accurate, and cost effective way of obtaining blood group information from a sample [291].

1.10.3. Low level DNA analysis

Trace latent DNA samples, which undergo a DNA extraction process prior to PCR amplification, contain, by definition, low levels of DNA and have a low success rate for producing informative DNA profiles. The term informative profile refers to a profile a laboratory would consider up-loadable to their justice database for comparison; the number of alleles required to deem a profile informative is dependent on individual laboratory's guidelines.

The ESR, NZ, do not process many of the touch samples submitted to them, due to poor success rates, as time and money are invested in the analysis of such samples with limited success [106]. An internal study showed that the type of latent DNA, including touch DNA, and sample type had an effect on the number of alleles present in a resultant profile. Handled items, processed by standard PCR post-extraction workflow, produced the lowest quality DNA profiles with 69% of samples analysed presenting no result at all [106].

One of the benefits, stated in publications, of direct PCR is that the process greatly increases the sensitivity of the amplification process [193, 199, 258, 289]. Additionally, there is no reported increase in amplification of artefacts using direct PCR with standard cycle numbers, while sensitivity is still increased providing more genetic data without increased profile analysis complexity due to stochastic effects [263, 264], when compared to LCN [220, 221]. As no increase to cycling conditions are required for direct PCR amplification, stochastic effects found in the resultant DNA profiles, from low-level DNA samples, are not any more prominent when direct PCR is employed than when processed by standard extraction-based methods [263, 264], depending on any present inhibitor's

effects to PCR. Recent improvements in the buffers provided within STR kits have allowed for the use of manufacturers' protocols to be used throughout the amplification [248]. Central to this is that the PCR cycle number remains the same for both post-extraction PCR and direct PCR workflows; cycle numbers are as validated by the manufacturer or in-house, where manufacturers suggest internal validation for individual optimisation of cycling conditions. When compared to the use of personally designed buffers or altered cycle numbers, the use of standard PCR protocols facilitates simplified implementation of direct PCR by accredited forensic laboratories as in-house validations of the utilised commercially available kits would be completed by the laboratories for their usual extraction-based amplification procedures and no additional validation would be required, with respect to kit used [3, 285].

1.10.3.1. Use of forensic exhibit as the DNA template in direct PCR

Since the successful amplification from bloodstained and blood/saliva stained FTA™ [257, 285] many sample types including hair [3, 262, 263, 292], fingernails [195, 260, 293], fibres or fabrics [3, 195, 259, 294], and tissue samples [295] have been processed using direct PCR [3, 257, 259, 260, 262, 265, 285, 293, 294]. Low-level blood and saliva stains on a number of forensically relevant substrate types, including substrates known to cause PCR inhibition, were analysed by three Y-STR amplification kits under different direct PCR pre-treatment workflows [296]. Direct amplification from blood using the Yfiler® Direct kit yielded 71 full DNA profiles out of a possible 120 with PowerPlex® Y23 and Yfiler® Plus generating 69 and 61 full DNA profiles, out of a possible 120, respectively. This study showed blood and saliva deposited on items such as cigarette butts, woodchips, leaves, and fabrics can be amplified directly [296].

In 2010 Linacre *et al.* examined four fabrics, being polyester, cotton, nylon, and denim, for their ability to facilitate direct PCR amplification of cellular material deposited by touch [259]. Volunteers washed their hands and rubbed the fabric between their thumb and first finger for 5 s. A 2 mm^2 cutting was taken and placed into a 0.2 mL PCR tube for direct PCR amplification using PowerPlex 16 or SGM Plus®. Full profiles were obtained when SGM Plus® was utilised (20/20 alleles) for all samples, however more alleles were generated using PowerPlex 16 (average 24.75/32). Differences in amplification success between fabrics was observed with PowerPlex 16 amplification; touch DNA on nylon samples resulted in the best amplification by direct PCR (average 96% alleles amplified), and denim resulted in the poorest amplification (average 49% alleles). Previous research indicating dyes can cause an inhibitory effect to PCR [297] were also supported [259]. Blackie *et al.*

showed it was possible to produce STR profiles by directly amplifying single fibres from an item of worn clothing with 81% of 35 directly amplified fibre samples being considered informative from this study [294].There is potential for multiple fibres to be taken from the same area of the item of clothing and amplified at different times allowing the same evidence type to be re-tested if needed. However, as there may be some difference in the DNA profiles observed when two different fibres are amplified due to differing DNA quantities or qualities on each fibre, this would not be an absolute true replicate of the original sample.

In 2014, Tie and Uchigasaki analysed 1.5 mm × 0.5 mm fingernail fragments by direct PCR; these were both fresh and from storage between 1 and 10 years [293]. Both the GenePrint® SilverSTR® III System and Identifiler® primer sets were utilised with a personalised buffer system. STR profiles were generated using both amplification mixtures, although the number of samples successfully amplified, the average number of alleles per sample, and the results obtained from each fingernail length analysed were not disclosed. Ottens *et al.* also analysed fingernail clippings by direct PCR in 2014 [260]. This study involved 4 mm^2 nail clippings from 40 fingernails being amplified with NGM™. Of the 40 samples, 38 returned profiles with 17 exhibiting full STR amplification. In addition to amplification success, PCR constituents and conditions remained as stated in the manufacturer's protocol. These studies have shown that only a small portion of the fingernail is required for direct amplification to obtain informative DNA profiles [260, 293]. Because of this, it may be possible to amplify another segment of the same fingernail and obtain a concordant DNA profile. The same issue with the lack of reproducibility is true, with respect to re-testing a sample, when a portion of fingernail is used in a PCR for amplification as with targeting multiple fibres, as stated above. The resultant profiles from two samples taken from the same exhibit (replicate samples) may not be identical, as ineffective amplification or drop-out may occur at different loci among replicate samples, however the genotypes present should be concordant as they originate from the same donor(s), and this exhibit would be able to be re-tested if required by or accreditation guidelines. Concordance of the donor's DNA genotypes may become complicated where two or more donors are present within a fingernail sample as variations in resultant profiles may be produced between replicate samples if there is DNA superficial to the person of interest either present, or absent, at the area of nail tested by direct PCR.

Some sample types that are used directly in the PCR are not able to be re-tested in any way as they are entirely used in the reaction, for example hairs. Ottens *et al.* showed that successful STR amplification is possible from a single hair [262] with Mohanned-Geba supporting this with a

comparison between direct PCR and standard extraction based PCR methods [265]. Ottens *et al.* placed a single root tip of either telogen or anagen hairs into the PCR and was able to generate full profiles from 6 of 30 telogen hairs and all 30 anagen hairs using NGM™ or NGM SElect™. Mohanned-Geba found that when 1 or 3 hairs underwent STR typing, direct PCR amplification gave better results than amplification post-extraction. When 5 or more hairs were added to the PCR, RFU values of peaks produced by direct PCR amplification dropped below that of samples processed by amplification post-extraction. The conclusion was made that with low sample input (1 and 3 hairs) direct PCR amplification resulted in better quality STR profiles than the same sample input through the PCR post-extraction workflow; this is in contrast to high sample input (5 hairs), where PCR post-extraction produced peaks with higher RFU values than those produced after amplification by direct PCR. Although the individual hair cannot be re-tested, direct PCR allows singular hairs to be treated as separate exhibits and increases the chance of obtaining an informative DNA profile if a number of hairs are submitted as one group. In a similar way, processing a small section of a biological stain from fabric may enable multiple cuttings to be taken and processed separately if repetition of sample analysis is required.

Although the use of the exhibit itself allows all the DNA on a sample to be used as template for PCR, this is not always possible. An example of this is in the analysis of touch DNA on exhibits where collection, by either a swab or a tapelift, is required prior to amplification.

1.10.4. Touch DNA: samples taken from exhibits and analysed by direct PCR

Touch DNA, often deposited as a fingermark or by handling an object, has been a challenging evidence type from which to generate genetic data. Touch DNA has the potential to contain both cellular DNA and cell-free DNA, both of which can act as template within a PCR [108, 111, 112, 149, 150, 298]. In 1997 a landmark paper showed that fingermarks possess DNA in sufficient quantities to generate an STR profile [108]. The method employed used an extraction prior to amplification and opened the way for a new type of evidence to be considered for examination; this being touched items. The extraction process was however reported to remove cell-free DNA eliminating this as a possible template source for PCR [114]. Theoretically, direct PCR has the benefit of retaining cell-free DNA as well as cellular DNA from a sample to increase the potential template available for PCR therefore facilitating an improvement in the quality and quantity of the data within resultant DNA profiles. Many institutions, such as the ESR, limit the number of trace or low-level DNA samples they process to balance the expense of DNA analysis with the reduced likelihood of obtaining informative

DNA profiles [131, 291]. The recent application of direct PCR methods to forensic practice may now allow investigation into its efficacy in touch DNA analysis.

Forms of touch DNA were categorized by Cavanaugh and Bathrick into porous and non-porous touch DNA samples [204]. These include samples such as items of clothing that make skin contact through being worn, which have been shown to produce higher DNA yields, on average, than samples that have been handled [130, 299]. Touch DNA samples produced through deposition by touch or handling an item also fall into these categories. It has been advised that the recommended sampling technique for each category is different; tapelifting or direct use of the exhibit is advised for porous sample types [259, 294, 300] while swabbing is the preferred method for DNA recovery from non-porous sample types [204, 264].

1.10.4.1. Use of swabs of a forensic exhibit as the DNA template in direct PCR

Following van Oorschot and Jones' successful STR profiling of fingermarks [108], the idea that touch DNA could be used in forensic casework was introduced. Since this study, touch DNA has been analysed with varying degrees of success using extractions and various forms of low template DNA typing. Recently, fingermarks were shown to produce interpretable profiles in 71% of the fingermarks tested when processed by direct PCR [264]. These were deposited onto pre-cleaned glass microscope slides. A subsequent study included a wider range of surface types including wood, masking tape, and unfired brass, nickel and aluminium bullet casings [193]. In an attempt to mimic real-world scenarios these substrates were not cleaned prior to DNA deposition, by a known individual, touching these items for less than 15 s. No STR alleles were present from the brass cartridge cases, however nickel and aluminium cases produced an interpretable profile in 60% and 53% of samples respectively. Wood that had been touched produced interpretable profiles in 53% of samples while masking tape produced interpretable profiles in 73% of samples. Different environmental factors were also investigated leading to the conclusion that short exposure to ultra-violet light or rain does not adversely affect DNA recovery, however a relatively small sample size restricted the power of these conclusions [193].

Touch samples from surfaces such as keyboards, door handles, and computer mice have also been recently analysed by employing direct PCR [253]. There were no time frames associated with the touching of these items as they were collected from a private desk area. Only between two to four of each item were analysed with varying success. Partial, single-sourced DNA profiles were obtained from three of the four keyboards, and a full profile was obtained from the other. Full profiles were obtained

from both computer mice, one being a mixture, and partial profiles were obtained from all door handles [295].

Fired and unfired ammunition has been an exhibit type from which amplification of STR alleles has been difficult. As such, much research is being conducted to improve recovery of DNA and amplification of available template [135, 136, 138, 202, 301, 302], however direct PCR has not been applied to these samples frequently [136]. Studies have shown that alterations to sample pre-treatment and collection [135], collection and extraction methods [202, 302], extraction method alone [301], and amplification kit [138] can impact the success of STR typing from both fired and unfired ammunition. A recent review on the amplification of genetic material from ammunition, both fired casings and unfired cartridges, discussed the potential for successful DNA testing by examining a variety of collection, extraction, and amplification kits [303]. Conclusions made within this paper were that an optimal collection method and workflow, with respect to extraction and STR amplification kits utilized and PCR amplification post-extraction or by a direct approach, cannot be definitively identified; however the suggestion of EDTA addition to collection mediums, pre-amplification concentration, and direct PCR amplification have been suggested [303]. Thanakiatkrai and Rerkamnuaychoke performed a comparison between direct PCR and PCR post-extraction, using Identifiler® Plus, for the analysis of fired bullet casings (9 mm) after being touched for 15 s by a volunteer [136]. Using their methods, they achieved an average of 3 alleles using direct PCR methods and 3.5 alleles using conventional extraction-based methods. These results do not indicate any significant advantage of either method when examining this sample type; the benefits of time and cost are still relevant to direct amplification from the 9 mm bullet casing when considering these results [303].

In a recent study a number of items, typical of those submitted for forensic examination, were touched for a period of up to 15 s [289]. These were comprised of the following: a glass slide for comparison with previous literature, an unfired aluminium cartridge case, insulated wire, small circuit board, ziplock bag, mobile phone, and SIM card. Results from this study found that 86% of fingermarks produced informative profiles and is in line with previous research which found that 71% of fingermarks produced informative profiles [264, 289]. This minor discrepancy may be due to the different swabs used, a 2 mm^2 cutting from a nylon FLOQ swab (Copan Industries) versus a nylon ultra-fine micro-applicator (City Dental, Adelaide), as Templeton *et al.* showed the use of different swabs cause differences in the quality of the profiles generated from challenging samples [199]. The DNA

amplification kit used may also play a role in the quality of the profiles generated as the sensitivity of the kits and the ability of the buffers to cope with inhibitors has improved as new kits are developed by the commercial suppliers [296, 300, 304]. The study by Templeton *et al.* [193] highlighted the advantage of direct PCR over extraction-based PCR methods for niche application with sample types similar to those tested where touch DNA may be free from PCR inhibitors. Additional studies performed into the difference in the rates of profiling success between samples processed by extraction and those processed by direct PCR methods also show support for direct PCRs application into evidentiary sample analysis to increase the potential for detecting alleles in resulting STR profiles [3, 143, 193, 258].

Data also suggested that different STR kits produce different quality profiles from the same items on which touch DNA was deposited [289]. Samples processed with GlobalFiler® produced informative profiles from a total average of 77% of samples while samples amplified using Identifiler® Plus produced informative profiles on an average of 90% of samples tested [289]. This changed slightly with sample type, but not significantly. There were clear differences in the number of alleles observed per profile between individuals indicating a variation in people's propensity in DNA deposition. This is in line with previous research which showed an association between an individual's shedder status and the STR profile obtained from touch DNA [155, 163, 305].

1.10.4.2. Use of tapelifts of forensic exhibits as the DNA template in direct PCR

An alternative to the swabbing technique used in the recovery of trace DNA is tapelifting. Tape-lifts are routinely taken from evidential items such as clothing. The ability to use tapelifts in direct PCR opens an avenue for different exhibit types to be analysed with this method, which cannot be used in the reaction entirely or swabbed efficiently. Tape-lifting as a technique for touch DNA collection has been investigated but limited in its application to a range of sample types. It was found tapelifting was advantageous over swabbing on a number of fabric types and that the type of tape used can influence the DNA obtained from the item and released into the extraction pool [198, 200, 306]. Haines and Linacre found that latent DNA, such as touch DNA, could be visualized on tapelifts through the use of a fluorescent dye [307]. Direct amplification was performed from tapelifts of samples on which biological material had been deposited 12 months prior to collection. It was found that visualisation of cellular material on the tapelift allowed for a targeted approach for direct amplification to increase the potential for informative DNA data to be produced [307].

A recent study by Kanokwongnuwut *et al.* has shown that staining a tapelift sample with a fluorescent nucleic acid dye allows for effective visualisation of the cellular material present on the tapelift and available for subsequent amplification [308]. Ten semi-adhesive tapes were tested for their recovery rate, inhibition to direct PCR and the background fluorescence observed under microscopy following staining. Of the ten tapes tested, three were deemed suitable for use in conjunction with the fluorescent staining technique, and direct PCR. The same group recently published a study in which they applied this fluorescent staining coupled with direct PCR to the analysis of substrates representation of exhibits found during wildlife crime investigations [192]. Tapelifts were taken from six substrates, being polyester rope, PVC insulated wire, steel wire, stainless steel wire, and jute, with a subset being left outside for 7, 14, or 28 days. Stained cellular material observed on the tapelifts allowed for the targeting of an area of tape exhibiting the highest density of stained cells for subsequent direct PCR amplification. Following the method described cells could be visualized and full STR profiles could be generated after 28 days.

Tape-lifts have also been applied to direct PCR methods in a study analyzing fibres, where all tapelift samples from five fabric compositions produced DNA profiles comprised of 15 alleles or more [294]. A number of profiles were mixtures including alleles from individuals that co-habit with the wearer of the fabric item. Non-wearer DNA can be routinely and innocently found on items of clothing and must be considered with analysis of any profiles obtained [107, 165, 309].

1.10.5. Implementation of direct PCR to evidentiary samples

As previously discussed, there are many factors that influence the quantity and quality of the data obtained using PCR techniques; both direct PCR and extraction-based PCR workflows. These include the swab or tapelift tape used [198, 199, 252], the STR kit employed [289, 296, 300], the surface of the exhibit [193-196, 289, 296, 300], the propensity of the donor to deposit DNA [150, 163] and environmental factors influencing the exhibit post DNA-deposition [310]. Some of these, such as the type of sampling and analysis method used, can be controlled to some extent by the examiner while others, including the donor's propensity to deposit DNA, the presence and persistence of nondonor DNA and environmental factors, are not able to be controlled and add to the complexity of low DNA template analysis.

There are additional limitations inherent within direct PCR methodology and its application to forensic analysis. The process of DNA extraction plays an important role in inhibitor removal to facilitate efficient PCR. As direct PCR methods circumvent this process there is the potential for

increased inhibition within the PCR, depending on the source of the sample, therefore it may be only applicable to template assumed to be free from inhibitors. A laboratory may also need to implement a workflow for evidentiary samples undergoing the direct PCR protocol separate and distinct from samples processed using a standard automated extraction process. Separate workflows, with separate standard operating procedures (SOPs), may not be appealing to many laboratories. In the cases where laboratories are utilizing direct PCR methods for reference samples, this is not an implementation restriction to the incorporation of direct PCR of evidentiary samples into practice.

As there is a mandated requirement of DNA quantification in many operational forensic laboratories, due accreditation guidelines, and this is performed post-extraction, direct PCR may not be employed in routine forensic investigation until changes are made within these guidelines [273, 274]. As direct PCR often uses the entire sample in a single reaction to achieve informative results, it does not allow for secondary testing of the sample if this is a requirement for admissibility or accreditation [273, 274]. Increased sensitivity, due to all DNA within a sample being used as amplification template, can also lead to more complex mixtures increasing the complexity of analysis and inferences about the transfer of biological material to the exhibit [264]. Many studies have been performed into the presence of non-self DNA on an individual's body or clothing as well as the potential for DNA to be present on an exhibit or sample circumstantially [165, 281, 282, 311, 312]. This, combined with the increased sensitivity of direct PCR methods, increases the potential to observe DNA data within a profile from an individual who had no contact with the exhibit or sample being examined, thus increasing the analysis complexity of resulting profiles. With this understanding, care needs to be taken in exhibit selection for direct PCR amplification.

1.10.6. Intelligence samples

One area where the need to follow accreditation guidelines is decreased or perhaps unnecessary, is the analysis of samples collected covertly or for intelligence purposes only, where the data collected are not going to be presented in a statement submitted to a court. Under such situations speed and increased sensitivity from touched items may be paramount; the direct PCR approach is ideal in meeting both of these conditions.

Studies into IED components by direct PCR have varied success rates [142, 267, 313]. In 2019 Taster *et al.* performed a comparison between direct PCR and amplification post-extraction on post-detonated IED substrates spiked with epithelial cells [314]. They report that samples subjected to an extraction prior to amplification, with GlobalFiler®, generated DNA profiles with significantly higher

allelic drop-out when compared to samples that underwent direct PCR amplification. Furthermore, they suggested the sample type influenced the amount of genetic data present within resultant profiles. A recent study amplified post-detonation touch DNA samples on a range of substrate types; these being insulated wire, plastic, battery coating, aluminium, and electrical tape [313]. Direct PCR amplification, with VeriFiler™ Plus, of 37 recovered items or fragments, resulted in STR profiles of 28 samples containing autosomal alleles that matched the donor with 18 (49%) resulting in informative profiles (containing ≥ 12 autosomal alleles). This compared with amplification post-extraction where amplification of only one sample, of the 11 amplified that returned a quantification value post-extraction from the 27 that underwent extraction, resulted in an informative profile [313].

Triaging of samples known to contain DNA would also be beneficial, for both intelligence samples and in regular casework. Fluorescent dyes that bind to DNA have been used in direct PCR methodologies previously [163] and recently Tonkrongjun *et al.* incorporated this technique into their direct PCR workflow for the analysis of IED samples [142]. They reported that more alleles were obtained in 98.6% of samples when DNA was visualised prior to collection compared with a 'blind swabbing' approach [141]. Martin *et al.* used a nucleic acid binding fluorescence dye to visualise cell loss as a result of detonation by comparing images taken pre- and post-detonation [313]. This also facilitated the conclusion of substrate-specific inhibition to their direct PCR workflow from plastic and insulated wire samples; cells were observed in large quantities however amplification was unsuccessful for samples that underwent detonation. The use of fluorescent dyes was also discussed for its triaging potential and a workflow of fluorescent visualisation and direct PCR amplification was suggested [313].

In cases where forensic analyses are performed for intelligence rather than use in a court, such as an IED event or campaign in a remote or hostile environment, the rapid sampling of items may be of priority to aid in swift data collection for application to the relevant case or situation. With the removal of extraction, through the utilisation of direct PCR workflow, reduction in sample processing time ensues. Additionally, the requirement for equipment such as centrifuges, incubators, and quantification kits and machinery are removed offering the potential to perform genetic analyses in the field. By employing direct PCR in field-based analytical units there is no need for an extraction process, only a PCR machine and a means to analyse the PCR products.

1.10.7. Conclusion

The use of direct PCR for the analysis of evidentiary casework samples should not be viewed in contest with currently employed methods for DNA analysis, rather it is an additional type of DNA profiling with niche applications. There are, however, a number of benefits direct PCR has over extraction based methodology including: the potential increase in genetic data available for analysis from trace samples [134, 143, 193, 259, 260]; lower opportunity for contamination due to fewer tube changes [193]; decreased cost due to the omission of the extraction-based consumables [196, 290]. As direct PCR can allow increased data to be obtained from challenging samples [141, 267, 313, 315] its application to some evidentiary samples, which would not produce informative data through extraction-based PCR methodologies, should be considered. The technique has been shown to be applicable to a range of exhibit types, both through the use of the exhibit as template such as through fabric, hair, or nail analysis, or through the use of a swab or tape lift from a range of sample types as PCR template. Any recommendations as to sampling, analytical kit choice, processing, or analytical guidelines, with respect to non-self DNA potential and mixture analyses, made through the literature should be considered before implementation into forensic workflow as exhibit types often change the way direct PCR is applied or sampling occurs; there is no uniform method for all sample types.

With the incorporation of tapelifts into the sampling of additional surface or exhibit types there is potential that an exhibit can be tested more than once through the careful segregation of the tapelift into separate reaction tubes. Investigation into whether there is any reproducibility or sample splitting capability when swabbing is advised if there is an accreditation requirement for more than one reaction to be able to be performed. There is also difficulty with the incorporation of direct PCR in current laboratory workflows where alternate equipment set-up may be required, and automation of direct PCR may be challenging. As an additional tool, to other techniques or evidence recovered, direct PCR offers an extra avenue for investigation and data development. It is an applicable workflow to certain niche sample types where traditional extraction-based methods may not yield informative results.

CHAPTER II:

OPTIMISING METHODS FOR DIRECT PCR IMPLEMENTATION

Manuscripts enclosed:

Martin, B., Taylor, D., Linacre, A. (2022). "*Comparison of six commercially available STR kits for their application to touch DNA using direct PCR*" Forensic Science International: Reports

2.1 PREFACE

There is no current representation or discussion about how kit choice influences direct PCR amplification success of latent DNA material deposited by touch; there are validation studies and STR kit comparison literature for reference material or other high template DNA sources post-extraction. This research involves the comparison of multiple commercially available STR amplification kits for their application to direct PCR and presents quantitative data to laboratories, both research-based and operational, for consideration. The data presented provides empirical data on which to base, and facilitate ease with, any decision in the implementation of direct PCR into workflow for operational or research-based laboratories. Results are reported in a manuscript currently accepted pending revision in *Forensic Science International: Reports*.

Of note, and not included in the manuscript, the time between cellular deposition and swabbing changed depending on sample availability and batching. This range was 1 day-3 weeks. All samples were stored at room temperature and away from light between deposition and sampling to ensure minimal bacterial degradation and UV damage. This was the case for work presented in subsequent chapters also. Although not thoroughly explored, the use of Prep-n-Go™ in previous literature has been suggested to improve STR amplification for direct PCR workflows [134]. As such, Prep-n-Go™ was utilised in the PCRs in this and all subsequent chapters, unless specified otherwise.

2.2 IMPACT STATEMENT AND AIMS

This research provides companies with feedback as to how their kits are performing against other providers and areas in which improvements can be made. The data also provides operational and research laboratories with data to align their STR kit choice with their desired outcomes; these may include ease of integration while understanding limitations or maximizing allelic yield. Discussion around the benefits and limitations of each kit, with respect to direct PCR, offers an important foundation for understanding DNA typing processes and optimisation within this workflow.

2.2.1 Aims

"*To provide empirical data from which laboratory groups can make educated decisions as to the ideal STR kit to utilise within their direct PCR workflows*"

2.3 PERFORMANCE COMPARISON OF COMMERCIALLY AVAILABLE STR KITS

2.3a Manuscript: Comparison of six commercially available STR kits for their application to touch DNA using direct PCR

Manuscript published: *Forensic Science International: Reports*, 2022

Statement of authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**85**	**100**	**70**	**100**	**55**		03.08.21
DT	-	-	30	-	35		03.08.21
AL	15	-	-	-	30		05.08.21

2.3a.1 Abstract

With an increase in the application of direct PCR to items of forensic relevance, as well as the array of STR kits available for amplification, the need for a comprehensive investigation into the optimum STR panel for this workflow has arisen. Here we examine the relative STR amplification success of touch DNA on a range of substrates, with surface properties typical of those found in forensic investigation, using six commercially available STR kits: GlobalFiler®, Identifiler® Plus, Identifiler® Direct, VeriFiler™ Plus, Investigator® 24Plex QS, and PowerPlex® 21. We report on the percentage of possible donor alleles amplified per profile, and the number of samples that resulted in informative genetic data (≥12 autosomal alleles). We also include comment on the ease of interpretation of the resulting EPGs for each of these six STR kits, when applied within a direct PCR workflow.

Donors of known shedder status deposited DNA by handling one of five substrates (glass slide, matchstick, insulated wire, circuit board, and a plastic ziplock bag) for 15 s, 15 mins post-handwashing with water. Each item was touched in triplicate by each volunteer for amplification with each STR kit resulting in a dataset of 720 samples.

The difference in the number of profiles considered to be informative was found to be statistically significant when comparing STR kits (p= 0.0011) and donors (p= $2x10^{-7}$), but not when considering substrates (p= 0.15). Identifiler® Plus amplification resulted in the highest profile coverage and second highest percentage of informative profiles. VeriFiler™ Plus generated informative profiles in the largest number of samples (94%) with PowerPlex® 21 amplification resulting in the fewest (79%). There was no significant difference between Investigator® 24Plex QS and GlobalFiler® in any empirical consideration; however, baseline noise and artefact presence made Investigator 24PlexQS profiles more difficult to analyse.

2.3a.2 Introduction

Reports detailing the application of direct PCR of forensically relevant samples and exhibits has expanded recently with studies using body fluids [257, 284, 291, 296, 316], reference samples [247-249, 298], and tapelifts [294, 315, 317] collected from exhibits. A variant on placing a sample taken from a substrate, such as swab or tapelift, into the direct PCR workflow is to place a substrate, directly into the PCR master mix [259, 260, 262, 263, 265, 292, 293, 295, 318]. There are two workflows referred to in the literature as 'direct PCR'[204, 319]; these include the use of an incubation/pre-treatment step prior to PCR, as with reference samples [247, 248, 254, 320], and the direct addition of the sample or item into the PCR tube [254, 262, 314]. For the purpose of this work, the second definition of direct PCR was examined. A considerable portion of the current research is focussed on the prospect of obtaining DNA profiles from 'touch DNA' or from sample types where the integrity of the DNA within PCR might be compromised if it is not subjected to an extraction process: such sample types include fired bullet casings [321] and IED components [136, 142, 314, 322]. The ability to obtain informative STR profiles using standard means that involve the use of an extraction process from samples with DNA deposited by touch is very limited [106, 133, 299, 323], however the application of direct PCR has shown an improvement in some circumstances in resultant profiles [193, 264, 289, 322, 324]. As laboratories are starting to perform direct PCR analyses within their workflows, or are considering this move, the data presented in this paper aims to provide information regarding the ideal commercially available STR kit for direct PCR to allow integration into laboratory practice.

The direct PCR workflow differs from traditional workflow through the circumvention of the DNA extraction process. Although the extraction process removes potential PCR inhibitors, the loss of DNA template is significant and can be as much as 76% [2]; selection of specific kits [7]and modification or optimisation to protocols has been known to increase extraction efficiency [325]. This template loss

is not a concern where the DNA template is readily available in large amounts, however, with 'touch' DNA samples the initial amount of DNA template is inherently low so any loss through extraction considerably reduces the potential for an informative DNA profile to be generated [323]. Direct PCR removes this extraction step and therefore any subsequent loss of DNA is avoided; this is ideal where low DNA template exists within a sample [326]. There have been a number of studies that concluded that the direct PCR workflow has advantages over extraction-based workflows with respect to STR DNA profiling, both for high template samples [247, 284, 291, 295, 316] and low template samples [193, 264, 289, 294, 315]. Disadvantages of direct PCR include: any inhibitors of PCR are not removed due to the omission of any extraction/purification process; there is no opportunity to quantify the mass of DNA collected; and replicate amplifications cannot be performed due to the use of the entire sample in the PCR process [204, 289]. Caution must be taken when considering samples where PCR inhibitors may be present, for example haem within blood samples [268, 269]. As fewer inhibitors are present in 'touch' DNA, which consists predominantly of corneocytes and keratinocytes with an uncharacterised concentration of cell-free DNA [112, 113, 326], direct PCR application is a viable workflow option in the analysis of these sample types.

Multiple STR amplification kits have been used in direct PCR methodologies. Initial studies utilised SGM Plus® [259], PowerPlex® 18D [249] and NGM SElect™ [193, 262, 264, 294], while more recent studies have amplified template using GlobalFiler® [195, 247, 289, 316], Identifiler® Plus [289], and VeriFiler™ Plus [313]. Over the course of research into the application of direct PCR to forensically relevant samples, companies have not only increased the number of loci available for amplification, but the ability of the buffers to overcome inhibitors and enzymatic function has also increased [193].

Previous research has shown that the data produced in the analysis of latent DNA, in the form of 'touch DNA', is influenced by the amplification kit used [249, 264, 289]. Myers *et al.* compared PowerPlex® 18D (PP18D) and Identifiler® Direct (IDFD) systems for the direct amplification of reference samples concluding that IDFD generated profiles with higher peaks than PP18D, leading to saturation and increased pull-up. When comparing the same injection time of 5 s, the success rates of a profile passing set guidelines were comparable between kits, at 95% IDFD and 96.25% PP18D [249]. Templeton and Linacre compared DNA profiles taken from fingermarks and amplified using NGM SElect™ and Profiler Plus® with the reported success for obtaining informative profiles being 66% with Profiler Plus® and 74% with NGM SElect™ [264]. Martin *et al.* compared GlobalFiler® and Identifiler® Plus when swabs were taken from a range of sample types. They reported that although GlobalFiler®

produced an average allele count of 36 alleles (of 46) while Identifiler® Plus produced an average of 31 alleles (of 32), Identifiler® Plus was the preferred kit for direct PCR amplification [289]. This was due to the standard deviations of ± 15 alleles with GlobalFiler® and ± 1 allele for Identifiler® Plus; DNA profiles were more consistently data rich when using Identifiler® Plus.

The intent of this body of research was to perform direct PCR on a variety of substrate types using a comprehensive range of STR amplification kits commercially available, used both routinely and infrequently in laboratories, to determine which kits performed most effectively for the analysis of touch DNA. The kits tested within this body of work, and the loci they amplify, are given in Supp. Table 2.1. The percentage of profile coverage, number of donor alleles amplified, and ease of interpretation was recorded to provide data to laboratories to support or advise their STR kit choice in any analytical work relating to touch DNA by direct PCR. The purpose of this work is not as a validation study, rather it is as an informative tool to laboratories as to the effectiveness of their currently employed kit to the application of direct PCR, as such the allelic amplification rates between kits is the focus. This is the largest comparison of commercial kits for the application of direct PCR on touch DNA; in fact for any template source, with the exception of a paper by Westen *et al.* (2014) investigating six commercial kits for Dutch allele frequency database development from blood samples [327] and a paper exploring a pre-treatment direct PCR approach to 13 STR kits using saliva samples [320], other kit comparisons within literature consist of two [249, 264, 289] or three kits [328-331].

2.3a.3 Methods

Ethics approval

Approval was obtained prior to sample collection from the Social and Behavioural Research Ethics Committee (SBREC) (reference 8109) of Flinders University.

Exhibits and volunteers

Eight volunteers (designated IND 01-08) were used to deposit DNA on these substrates: IND 01-04 were males, and IND 05-08 were females. The shedder status of all volunteers had been previously determined, following methods outlined in Kanokwongnuwut *et al.* 2018, to ensure representation of a range of shedder status was used within the dataset [163]. IND 01, IND02, IND04 were recorded as high shedders, IND 03, IND 07, IND 08 were recorded as intermediate shedders and IND 05, IND 06 were recorded as low shedders.

Five items were chosen to emulate potential real-life exhibits which comprised: glass slide, matchstick, insulated wire, circuit board, and a plastic ziplock bag. Each item was prepared in eighteen replicates by each volunteer giving a total of 720 samples. The eighteen replicates were separated at random into six groups for a total of three replicates per volunteer per group. These groups were analysed by different STR kits being GlobalFiler®, Identifiler® Plus, Identifiler® Direct, VeriFiler™ Plus (ThermoFisher Scientific, VIC, AUS), Investigator® 24Plex QS (QIAGEN, VIC, AUS) and PowerPlex® 21 (Promega Corporation, VIC, AUS).

Deposition of DNA

All items were cleaned with 3% bleach, wiped, and allowed to air dry in an isolated clean room to ensure no DNA was present on the items prior to the deposition of DNA by the volunteers. Negative control samples were collected from a set of cleaned items. Negative controls were performed in triplicate from each item. Deposition of DNA was performed by touching each sample for 15 s, 15 min post handwashing, as described in Martin *et. al,* 2018 [289]. Between depositing DNA onto a substrate and beginning the next deposition rotation, of handwashing and a 15 min wait, there was no instruction as to the time interval required.

Collection of DNA from exhibits

DNA collection by swabbing was performed for the glass slide, insulated wire, circuit board and ziplock bag as described in Martin *et al. 2018* [289]. Each swabbed sample was double-swabbed using a nylon ultra-fine micro-applicator (City Dental, Adelaide, AUS) premoistened with 0.1% Triton™ X-100 (Sigma, Australia). DNA collection was performed by tapelift for the matchstick samples following the method described in Martin *et al.* 2019 *("Sampling was performed using a strip approximately 25 mm × 30 mm of 25 mm width Sellotape brand adhesive tape. An area of approximately 3 mm^2 was cut from the tapelift and placed into a 0.2 mL thin-walled PCR tube.")* [315].

DNA Amplification

Direct PCR was performed on each sample using either the GlobalFiler®, AmpFLSTR® Identifiler® Plus, AmpFLSTR® Identifiler® Direct, VeriFiler™ Plus, Investigator® 24Plex QS, or the PowerPlex® 21 by removing the two swab heads, with a sterile scalpel blade, directly into the same 0.2 mL thin-walled PCR tube. From this point GlobalFiler® will be referred to as GF, AmpFLSTR® Identifiler® Plus as IDFP, AmpFLSTR® Identifiler® Direct as IDFD, VeriFiler™ Plus as VERP, Investigator® 24Plex QS as I24, or the PowerPlex® 21 as PP21.

Amplifications were performed in 25 µL following the manufacturer's protocol with exception of 2 µL of Prep-n-Go™ (ThermoFisher Scientific) and Low TE Buffer (ThermoFisher Scientific) replacing water. The exception to this was the IDFD kit which where amplifications were performed in 27 µL, due to the additional 2 µL Prep-n-Go™ added to the master mix. The addition of Prep-n-Go™ has been widely incorporated into direct PCR methods [292, 313, 332] and has been shown to increase genetic data yield [134]. All amplifications were performed on a ProFlex™ thermal-cycler (ThermoFisher Scientific). PCR product (1 µL) was added to the appropriate volume and designated size standard, according to manufacturer's instruction, and separated on a 3500 Genetic Analyser (ThermoFisher Scientific). Kits were chosen based on their previous literature use in direct PCR analysis, or current use for HID in forensic laboratories for casework samples within Australia; specialised low template kits were not chosen, with the exception of IDFD, as operational laboratories are unlikely to perform validation studies for the inclusion of additional kits where the need may not exist to do so.

Data analysis

Allelic Data Analysis

Allele data were analysed using GeneMapper® ID-X (version 1.4). The quality of the profiles, with respect to peak morphology and artefact incidence were observed and the number of alleles present from the donor were recorded. Peaks were recorded if they were of 50 RFU or above. Peaks were considered for homozygosity if they were ≥ 150 RFU. Samples were considered 'informative', or up-loadable to the Australian NCIDD, if they contained 12 or more amplified autosomal alleles [3, 70, 193]. The percentage of a donor's profile amplified was calculated by dividing the number of alleles present in a profile with the maximum number of alleles capable of being amplified.

Comparison of Genetic Data

Of the five kits amplified, the maximum number of donor alleles able to be amplified varied between 32 (IDFP and IDFD) and 49 (VERP). For this reason, assessments were made to consider different factors influencing the overall performance of each kit, these being: 1) the percentage of a donor alleles present in the profile; and 2) the number of donor alleles present per profile. For the statistical analyses, including Wald chi-squared test, linear and logistic regressions, R software was utilised [333].

2.3a.4 Results and Discussion

All negative controls taken from each item retuned blank profiles or profiles with no more than a single peak allele drop-in at low RFU (<80 RFU).

Each section below analyses EPG data in different ways to address various performance metrics of STR kits. Qualitative observations relating to data produced with each kit are discussed broadly to introduce potential user experiences when analysing touch DNA by direct PCR. The number of alleles amplified, as a discrete total or average value, between kits is described to discuss the potential 'donor match' statistical weight obtainable though direct PCR touch DNA analysis with each STR kit. To account for variability in the maximum number of loci amplifiable between STR kits, the percentage of the profile present, when compared to this maximum value, was calculated and discussed as 'profile coverage'; this allows an indication as to PCR efficiency due to kit constituents between STR kits rather than a discrete allele number.

Percent of donor profile amplified: STR kit design

To assess confidence in the ability of each kit to amplify genetic data in the presence of inhibitors or degraded DNA from direct PCR amplification of touch DNA, the observed profile percentage was calculated for each sample, hereafter referred to as profile coverage. For example, PP21 amplifies a maximum of 42 alleles while VERP amplifies a maximum of 48, therefore if 16 alleles are present this corresponds to 38% and 33% profile coverage respectively. Due to the size of the dataset and random deposition allocation it is not believed that the difference between intra-personal depositions would cause the performance of specific kits to be inadvertently increased. If all buffers were able to manage the presence of any inhibitors equally and all enzymatic activity was the same between all kits, the relative profile coverage observed should be the same across all kits; this was not observed, and kit type was found to be significant to the percentage of the donor profile observed ($p= 0.0011$); IDFP outperformed the other five kits. This was also observed by Lin *et al.* (2017) where IDFP portrayed a better tolerance to tannic acid than GF did [331]. IDFP and VERP amplification produced an average of 91% and 88% profile coverage respectively, which is significantly more than the other four kits (Figure 2.1). The inference is that the buffer composition and/or enzymatic activity within IDFP and VERP allowed for more effective direct PCR amplification of touch DNA than the other four kits tested. Kits with a higher standard deviation in these data may also have higher cellular input requirements [326].

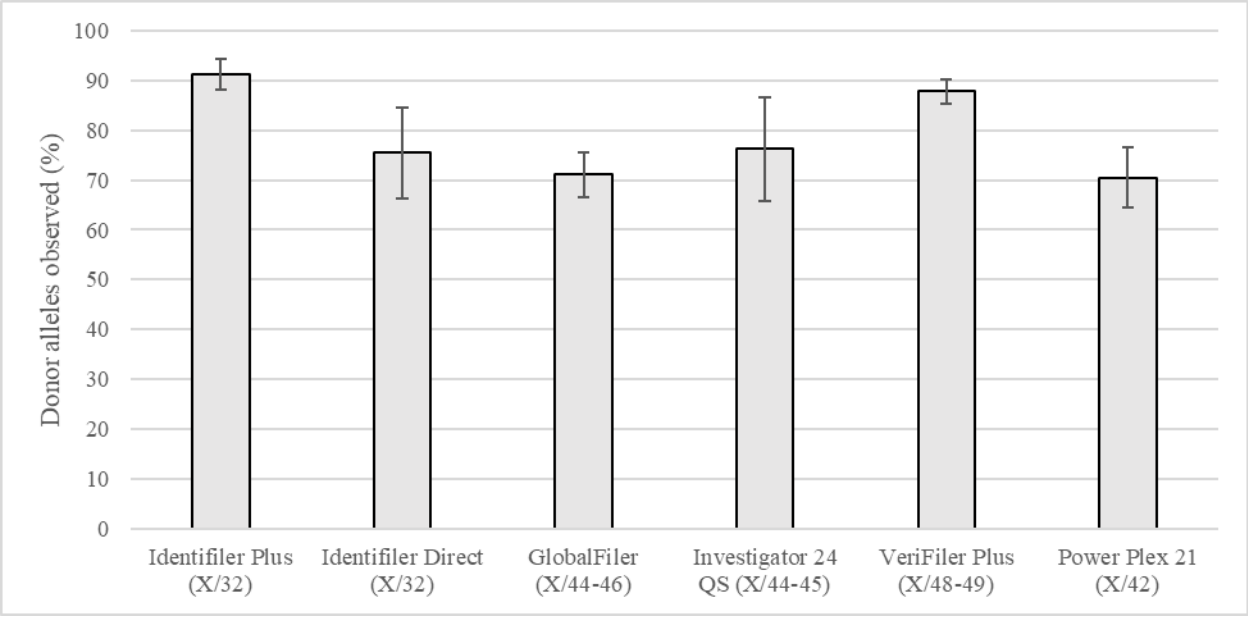


Figure 2.1. Average percentage of donor profile observed of all volunteers on each substrate type, per kit. Error bars represent the standard deviation in profile coverage across the triplicate samples of all substrates (N=120 for each kit).

Caution is noted when making an inference that amplification success is affected by a kit's constituents as the number of loci amplified differs between kits, as does the size of the amplification products for each loci differ. As STR amplicons of higher mass are more likely to be lost to degradation or amplified inefficiently due to inhibitor presence compared with amplicons of lower mass, profile coverage can be influenced by the locus location design of the kit. Some STR kits have a larger allelic rages than others, VeriFiler™ Plus amplifies products between 80-430 bp while SGM Plus® lies within 90-360 bp [42, 43], while others design their kits to have the highest percentage of loci in the smaller amplicon side of the EPG [334]. For example, the average number of donor alleles amplified per profile is significantly lower when amplified with IDFD (<360 bp) than with any other kit ($p= 5.03x10^{-7}$), however, it presented an average of 75% profile coverage, which is higher than both GF (<460 bp) and PP21 (<460 bp). Because of this, comparison needs to be made between kits with a similar maximum product mass, these being GF, INV24, PP21 and VERP. GF, INV24 and PP21 performed similarly, with no statistical difference with respect to the average profile coverage per profile; Lim *et al.*(2017) showed that for humic acid and collagen there was no difference between INV24 and GF for most concentrations and for tannic acid INV24 was more robust [331]. These kits all presented significantly lower profile coverage than VERP ($p=1.05x10^{-5}$), which even contains additional loci. The inference from these data is that the constituents of the VERP kit facilitated direct PCR amplification of touch

DNA STR alleles more effectively than the other three kits listed, even considering the amplification of alleles with comparatively larger masses.

There was no significant difference observed with the average profile coverage between different substrate types when considering all kits (Supp. Figure 2.1) (p= 0.15). Individual kits resulted in subtle differences between the substrates, but these was not significant for any kit (Supp. Figure 2.2) even considering the glass slide with IDFD, substrate was not significant (p= 0.10). The matchstick presented the largest variability within the data, with the ziplock bag and circuit board presenting profiles with a more consistent profile coverage between sample replicates and kits.

Individual volunteers differed in profile coverage across all kits (Supp. Figure 2.3). Their deposition as described by their shedder status did not, in all cases, correlate to the amplification success as observed in their STR profile. The quality of the DNA within the touch deposit is not identified using the shedder status determination method chosen, in addition to this there is limited knowledge surrounding the intrapersonal deposition stability over time. It has been postulated that a single designation of an individual's shedding ability should not be used [335], although for research purposes where the shedder status is not a paramount variable from which assessments on other data is made this is not necessary. IND 04 provided a touch deposit that resulted in consistently high-profile coverage, with an average of 95% alleles amplified. IND01, IND02, IND03, and IND06 presented similar median percentages of profile coverage, with an average ranging between 80-90% alleles. IND05, IND07 and IND08 provided touch deposits that resulted in significantly lower profile coverage than the other volunteers, 66%, 75% and 57% respectively. Additionally, these volunteers exhibited the greatest variation between samples in profile coverage; their propensity to deposit DNA is lower than the other volunteers and this varied greatly between deposition events. This is in line with previous research, which hypothesises activity plays a role in low shedders' deposition more so than for high shedders, who deposit enough DNA for a full profile more consistently [149, 163].

Number of donor peaks amplified: statistical weight

The total number of donor alleles amplified give an indication of the strength of potential match probabilities that can be obtained with each kit. There were significantly more donor alleles amplified per profile when VERP was utilised than with any other kit ($p= 2.73x10^{-11}$) (Figure 2.2). VERP amplification resulted in an average of 43 donor peaks per profile. This compared with 29 by IDFP, 24 by IDFD, 32 by GF, 35 by INV24, and 30 by PP21. In a recent study on the sensitivity of three forensic autosomal kits, VERP was shown to be far more sensitive than INV24, with 50% profile coverage

compared with 4% when 8 pg of DNA was added [330]. A higher number of donor alleles would indicate the optimum STR kit for the amplification of touch DNA by direct PCR; this is VERP based on these data. After VERP, IDFP followed by INV24 and GF are the kits that provide the highest likelihood of obtaining an informative profile (dashed line in Figure 2.2) however INV24 and GF amplification resulted in the highest number of donor alleles per profiles of these three.

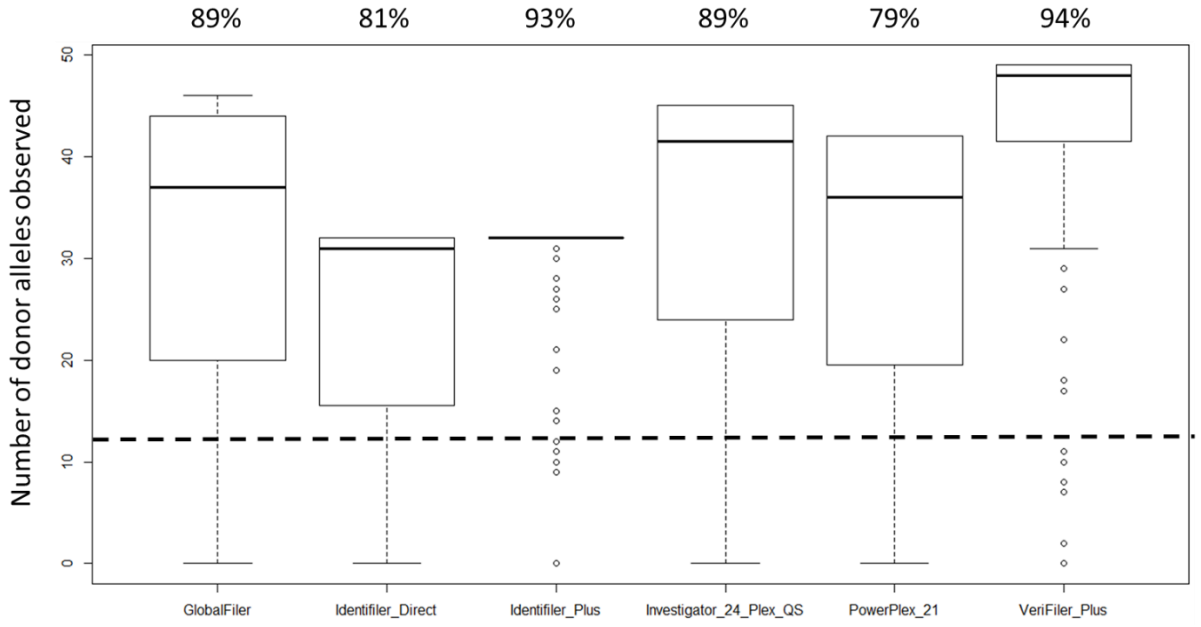


Figure 2.2. Number of donor peaks obtained per STR kit, observed for all substrates from all volunteers. The dashed line represents 12 alleles and the value at the top of the boxplots is the percentage of data above this line (N= 120 per kit).

Ideally an STR amplification kit has a high average number of donor alleles amplified, and low resultant data spread between replicate sample results to give confidence in the consistency of its amplification. Although the replicates performed are not technical triplicates, rather they are biological replicates, due to the random assignment of the deposits from the donor between kit amplification choice the assertions described by the trends retain their value. Additionally, the use of a range of shedders mitigates the variance in deposition as having a large effect on simplification success. These data show that in the number of amplified donor alleles per profile IDFP and VERP have the lowest variation between analysed samples (Figure 2.2). Due to this low variation in amplified alleles, an estimation as to the quality of profile that should be obtained from evidentiary samples, similar to the substrates examined and providing touch DNA is present, can be made. Amplification

with the other kits cannot provide the same confidence in the number of donor peaks expected in a profile, given the same conditions.

As the lowest standard deviation in the dataset pertaining to the number of donor alleles amplified was observed with IDFP, both as an average of all volunteers and within volunteer datasets, this kit may be preferred over INV24 and GF even though these two kits amplified more donor alleles on average. This variation in the number of donor alleles amplified is likely due to the variable nature of touch DNA depositions. Depositions are not identical between events as pressure [144, 172] and activity [2, 148] can influence them. VERP has a low standard deviation and a high average number of donor alleles amplified therefore it consistently produced profiles with a high number of donor alleles, providing the best opportunity for high match probability statistical strength with a potential match found with a database search. In casework, additional PCR inhibitory factors can reduce the efficiency of amplification when compared to the touch DNA deposits analysed for this study, therefore a reduction in the number of donor alleles within a profile would be expected.

As data for each substrate amplified per kit clusters closely with respect to informative profile observations, with the exception of IDFD and INV24, each kit performs similarly when amplifying each of the substrates (Supp. Figure 2.2); the relationship between substrate and informative profiles for the glass slide using IDFD showed p= 0.0502 and INV24 showed p= 0.0163. With the exception of IDFD (informative profile p=0.006) and INV24 (number of alleles p= 0.035) there is no significant difference in the number of donor alleles amplified or the likelihood of an informative profile being produced from any substrate tested when amplified with the same kit for the remaining kits.

The spread of data between donors, within kits, differs (Supp. Figure 2.4). IND04 presents low variance in the number of amplified alleles from their touch deposit in all kits, while IND05 and IND08 show much variance in most kits. IND03, IND04 and IND06 consistently presented more donor alleles per profile than the dataset average and IND08 was consistently below (Supp. Figure 2.5). Other volunteers show variable variance in the number of donor alleles amplified, across all kits. This indicates the variable nature of deposition as on occasion amplification of a low shedders' DNA was more successful than that of a high shedder.

Interpretation Challenges

The interpretational challenges listed below are not the focus of this work, rather they are included to inform laboratory or research staff as to potential issues or considerations that may arise due to direct PCRs application to touch DNA samples with these kits. As individual laboratories

perform their own internal validations, and these would be more suited to the sample load they are utilising, the data and observations explained below is not as in depth as traditional kit validations; these may not be translatable to other users' experiences.

Baseline

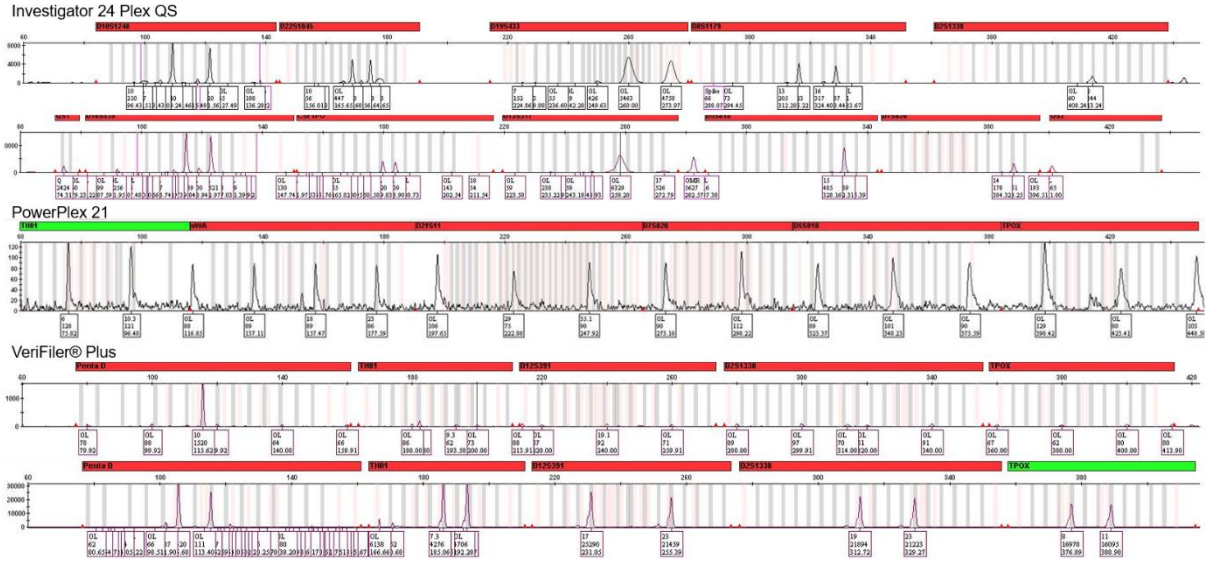


Figure 2.3. Examples of dye lane anomalies observed multiple times in the analysed datasets. Investigator 24 Plex QS (top title), PowerPlex21 (middle title), and VeriFiler® Plus (bottom title) show large baseline noise, often in lower half of electropherogram. PowerPlex21 (middle title) and VeriFiler® Plus (upper tile) show non-allelic peaks at consistent distances across the black or purple dye lane, respectively. The PowerPlex 21 black dye lane is from a negative control sample; all other dye lanes were negative. The Investigator 24 Plex QS and VeriFiler® Plus dye lane images are all from separate single-source profiles.

The baseline of multiple INV24 dye lanes was subject to greater noise when compared to other kits (Figure 2.3). Alleles with low RFU were difficult to designate and would pose real challenges to casework samples; as the donor alleles were known for these research samples, interpretation was not an issue, however casework profiles cannot be treated in the same way. The baseline of the purple dye lane in VERP profiles often exhibited greater noise than other kits, the exception being INV24. This noise was most common around the loci with the lower mass and could be easily distinguished from real alleles (lower panel of 'VeriFiler® Plus' in Figure 2.3). The RFU of amplified donor alleles were significantly higher than the baseline noise observed in most samples, therefore these artefacts did not pose difficulty with profile analysis, however if amplification resulted in true alleles of similar peak

heights to these anomalies, or the baseline noise, analysis was challenging and may be impossible at some loci in casework scenarios. Tay *et al*. (2019) showed a similar trend between the baseline of VERP and INV24, whereby VERP exhibited lower levels in the presence of DNA. They concluded that for the amplification of latent DNA, VERP provided a higher level of confidence in the differentiation of alleles from noise related to artefacts [330].

The black dye lane within many PP21 profiles was also subject to peak anomalies, whereby peaks appeared at a consistent distance from one another along the entirety of the dye lane (Figure 2.3). This made interpretation of true alleles difficult as the false peaks were often a similar RFU to true alleles. These peaks were also observed in some reference profiles from good quality extracted DNA; therefore, it is not an anomaly due to the direct PCR amplification of touch DNA. The purple dye lane of VERP was also subject to these peak anomalies along all loci at consistent mass differences (upper panel of 'VeriFiler® Plus' in Figure 2.3). Overlaying of the size standard lane with these supported the conclusion that these peaks were due to pull-up from this size standard dye. Re-running the sample with a lower size standard addition reduced these peaks observed in the profile.

Additional Anomalies and Artefacts

Increased pull-up and stutter were observed in INV24 and PP21 profiles when compared with IDFP, IDFD, GF and VERP, data not shown. The increase in pull-up in INV24 and PP21 profiles was more apparent in loci with lower amplicon mass; it occurred from amplicons with RFUs that would not indicate saturation for many loci in INV24 profiles. Profiles generated from amplification by GF and IDFD produced increased incidence of split-peaks when compared to other kits. INV24 was subject to PCR efficiency reduction for loci with larger mass, due to inhibitory effects from the substrate or the quality of the touch DNA template, more frequently than with other kits. The loci with smaller masses were amplified far more than those with higher masses where a 'ski slope' was observed in many profiles.

Additional Comments

One allele designation was discordant at Penta D for IND02 between VERP and PP21, where their amplified allele was called 9.4 in all samples amplified by VERP and 10 in all samples amplified by PP21; this was tested again with reference samples from the individual and the same was observed.

Mixtures within profiles were similar across kits, with an average of 34% samples presenting mixed profiles. This is in line with previous research [264, 289], non-donor peaks could be attributed

to cohabitants of the donor. Heterozygous peak imbalance occurred in all kits, more severely and more often in GF and INV24, data not shown. This is not unusual for latent DNA analysis, such as the touch DNA template analysed by direct PCR here [41, 336]. A paper comparing GF, INV24 and PowerPlex® Fusion 6C found that GF presented better heterozygous balance and intra-colour signal balance than INV24 [331].

Although the baseline of INV24 and VERP was often subject to greater noise, when compared to other kits, these kits also amplified the largest number of donor alleles per profile, with INV24 amplifying an average of 34 donor alleles and VERP amplifying an average of 43 donor alleles. There is therefore a trade-off between increased manual profile interpretation and allelic data generation that may be advantageous. The QS peaks in the VERP and INV24 profiles provide information into the mechanics behind allele amplification success; they can indicate DNA degradation or inhibition from a substrate or template source. Use of these kits for challenging sample types, such as discharged ammunition or detonated IED components, would give information as to the potential requirement for extraction if direct amplification failed and inhibition is indicated by the QS peaks.

2.3a.5 Conclusion

The data provided in this study is designed to provide comment on the optimum STR panel for the amplification of touch DNA by direct PCR and inform laboratories on the benefits and limitations of each kit before incorporation into workflow ensues. Successful direct PCR amplification of touch DNA from a range of substrates was performed with all six STR kits. Laboratories need to consider what they view as important to determine their individual conclusion as to kit performance in the context of their workflow (Table 2.1)

Table 2.1 Summary of main findings related to STR kit performance with respect to collective data of all substrates and donors.

Parameter	High performance	Mid performance	Low performance
Profile Coverage	IDFP, VERP	GF, INV24, PP21, IDFD	
Number of Alleles	VERP	GF, INV24, PP21, IDFP	IDPD
Baseline Anomalies	IDFP, IDFD		VERP, INV24, PP21
Pull-up (P), Stutter (S), Split peaks (SP)	VERP, IDFP		INV24 (P/S), PP21 (P/S), GF (SP), IDFD (SP)

The kits that produce informative profiles more frequently may be favoured to ensure that the maximum number of evidentiary samples can be compared to a DNA database. IDFP, GF, INV24 and VERP resulted in informative profiles more often than IDFD and PP21. The difference in the number of informative profiles within the dataset was found to be significant when considering donor and kit choice, but not the substrate. VERP, INV24 and GF amplified the highest number of donor alleles on average. Significance in the number of donor alleles amplified was only observed with VERP when compared against all other kits. The average number of donor alleles amplified may be of more importance, to increase the statistical power of any potential match, even if samples are able to be uploaded for comparison less frequently.

Aligned with these considerations, a kit's robustness may be advantageous when amplifying samples by direct PCR, with increased inhibition potential and/or degraded template presence. In this case, the kits with the highest profile coverage are preferential. VERP and IDFP presented the best amplification for touch DNA samples by direct PCR with respect to profile coverage, with INV24 the lowest.

VERP outperformed all kits in each of the three parameters examined, with the exception of IDFP in profile coverage, while IDFD under-performed significantly with respect to the average number of donor alleles amplified per profile. Of the other kits, there is no clear advantage one has over the others. IDFP amplification resulted in the highest percentage of profile coverage and second highest in percentage of informative profiles, however its average number of alleles was lower than all kits, with the exception of IDFD. GF, INV24 and PP21 could not be separated significantly in any empirical data observed. Although there is no clear advantage between these three kits in terms of the empirical measurement considerations discussed, ease of interpretation did vary. INV24 and PP21 presented multiple interpretational challenges with touch DNA analysis, therefore these may not be favoured if trace analysis is routinely performed or if inhibitors are likely to be encountered when a direct PCR approach is used.

Consideration of a laboratories budget and locus concordance with current databases will be the most important factor when selecting one of these kits for implementation or continued use. The trends discussed from the analysis of this dataset consider touch DNA amplified by direct PCR only. Alternative template sources and workflow may result in different trends to those observed here.

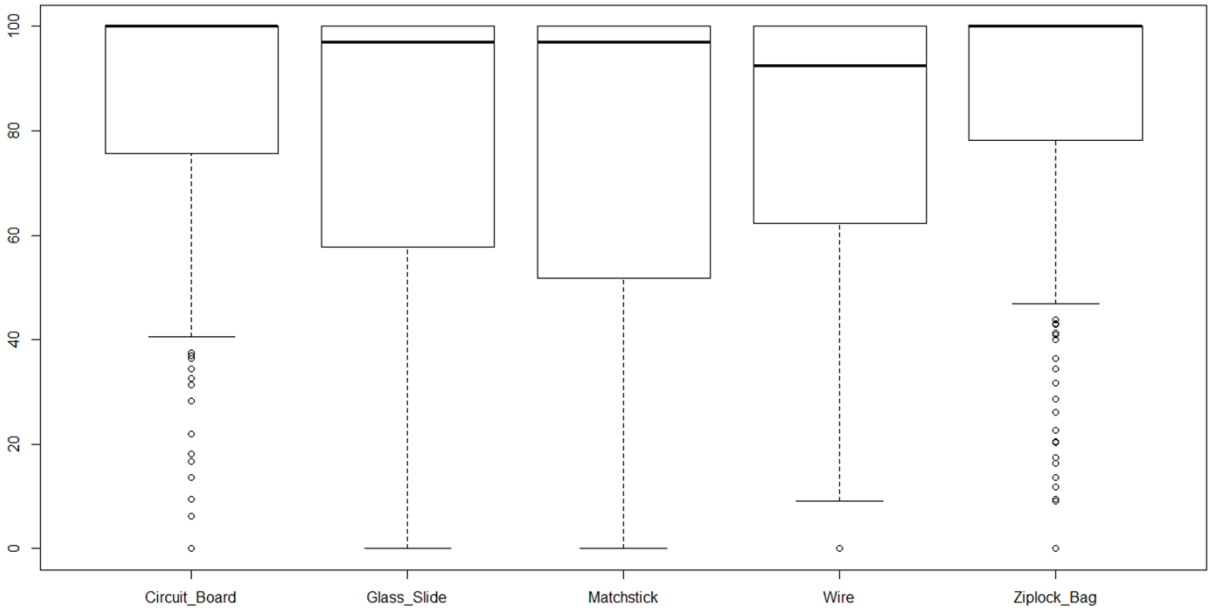
2.3a.6 Acknowledgements

This research is supported by the Attorney General's Department through the Ross Vining Memorial Research Fund. We would also like to thank all volunteers that participated in this study.

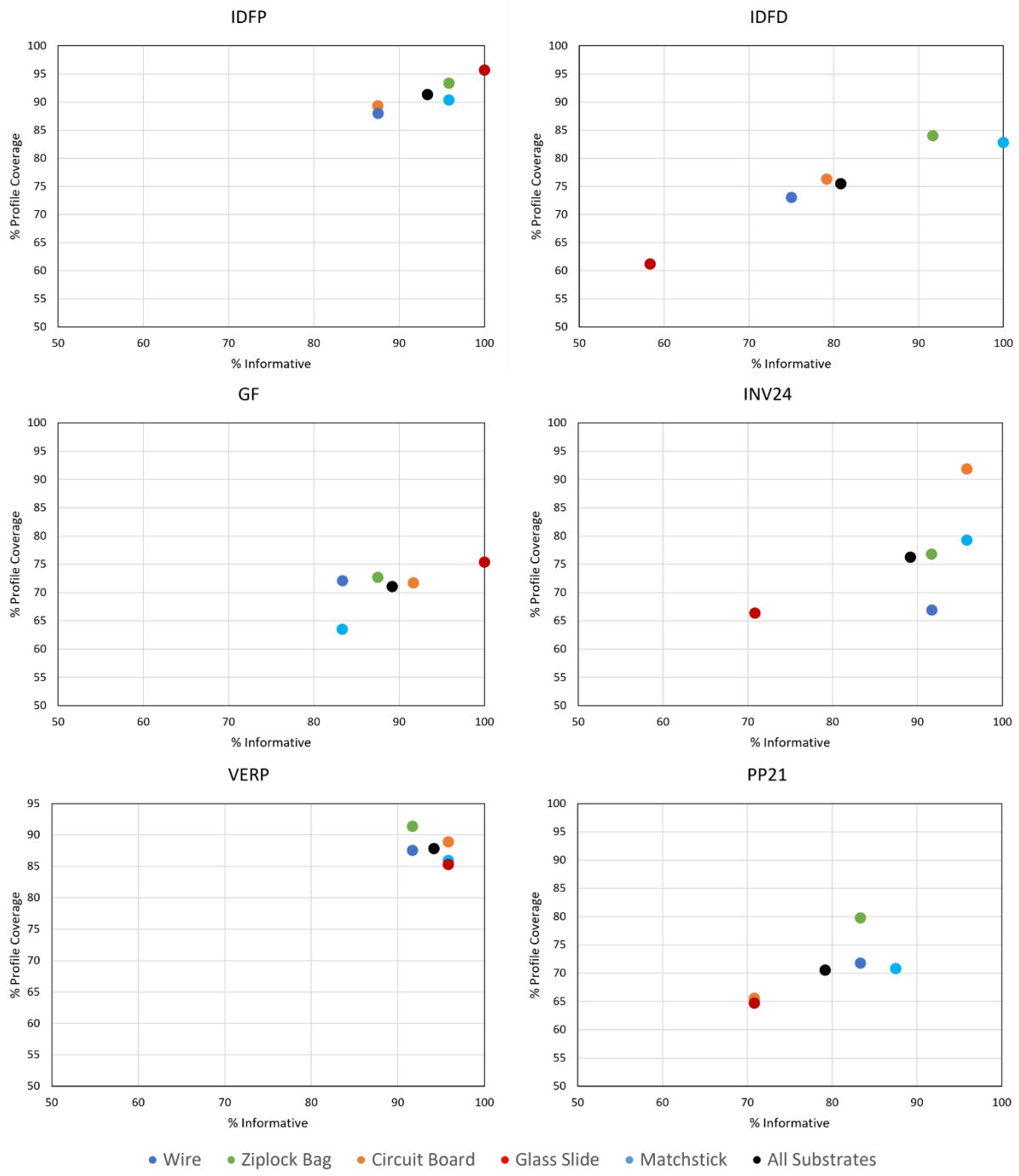
2.3a.7 Conflicts of Interest

The authors declare no conflict of interest.

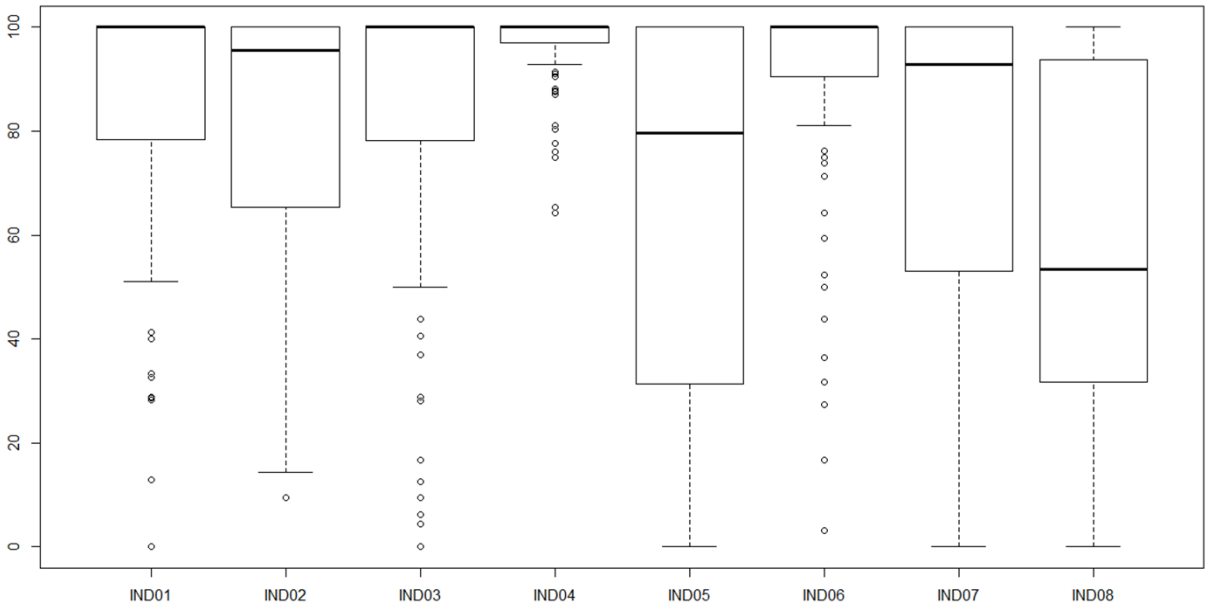
2.3a.8 Supplementary Materials



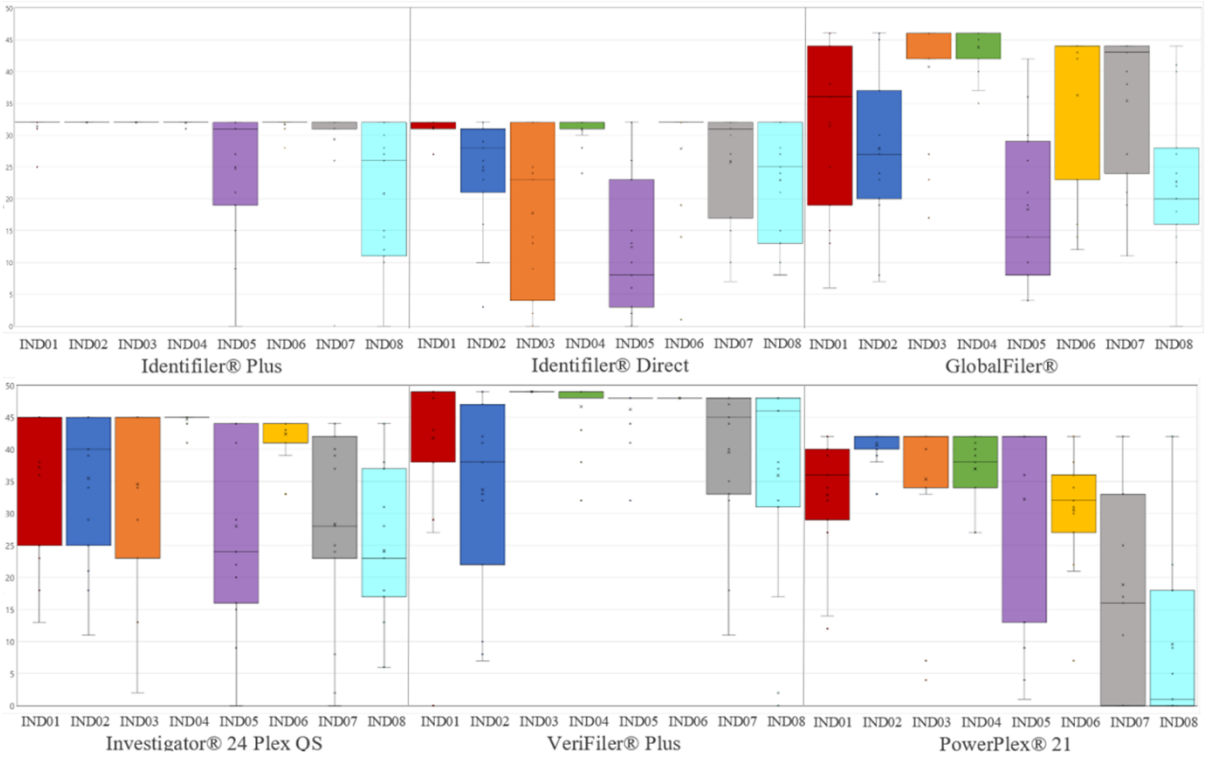
Supp. Figure 2.1. Profile coverage obtained per substrate, observed for all kits for all volunteers. Each volunteer touched each of the five substrates, with triplicate samples amplified by each of the six STR kits analysed, for a total dataset of 144 per substrate kit. STR kits were Identifiler® Plus, Identifiler® Direct, GlobalFiler®, Investigator® 24 Plex QS, VeriFiler® Plus, and PowerPlex® 21.



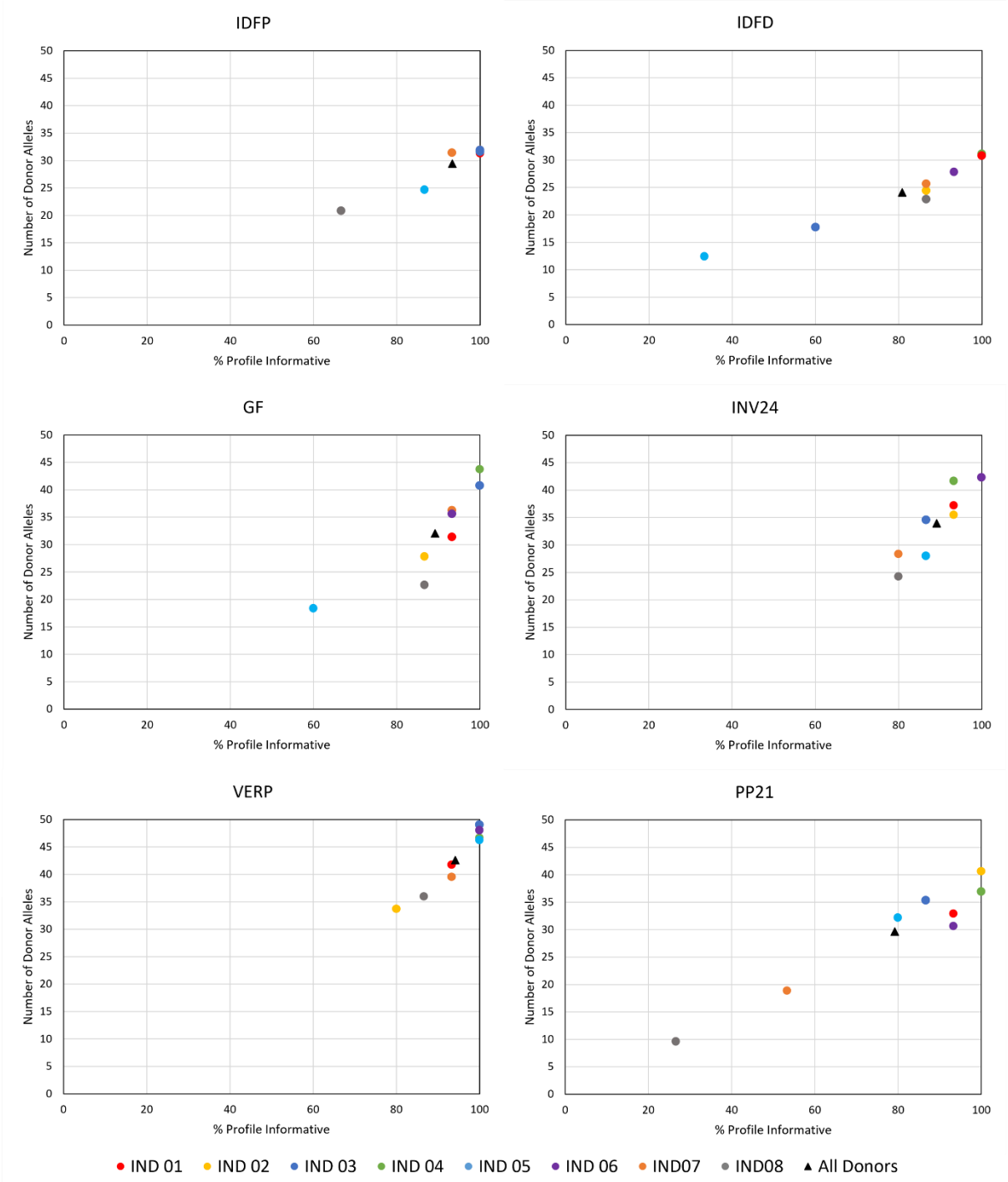
Supp. Figure 2.2. Amplification success per substrate for each kit, as an average across all volunteers, observing the percentage of informative profiles (x) against the profile coverage (y). Each volunteer touched each of the five substrates, with triplicate samples amplified by each of the six STR kits analysed (N=144 per kit, N=24 per substrate data-point).



Supp. Figure 2.3. Percentage of profile coverage across all kits (y) against volunteer (x). Each volunteer touched each of the five substrates, with triplicate samples amplified by each of the six STR kits analysed, for a total dataset of 90 per volunteer. Substrates were a circuit board, a glass slide, a matchstick, an insulated wire, and a ziplock bag. STR kits were Identifiler® Plus, Identifiler® Direct, GlobalFiler®, Investigator® 24 Plex QS, VeriFiler® Plus, and PowerPlex® 21.



Supp. Figure 2.4. Box and Whisker plot containing data representing the number of donor alleles in each sample per kit, with respect to each donor (N=15 per boxplot). All eight donors are represented by a different colour. Horizontal line indicates median number of donor alleles observed in profiles for each dataset. Whiskers indicate the maximum and minimum number of alleles observed in a profile within each dataset. Four volunteers touched five items (insulated wire, ziplock bag, circuit board, glass slide and matchstick) in triplicate for each of the six STR amplification kits analysed.



Supp. Figure 2.5. Amplification success per donor for each kit observing the percentage of informative profiles (x) against the average number of donor alleles per profile (y). Each volunteer touched each of the five substrates, with triplicate samples amplified by each of the six STR kits analysed 90 per volunteer. Substrates were a circuit board, a glass slide, a matchstick, an insulated wire, and a ziplock bag.

Supp. Table 2.1: DNA profiling kits used in this study, and the loci they target

	Identifiler Plus	Identifiler Direct	GlobalFiler	VeriFiler Plus	Investigator 24 Plex QS	PowerPlex 21
D3	Yes	Yes	Yes	Yes	Yes	Yes
vWA	Yes	Yes	Yes	Yes	Yes	Yes
D16	Yes	Yes	Yes	Yes	Yes	Yes
CSF	Yes	Yes	Yes	Yes	Yes	Yes
D6				Yes		Yes
D8	Yes	Yes	Yes	Yes	Yes	Yes
D21	Yes	Yes	Yes	Yes	Yes	Yes
D18	Yes	Yes	Yes	Yes	Yes	Yes
D5	Yes	Yes	Yes	Yes	Yes	Yes
D2S441			Yes	Yes	Yes	
D19	Yes	Yes	Yes	Yes	Yes	Yes
FGA	Yes	Yes	Yes	Yes	Yes	Yes
D10			Yes	Yes	Yes	
D22			Yes	Yes	Yes	
D1			Yes	Yes	Yes	Yes
D13	Yes	Yes	Yes	Yes	Yes	Yes
D7	Yes	Yes	Yes	Yes	Yes	Yes
Penta E				Yes		Yes
Penta D				Yes		Yes
THO1	Yes	Yes	Yes	Yes	Yes	Yes
D12			Yes	Yes	Yes	Yes
D2S1338	Yes	Yes	Yes	Yes	Yes	Yes
TPOX	Yes	Yes	Yes	Yes	Yes	Yes
SE33			Yes		Yes	
Yindel			Yes	Yes		
DYS391			Yes		Yes	

2.3b Expanded Discussion of Manuscript

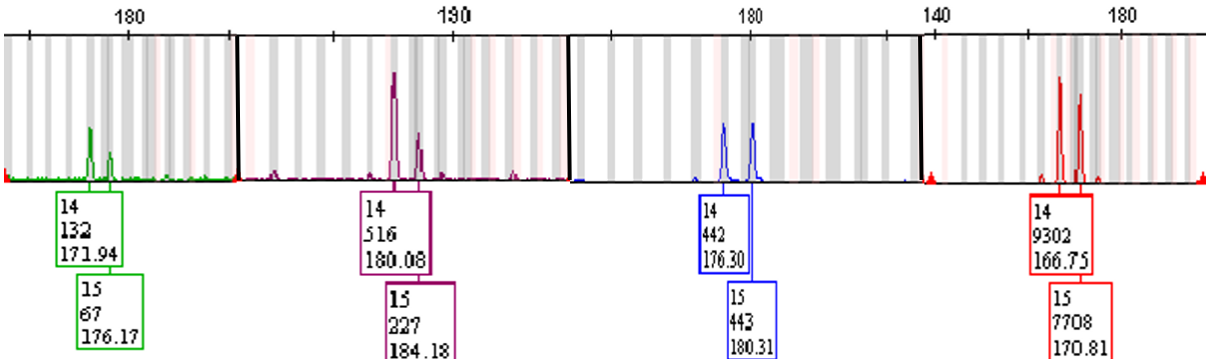


Figure 2.4. Example of peak balance or imbalance at locus D1S1656 in INV24, GF, PP21 and VERP (left to right). All samples are taken from IND 05. INV24 and GF present heterozygote peak imbalance, while PP21 and VERP's heterozygote peaks are balanced.

Heterozygote peak imbalance was observed across many profiles (Figure 2.4) and could complicate analysis of mixed profiles in casework application; this is a well-documented result of low template amplification [10, 220, 230]. To assess peak imbalance, the ratio between heterozygote peaks was considered for all concordant loci across all kits for each volunteer; as no significant difference between substrates is observed, only profiles from ziplock bags were considered for this assessment resulting in 1391 loci being analysed. Peak imbalance between kits, when considered as an average of all heterozygote loci in every profile, was fairly similar (Table 2.2); however, imbalance observed in IDFD was greater than in other kits with 24% of loci presenting imbalanced alleles. When considering the average ratio between all heterozygote peaks due to individual kit amplification IDFD, INV24, and GF presented the lowest balance. PP21, VERP, and IDFP profiles presented the highest average heterozygote peak balance ratio (85%, 83%, 82% balance respectively).

Table 2.2: Percentage of concordant loci displaying imbalanced heterozygote peaks, for all ziplock bag samples considering all volunteers, for each kit. Heterozygote peaks were considered imbalanced if the RFU ratio is <70.

Kit	Imbalanced Loci	Total Loci Analysed	Percentage Imbalanced Loci (%)
GLOBALFILER	39	198	20
IDENTIFILER PLUS	47	264	18
IDENTIFILER DIRECT	60	245	24
VERIFILER PLUS	42	243	17
POWERPLEX 21	32	217	15
INVESTIGATOR 24 PLEX QS	41	224	18

2.3b.1 Ability to Upload

By considering a profile as a) up-loadable or b) not up-loadable it standardises the data to profiles that either are, or are not, up-loadable to a DNA database. These data indicate which kits are advantageous for casework, to maximise the number of profiles considered up-loadable to a database the kit with the highest likelihood of returning an up-loadable profile should be chosen.

Because the maximum number of alleles amplified varies between kits, it would be expected that a larger proportion of profiles were up-loadable from STR kits that amplify more loci, this however was not always observed. Individual laboratories have different guidelines on what profiles are considered up-loadable to their DNA database; for the purpose of this study any profile with 12 or more autosomal alleles was deemed up-loadable. IDFP counterintuitively produced the second largest number of up-loadable profiles within the dataset at 93% (112/120 profiles), even though it is designed to amplify the fewest number of alleles. VERP performed significantly better than all kits (94% considered up-loadable, p= 0.0098) and PP21 significantly poorer (79%, p= 0.0044); there was no significant difference between IDFD (81%), INV24 (89%) and GF (89%). This gives an indication that IDFP and VERP are better suited to the analysis of latent DNA by direct PCR, closely followed by GF and INV24, as a larger proportion of samples amplified returned profiles that were considered up-loadable to a DNA database.

Comparison by logistic regression against IND01, with 96% of profiles generated being considered up-loadable, showed IND03 (89%, p= 0.035), IND05 (74%, p= $3.6x10^{-5}$), IND07 (83%, p= 0.011), and IND08 (72%, p= $8.4X10^{-6}$) produced template resulting in a significantly lower number of up-loadable profiles when amplified with any kit. IND02 (91%), IND04 (99%) and IND06 (97%) showed no significant difference. A Wald chi-squared test, considering the influence that STR kit, donor, and substrate type has on the likelihood of obtaining an up-loadable profile, was performed; there was a significant difference based on STR kit (p= 0.0011) and donor (p= $2X10^{-7}$), however no significant difference was observed depending on substrate type sampled (P= 0.15).

2.3b.2 Applications of Dataset for External Research or Operational Groups

With this dataset, it is possible to predict the probability of obtaining an up-loadable profile given a combination of factors. For example, the 95% confidence bounds on the probability of obtaining an up-loadable profile when IND02 touches an insulated wire, and this sample is amplified using PP21, is between 0.8 and 0.97. These data could act as a guide to predict the probability of obtaining an up-loadable profile on a research sample, or evidential exhibit, when utilising different

kits. To do this the sample would need to have a similar surface type as the substrates tested within this dataset, and the shedder status of the donor would need to be known and compared to a donor within this dataset.

Within this dataset there is the possibility to extrapolate theoretical data output given a range of parameters, including donor shedder status, kit choice and substrate surface type. An estimation as to the quality of the genetic data obtainable through amplification between kits can also be made. As a result, laboratories could predict the success they might expect if they moved to an alternate kit by comparing the data currently obtained within their laboratory to the dataset here from alternate kits.

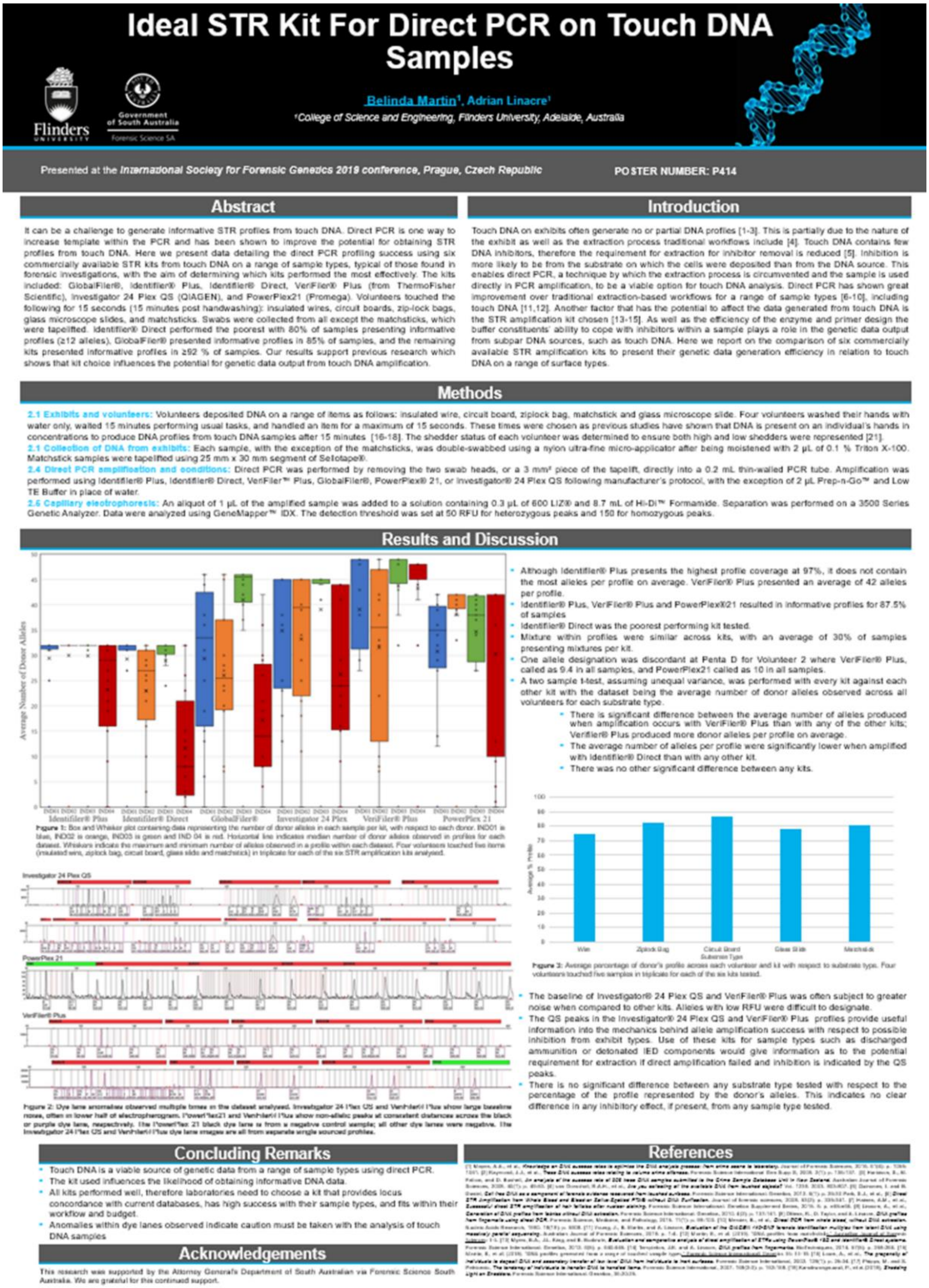
2.4 CONCLUDING COMMENTS

As each step of the analytical process affects the EPG obtained from a sample, it is vital that each are assessed separately for their influence on direct PCR amplification of latent DNA before the technique is widely used for forensic work. This chapter represents an important step in addressing how the PCR process affects direct PCR workflow results and allows consideration for STR kit choice through the comparison of six commercial kits. The data presented within this chapter provide the most comprehensive comparison of extensively used STR kits, currently available. When comparing four 6-dye STR kit systems, comprising five data dye lanes and an internal lane standard lane, four key metrics were used; profile coverage, the number of alleles amplified per profile, baseline anomalies, and profile artefacts. It has been shown that VeriFiler Plus™ was considered a high-performance kit in all metrics with the exception of baseline anomaly presence (low). GlobalFiler®, Investigator® 24-Plex QS and PowerPlex® 21 were inseparable to one another overall; these three kits presented medium-low performance in each metric measured leading to the conclusion that the largest driving factor for STR kit choice for direct PCR amplification from latent DNA within operational forensic facilities is financial. The aim "*to provide empirical data from which laboratory groups can make educated decisions as to the ideal STR kit to utilise within their direct PCR workflows*" has been addressed within this work. As additional kits are produced, analysis as to their performance and application potential to the direct PCR workflow should be performed in a comparative fashion to the currently available dataset.

2.5 APPENDICES

2.5.1 Poster presentation at "HIDS" conference, Kobe, Japan, 2019, 'Maximising the DNA Profile from Touch DNA'.

2.5.2 Poster presentation at "The International Society of Forensic Genetics" conference, Prague, Czech Republic, 2019. 'Ideal STR Kit for Direct PCR on Touch DNA Samples'.



CHAPTER III:

EXPLORING FACTORS INFLUENCING STR AMPLIFICATION SUCCESS FROM TOUCH DNA DEPOSITS

Manuscripts enclosed:

Kanokwongnuwut, P., **Martin, B.**, Kirkbride, P., Linacre, A. (2018). "*Shedding light on shedders.*" Forensic Science International: Genetics

Martin, B., Blackie, R., Taylor, D., Linacre, A. (2018). "*DNA profiles generated from a range of touched sample types*." Forensic Science International: Genetics

Kanokwongnuwut, P., **Martin, B.**, Taylor, D., Kirkbride, P., Linacre, A., (2021). "*How many cells are required for successful DNA profiling?*" Forensic Science International: Genetics

Martin, B., Kaesler, T., Taylor, D., Linacre, A. (2022). *"Analysis of RapidHIT application to touch DNA samples."* Journal of Forensic Sciences

3.1 PREFACE

In addition to STR kit choice, described in Chapter II, there are many factors that influence the success of DNA profile generation with respect to touch DNA that were previously unexplored. It is well understood that surface type plays a role in DNA deposition, persistence, and recovery; therefore, it is important to produce data relevant for a wider range of surface types than currently within the literature. Although previous studies utilising direct PCR amplification have been performed on a range of substrate surface types, there are many surface types that are yet to be investigated. As well as surface type, it is well understood that cellular deposition rates between individuals change, however the effect these deposition rates have on direct PCR amplification profiling success of fingermarks deposited by individuals from different shedder groups was previously unknown. Knowledge of different individuals' propensity to deposit cellular material differs is an important step in understanding the types of profiles able to be obtained from touched items. A DNA donor's shedding capacity offers different PCR template quantities for STR amplification. Data surrounding analytical threshold requirements of starting material is traditionally based off quantification data post-extraction and/or EPG analysis; this has scope to be improved. Knowing more exact template requirement thresholds for the amplification of touch DNA will greatly increase the triaging potential of casework samples and aid in project design or result discussion. With the introduction of rapid DNA profiling to reference samples the question as to the viability of this technology's application to latent DNA processing has been raised by the company, ThermoFisher Scientific.

This chapter is split into four phases, each of which examines multiple factors that affect STR amplification of touch DNA, as a form of latent DNA.

The first phase of the chapter explores the amplification of touch DNA by direct PCR on a range of sample types, typical of those encountered in casework, not previously examined. Direct PCR results were compared to extraction-based PCR with the individual depositing DNA also being investigated as to their effect on the success in STR profile generation following direct PCR amplification. This will increase the fluidity of direct PCR integration into practice as SOPs can be written on the basis of these findings without the requirement for multiple laboratories to partake in laborious validation studies. Results are reported in a peer reviewed publication in *Forensic Science International: Genetics.*

The second phase of the chapter considers how an individual's propensity to shed DNA can be easily identified and what role this plays in the success experienced in STR amplification, through

direct PCR, of a single fingermark. Results are reported in a peer reviewed publication *in Forensic Science International: Genetics.*

The third phase of the chapter explores, in depth, the relationship between the number of deposited cells and the resultant STR profile. Direct PCR and PCR post-extraction were performed following sample collection. Samples consisted of visualised saliva or touch DNA cells that were counted and distributed into discrete categories. Both swabbing and tapelifting were examined to determine whether any difference in amplification efficiency, given the same starting DNA quantity, existed between the collection methods. The number of visualised cells, either buccal or touch, required to produce a full DNA profile varied between cell type and workflow choice. Results are reported in a peer reviewed publication in *Forensic Science International: Genetics.*

The fourth, and final phase of the chapter explored the introduction of rapid DNA profiling, through the RapidHIT system, to latent DNA samples. Results are reported in a peer reviewed publication in *Journal of Forensic Sciences*.

3.2 IMPACT STATEMENT AND AIMS

The paper "DNA profiles generated from touch DNA on a range of sample types" has provided support for the incorporation of direct PCR for the analysis of latent DNA. The work has been cited in reviews describing DNA transfer [337], touch DNA deposits [113], and the recovery of DNA from metal surfaces and ammunition [338]. This work has been referenced by subsequent studies in light of the conclusions drawn with regard to ideal kit use for their workflow [141] and has been utilised to support direct PCR processing of ammunition [137], pipe-bomb [314], and firearms samples by other research groups. These data provide operational and research laboratories with increased understanding as to optimised latent DNA processing from a range of commonly encountered surface types.

The paper "Shedding light on shedders" has enabled a simplified, time and cost effective, method for obtaining highly accurate data pertaining to an individual's shedder status. This has revolutionised the way in which countless research groups are analysing their DNA donors and has identified a relationship between the number of cells present within a fingermark, those which are visualised, and the resultant STR profile that allows increased triage potential for casework samples. This work outlined the value in cellular staining for research applications and the monitoring of cellular material within experimental design [339] or DNA collection [340, 341]. This work has also been cited in continual debates surrounding the importance of shedder status to the forensic community [41,

154] or whether the technique can be used to identify an estimate of template concentration [342]. This has the potential to decrease financial waste through a simplified method for the identification of cellular material present and the probable strength of a resultant profile if processed.

The paper "How many cells are required for successful DNA profiling?" has enabled a demonstrable guideline as to cellular template targets for a number of cell types, sampling techniques, and amplification workflows. This allows laboratories, in both research and operational capacities, to triage samples appropriately. Where low template is expected, these data facilitate the determination of workflow choice, reducing financial waste through accurate triaging methods and robust results. This work also discusses the difficulties of cell-free DNA as a component of touch deposits and how its collection can be optimised. This work has been cited in discussions of DNA profiling success from touch deposits with cellular visualisation correlation [343, 344], and cell-free DNA within touch deposits [345]. The additional work conducted with the Forensic and Analytical Science Service (FASS) has also been used by their personnel to improve exhibit processing where appropriate (personal communication).

Following on from the tangible processing thresholds found through the previously described work, questions arose as to how rapid DNA technology would compare with respect to template requirements. This research, with the RapidHIT instrument, is only the second study to utilise the technology for latent DNA and results obtained provide important guidance to facilities considering its introduction to procedure. In addition to providing the data described above, a close relationship with ThermoFisher Scientific, in relation to their RapidHIT system, for potential improvements or considerations to subsequent iterations of consumables has been made.

3.2.1 Aims

Phase 1: "*To identify whether a relationship exists between the shedder status of an individual and the expected quality of an STR profile once a fingermark is processed*"

Phase 2: "*To investigate further the viability of the direct PCR amplification workflow to a wider range of exhibit surface and sample types*"

Phase 3: "*To discover the visualised cellular material thresholds at which an informative and full DNA profile are able to be obtained given different cell types and workflow choices*"

Phase 4: "*To analyse the potential use of the RapidHIT system for the amplification of latent DNA samples*"

3.3 PHASE 1 EXAMINATION OF A VARIETY OF SURFACE TYPES FOR DIRECT PCR AMPLIFICATION VIABILITY

3.3 Manuscript: DNA profiles generated from a range of touched sample types

Manuscript published: *Forensic Science International: Genetics*, 2018

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**55**	**100**	**75**	**100**	**55**		03.08.21
RB	35	-	5	-	5		05.08.21
DT	-	-	20	-	20		03.08.21
AL	10	-	-	-	20		05.08.21

3.3.1 Abstract

Direct PCR from touch DNA has a range of potential applications in the field of forensic investigation for exhibit examination that, under standard extraction methods, rarely produce informative DNA profiles. Previous studies from 'touch DNA' have focussed on fingermarks created under laboratory conditions. Here we report on successful STR DNA profiling from a range of touched items. Direct PCR, with no increase in cycle number, was performed after eight different sample types, typical of those submitted for forensic investigation, were handled by volunteers for a maximum of 15 s to deposit trace amounts of their DNA. Amplifications were performed using either GlobalFiler® or Identifiler® Plus following manufacturer's instructions. These two kits were chosen deliberately as many laboratories worldwide have adopted and validated them in their workflow, thus allowing for direct PCR to be incorporated within their practises easily. It was found that informative STR profiles were obtained from all eight substrates using both STR kits. Identifiler® Plus out-performed GlobalFiler® in terms of the percentage of alleles amplified using the direct PCR approach. Both generated informative profiles from all items and all individuals, at different rates, with Identifiler® Plus being informative in a larger percentage of samples. GlobalFiler® produced profiles with an average of 60% ± 24% (36 ± 15 alleles) alleles present while Identifiler® Plus produced profiles with an average of 96% ± 4% (31 ± 1 alleles) alleles present. A comparison was made between the direct PCR

approach and subjecting touched samples to a standard DNA extraction process, both using Identifiler®. An average of 4% of profiles were informative for samples that underwent extraction with 100% being informative from the same subset of samples amplified by direct PCR. Our findings further demonstrate the success of direct PCR to enhance the STR DNA profiles from touch DNA. Further, Identifiler® Plus was found to generate informative profiles more often than GlobalFiler®. Direct PCR is fast, simple, and non-destructive of evidence with the ability to generate informative genetic data where standard methods are likely to fail.

3.3.2 Introduction

As cellular material sheds from our skin, DNA will be deposited on a surface as touch DNA in circumstances such as: inserting a bullet cartridge into a firearm; using a mobile phone; inserting a SIM card into a mobile phone; opening and closing ziplock bags; and holding wires and circuit boards, which may be involved in a terrorist act. The ability to obtain informative STR profiles from items such as these, if handled for a short period of time, is very limited. However, the importance of obtaining DNA profiles from trace or touch DNA evidence is increasing, as DNA is considered to be the gold standard in forensic evidence, and touch DNA evidence may be all that is collected from a crime.

There is a growing interest in the use of direct PCR to maximise the amount of DNA profile information obtained from forensic evidence, particularly from trace or touch DNA samples. Since its first application in forensic science in 2010 [259], direct PCR has been applied to single hairs [262], nails [260], fibres [294], bullet cartridges [136], different surface types [143, 193], and more recently fingermarks [193, 264]. Direct PCR has also been the subject of a recent review article where its informative power and benefits in niche application were outlined [204]. The aim of direct PCR is to maximise the amount of DNA collected from the substrate and made available for PCR template, thereby increasing the sensitivity of DNA profiling from trace biological material, by omitting the DNA extraction process. Extraction protocols fulfil the function of removing inhibitors from a sample for the downstream PCR process, which may be essential, for instance in the case of blood where haem is a known inhibitor [268], but less essential for touch DNA, where fewer inhibitors are present and DNA can be found cell-free [112]. It has been reported that extraction methods can result in the loss of 76% of the DNA within a sample [2]. By omitting the DNA extraction step, the process from sample receipt to CE is faster, and cheaper (as no extraction costs, and has fewer tube changes). A consequence of this is that a quantification step is also omitted and there is no sample pool to re-test if the PCR fails or if required by another agency. This can be detrimental only if there is a requirement for the quantity of DNA to be recorded or if the provision for re-testing is mandatory.

Previous studies on direct PCR investigated a single commercial STR kit that was available at the time of their studies. The first use of direct PCR employed SGM Plus® [259] with further studies using NGM SElect™ [262, 264, 294]. As the commercially available kits have increased the number of loci available to amplify, so has the ability of the buffers used to overcome inhibitors [193] as well as the activity of the enzyme. GlobalFiler® is one of the latest commercial STR kits launched by Thermo Fisher Scientific and amplifies 24 loci, comprising 21 autosomal STRs, 1 Y-STRs and 1 insertion/deletion (INDEL). Recently, GlobalFiler® was used to compare the success rate of amplifying DNA from blood, saliva, and semen using direct PCR methods compared to standard DNA extraction processes [253, 258].

The use of a hot start enzyme, where the sample is heated to 95°C for 10 to 15 min, is to the benefit of direct PCR as this heating will break open any cellular material, releasing the DNA into the PCR matrix. By comparison to GlobalFiler®, the AmpFLSTR® Identifiler® Plus amplification kit has a hot start enzyme, and a buffer with different constituents potentially more adept to overcome inhibitors.

We report on data obtained using direct PCR after items, typical of those submitted as part of a forensic investigation, were touched for a short period of time. Eight sample types were chosen, which included a fingermark as a comparative control. The fingermark sample type allowed comparison with previously published results to ensure the data presented here was congruent with previous research [204, 264]. A comparison is made between GlobalFiler® and Identifiler® Plus, using direct PCR methods. Through analysis of the same sample types, and amplification with Identifiler® Plus, we further compare the process of direct PCR with using standard extraction processes.

3.3.3 Methods

Exhibits and volunteers

Four items were chosen to emulate potential real-life exhibits which comprised of: unfired aluminium cartridge case, insulated wire, circuit board, and a ziplock bag. Four volunteers (designated PRI 01, PRI 02, PRI 04, and PRI 05) were used for these tests, and each item was prepared in nine replicates giving a total of 144 samples. The nine replicates were separated, at random, into three groups for a total of three replicates per volunteer per group, these groups being: GlobalFiler® using direct PCR, Identifiler® Plus using direct PCR, and Identifiler® Plus using standard extraction methods.

A set of eight items, including the four previously tested were chosen to extend the number of exhibits. The four additional items were as follows: mobile phone, SIM card, fuse, and glass slide. Seven volunteers (designated PRI 01, PRI 02, PRI 03, PRI 04, PRI 05, PRI 06, and PRI 07) were used for these tests, and each item was prepared in triplicate for a total of 168 samples. PRI 01-04 were male, and PRI 05-07 were female; PRI 01, PRI 02, PRI 04, and PRI 05 are the same volunteers as above.

The shedder status of all volunteers had been previously determined to ensure there was a wide range of DNA deposition rates within the volunteers used. Shedder status was determined following the method of Kanokwongnuwut *et al.*, under review. PRI 01 was found to be a high shedder, PRI 02, PRI 04, and PRI 07 were intermediate shedders and PRI 03, PRI 05, and PRI 06 were poor shedders.

Deposition of DNA

All items were cleaned with 3% bleach, wiped, and allowed to air dry in an isolated clean room, to ensure no DNA was present on the items prior to the deposition of DNA by the volunteers. Negative control samples were collected from a set of cleaned items. Negative controls were performed in triplicate from each item. Participants were asked to wash their hands, without soap, to remove excess cellular and cell-free DNA. They then waited 15 min before touching the items, as per regular use, for a maximum of 15 s. During the 15 min intervals the volunteers conducted normal activities with the exception of wearing gloves or washing their hands again. These times were chosen as previous studies have shown that DNA is present on an individual's hands and profiles were obtainable after 15 min [149, 150, 264].

Collection of DNA from exhibits

Each sample was double-swabbed using a nylon ultra-fine microapplicator (City Dental, Adelaide). Each swab head was moistened with 2 µL of 0.1% Triton™ X-100 (Sigma, Victoria, Australia), with the exception of the unfired aluminium cartridge where 5 µL of 0.1% Triton™ X-100 was added to the exhibit prior to each swabbing action. Sampling area was dependant on sample type; the same areas were targeted with both swabs on small items, while on larger items each swab was used in a different location suspected of being touched.

DNA extraction

DNA extractions were performed using the DNA IQ™ System (Promega, Sydney, Australia) using the 'cotton swab' method, following the manufacturer's protocol, with a final elution volume of 30 µL.

DNA quantification

The DNA in all samples was quantified after the DNA extraction process using Qubit® dsDNA HS assay (Thermo Fisher Scientific, Melbourne, Australia). Quantification followed the manufacturer's protocol for High Sensitivity.

DNA amplification

Direct PCR was performed on each sample using either the GlobalFiler® kit (Thermo Fisher Scientific, Melbourne, Australia) or the AmpFLSTR® Identifiler® Plus kit (Thermo Fisher Scientific) by removing the two swab heads, with a sterile scalpel blade, directly into a 0.2 mL thin-walled PCR tube.

Amplifications were performed in 25 µL following the manufacturer's protocol, 30 cycles using GlobalFiler® or 29 using Identifiler® Plus, with exception of 2 µL of Prep-n-Go™ (Thermo Fisher Scientific) and Low TE Buffer (Thermo Fisher Scientific) replacing water. All amplifications were performed on a ProFlex™ thermal-cycler (Thermo Fisher Scientific). PCR product (1 µL) was added to 8.7 µL Hi-Di formamide and 0.3 µL 600 LIZ® (Thermo Fisher Scientific) and separated on a 3500 Genetic Analyser (Thermo Fisher Scientific).

All extracted samples were processed for STR typing. Amplification of extracted DNA samples were performed in 25 µL using Identifiler® Plus, following manufacturer's protocols, with 10 µL of the DNA extract added to the PCR.

Data analysis

Data were analysed using GeneMapper® ID-X (version 1.4).The quality of the profiles, with respect to peak morphology, peak balance and artefact incidence were observed and the number of alleles present from the donor were recorded. Peaks were recorded if they were of 50 RFU or above. Peaks were considered for homozygosity if they were of 150 RFU or above.

3.3.4 Results and Discussion

All negative controls taken from each item retuned blank profiles or profiles with single peak allele drop-in at low RFU.

Direct PCR vs extraction

To compare data obtained from the direct PCR approach to that obtained using a commonly used extraction process, four volunteers touched four items (unfired aluminium bullet, insulated wire, circuit board, ziplock bag) in triplicate. These 48 samples were analysed using GlobalFiler® or Identifiler® Plus STR kits employing direct PCR and Identifiler® Plus using the extraction method. Identifiler® Plus was used for all extracted samples as it was found to out-perform GlobalFiler® in the direct PCR trials. The difference in the average percentage of informative profiles between substrate types, considering all data for each of the four volunteers, can be seen in Figure 3.1. Each of the 48 samples submitted for extraction were amplified using Identifiler® Plus. Quantification was performed to inform approximate quantities of DNA within the touch DNA samples taken. Of the 48 samples submitted for extraction 17 were quantified as 20 pg/µL or more, which is the quantification threshold

of Qubit. Only 13 of the 48 samples produced any alleles; three of which were informative with 14, 15 and 21 alleles, including amelogenin, and a maximum of 9 alleles were observed in the remaining profiles. These alleles matched those of the volunteers. Insufficient DNA was obtained after the DNA extraction process for successful amplification for the remaining 12 samples. These results were not unexpected as it has been reported that up to 76% of the DNA collected by a swab is lost during an extraction protocol [2] (Supp. Table 3.1).

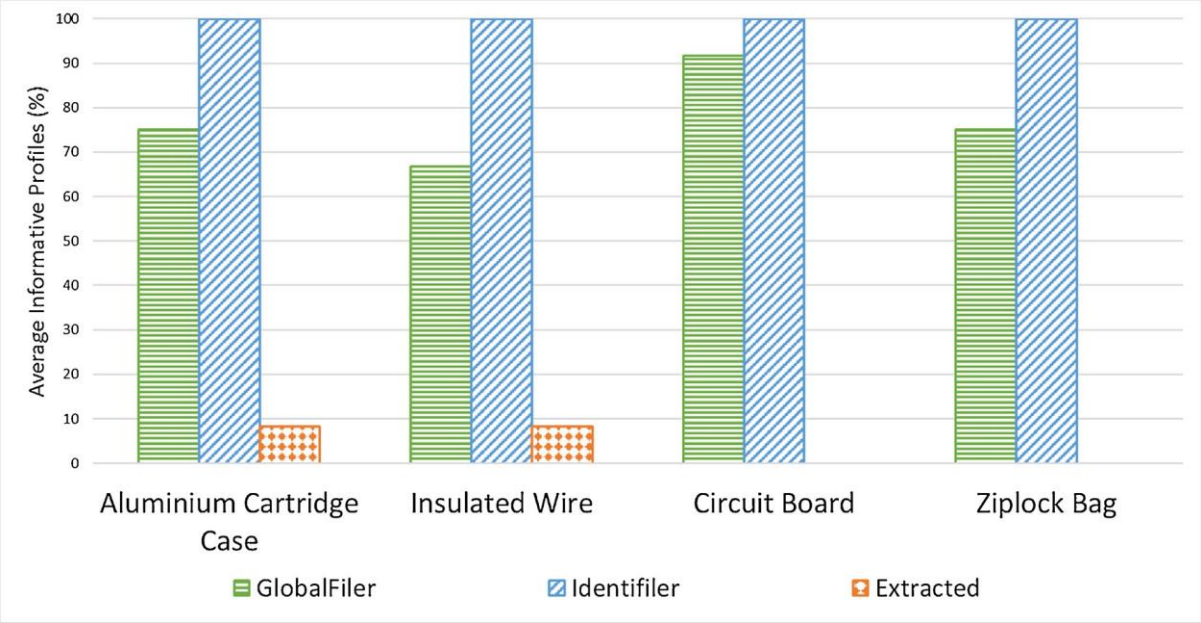


Figure 3.1. Average percentage of informative profiles, containing 12 or more alleles, excluding amelogenin, of all profiles obtained from each sample type. Each of the four participants handled each sample type in triplicate for each processing method. Comparison is between direct PCR using GlobalFiler® or Identifiler® Plus STR kits and the extraction-based profiling method using Identifiler® Plus. No informative profiles were obtained from the circuit board and ziplock bag sample types when an extraction was performed

The poor success of allele amplification after an extraction process contrasts with the direct PCR approach. An overall average of 77% of the 48 samples processed using GlobalFiler® produced informative profiles, compared with 100% of those processed with Identifiler® Plus, and only 6% of those processed with an extraction prior to amplification. As previously noted, samples processed using Identifiler® Plus produced more informative profiles than those processed with GlobalFiler®.

These data suggest that exhibits suspected to contain limited DNA, such as those touched for a short period of time, can be processed by direct PCR with either the GlobalFiler® or Identifiler® Plus STR kits and achieve significantly greater success rates than if the sample underwent extraction prior to amplification. While these data are supported by previous studies [6,7,14], our additional data supports the use of Identifiler® Plus for the amplification of touch DNA.

GlobalFiler vs Identifiler

STR data generated using direct PCR applying either the GlobalFiler® or Identifiler® Plus amplification kits, after four volunteers touched four items for a maximum of 15 s, are shown in Figure 3.2. All tests were performed in triplicate. Full Identifiler® Plus DNA profiles were obtained from all four items touched by volunteers 2 and 4. Data from volunteer 1 shows that full Identifiler® Plus profiles were obtained from 2 of the items (circuit board, ziplock bag) with an average of 98% of alleles being present from the aluminium cartridge case and 99% present from the insulated wire. The average percentage of alleles present, using Identifiler® Plus, for volunteer 5 was 88% for the aluminium cartridge case, 75% from the insulating wire, 91% from the circuit board and 94% from the ziplock bag. The data for Identifiler® Plus contrasts with that of GlobalFiler® where none of the samples touched by the four volunteers generated full DNA profiles. The highest percentage of alleles was an average of 92% for the circuit board (volunteer 4) with the least being an average of 20% for the aluminium cartridge case (volunteer 5).

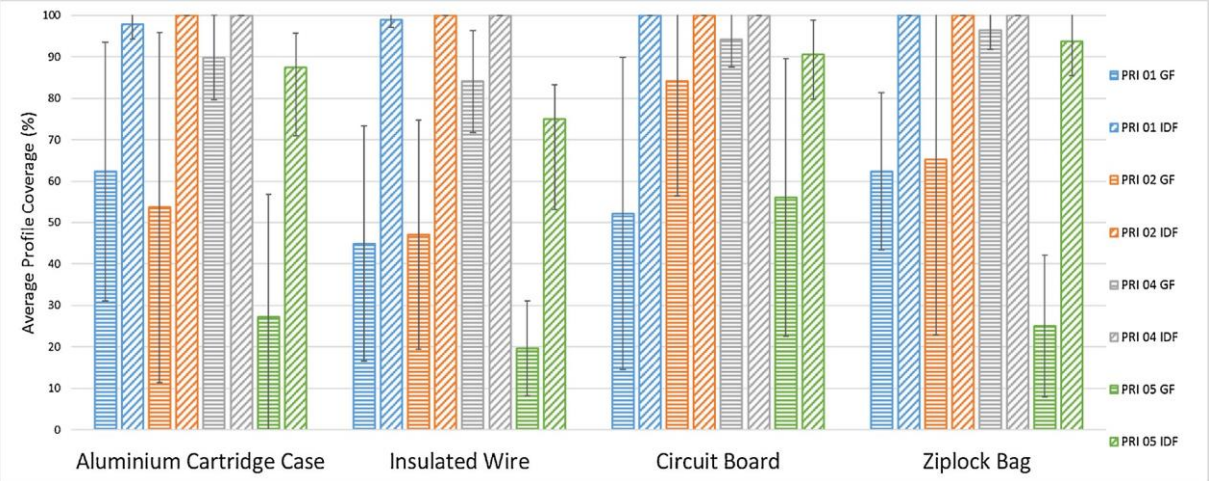


Figure 3.2. Difference in the average percentage of profile coverage (total number of alleles expected are 44 or 46 with GlobalFiler® or 32 with Identifiler® Plus) on each substrate, for each volunteer, using direct PCR between the GlobalFiler® and Identifiler® Plus STR kits. Error bars are the standard deviation in the percentage of profile coverage.

These data are shown using the percentage of alleles obtained considering a full profile. It should be noted that GlobalFiler® amplifies 24 loci, however this includes two Y-chromosome makers that will only amplify DNA from the male volunteers. The total number of alleles expected is therefore dependent on gender; 44 for female and 46 for male. This compares to the 32 alleles, from 16 loci, generated from a full Identifiler® Plus profile.

The profiles analysed using the Identifiler® Plus kit showed consistently higher quality, regarding profile completeness, when compared to those processed using GlobalFiler®. The variation

between replicates in allele presence was also greater when examining the GlobalFiler® samples compared with the Identifiler® Plus samples, shown by the standard deviations in Figure 3.2. It should be noted that an average of 36 (± 15) alleles were present in GlobalFiler® profiles while amplification with Identifiler® Plus generated an average of 31 (± 1) alleles. Although the average number of alleles per profile is higher in GlobalFiler®, compared with Identifiler® Plus, the standard deviation is also much higher therefore for increased consistency in the discriminatory power of the profiles generated it is advised that Identifiler® Plus be used. A comparison between STR profiles generated from one sample type using GlobalFiler® and Identifiler® Plus is shown in Supp. Figure 3.1.

As GlobalFiler® consists of a greater number of loci than present in Identifiler® Plus, a smaller percent of the profile is required to make the profile informative (considered up-loadable to the Australian DNA database). A profile is considered informative when there are 12 or more alleles, disregarding amelogenin, are present.

When considering the informative power of the profiles, Figure 3.3 shows a closer correlation between the kits used. Although the number of GlobalFiler® samples producing informative profiles is similar to the Identifiler® Plus samples in eight of sixteen comparative sets, between sample types per volunteer (Figure 3.3), the same trend is observed as previous; the GlobalFiler® kit is not as efficient at amplifying direct PCR samples as the Identifiler® Plus kit, even when considering the requirement of only 12 alleles. Between 30–100% of profiles produced, depending on the individual, were informative using the GlobalFiler® kit while 100% of profiles produced were informative using the Identifiler® Plus kit from all four volunteers.

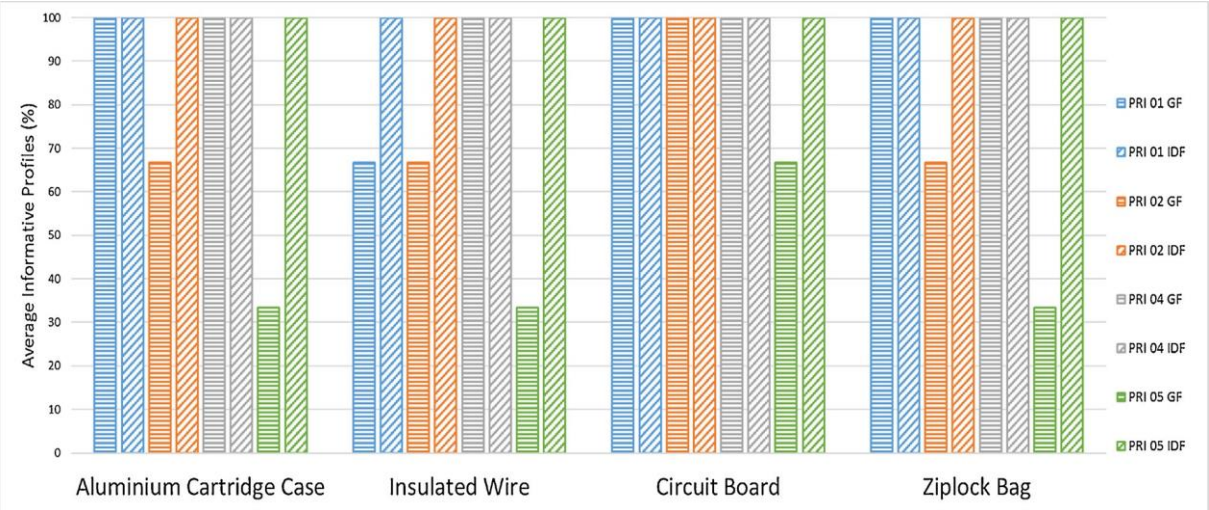


Figure 3.3. Difference in the average percentage of informative profiles on each substrate, for each volunteer, using direct PCR between the GlobalFiler® (left) and Identifiler Plus® (right) STR kits. Error bars are the standard deviation in the percentage of informative profiles. A profile is considered informative when 12 or more alleles, discounting amelogenin, are present.

These data suggest that there is a very high chance of generating informative STR DNA data from items touched for only a few seconds using direct PCR techniques. It is noted that the STR kit chosen has a significant effect on the potential for informative profile generation. The data indicates that Identifiler® Plus should be used to further increase the profiling success potential of touch DNA samples. The difference observed between the kits may be due to the superior buffer found in the Identifiler® Plus kit.

Identifiler substrate trials

Due to the superior data from Identifiler® Plus in the previous study, this kit was used to amplify an extended sample set including eight items touched by seven volunteers (additional items were a mobile phone, SIM card, fuse, and fingermark on glass slide). Cumulative data from these sample types, performed in triplicate, are shown in Figure 3.4.

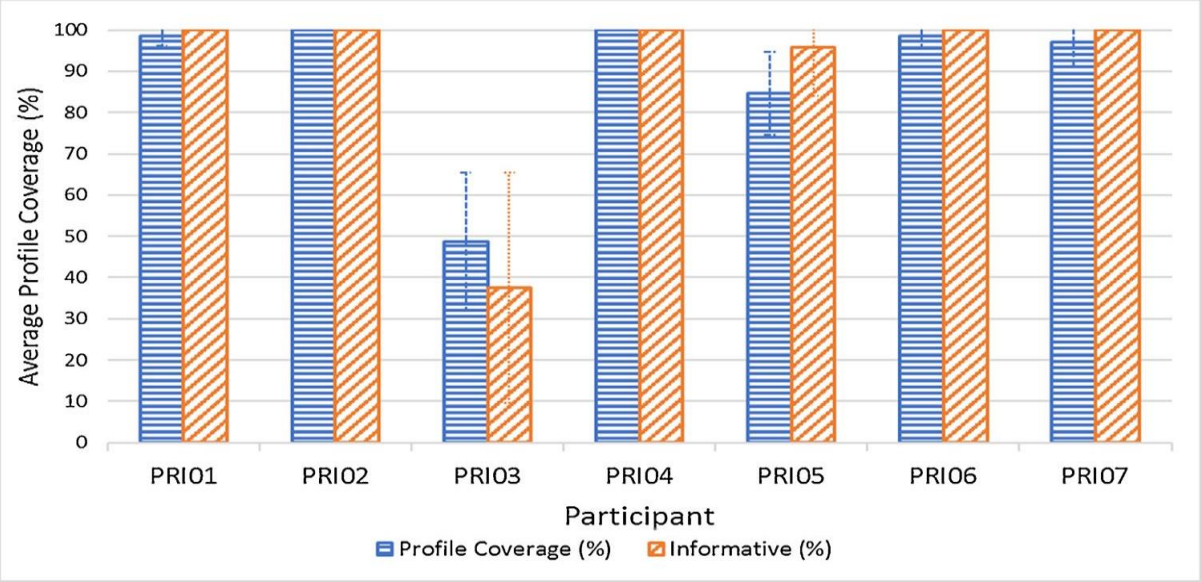


Figure 3.4. Difference in the average percentage of profile coverage (out of a total number of 32 alleles) of all profiles from each exhibit between the volunteers. PRI 0104 were male while PRI 05-07 were female. Each exhibit was performed in triplicate. Amplification was performed using Identifiler® Plus.

Touching these items for a maximum of 15 s resulted in full, or near full, DNA profiles for three of the four males (PRI 01, PRI 02, PRI 04). The exception was volunteer PRI 03, who was a poor shedder, where an average of 49% of alleles per profile were present and an average of 38% of profiles were informative. This is still far more successful that a total average of 4% when extractions were performed. Predominantly male donors presented higher profile coverage, or increased allele numbers, than those from female donors; standard deviations between replicates were also lower (Figure 3.4). This is not unexpected as previous research has shown that males, on average, shed more

DNA than females [281]. The cause of the significant drop in the amplification success of PRI03 is unknown but it may be due to their propensity to deposit DNA, activities performed before item handling, or difference in touching location to that which was swabbed.

PRI05 and PRI06 had been previously determined to be low shedders, therefore it is of note to observe informative profiles generated from items touched by these individuals; this further highlights the potential application of direct PCR to touched items.

Mixed profiles were prevalent in this study with 60% of the samples tested containing two or more contributors. This is in line with previous studies into direct PCR from fingermarks on glass, one of the sample types chosen in this study [8]. This was most likely due to the volunteer's actions having no restrictions during the 15 min post-hand washing, it is expected that a volunteer will touch items during this time that may have been previously handled by others. It has been shown that non-self DNA is present on an individual which would explain the prevalence of mixtures within this study, especially considering the sensitivity of the technique [149, 165, 281, 282, 312]. Mixed profiles often included only a few low-level non-donor alleles and where a larger number of non-donor alleles were present they exhibited a clear major contributor, with the exception of PRI03 whose profiles presented mixed profiles closer to a 1:1 ratio. The alleles generated within the mixed DNA profiles could be deconvoluted when compared against a database containing volunteers, office staff, and laboratory staff. The exception for this was that unaccounted for alleles were present in PRI03's profiles as profiles of individuals with which PRI03 shares common space with were not able to be obtained.

When all data from each volunteer are averaged dependant on sample type, the expected resultant profiles from each object can be assessed (Figure 3.5). Most of the variation seen was due to PRI03. Figure 3.5 contains data from this volunteer to account for the occasion of an equally poor DNA donor in the general population. Even with the lowered mean and increased standard deviations from the average profile coverage percentage, due to PRI 03, a sample processed via direct PCR using the Identifiler® Plus kit produced a profile with an average profile coverage of 82%-96%, dependant on sample type. Glass substrates, such as the fingermark on glass and the fuse, generated fewer loci when amplified than the plastic and metal substrates. The sample produced from the ziplock bag produced the most profile coverage on average. It should be noted that the swabbing method conducted in this study targets the inner surface of the bag opening and the seam along which the bag is closed. This may be in contrast to sampling a large surface area of the exterior of the bag, leading to the potential to collect DNA from a large number of donors if multiple people have had cause to handle the bag.

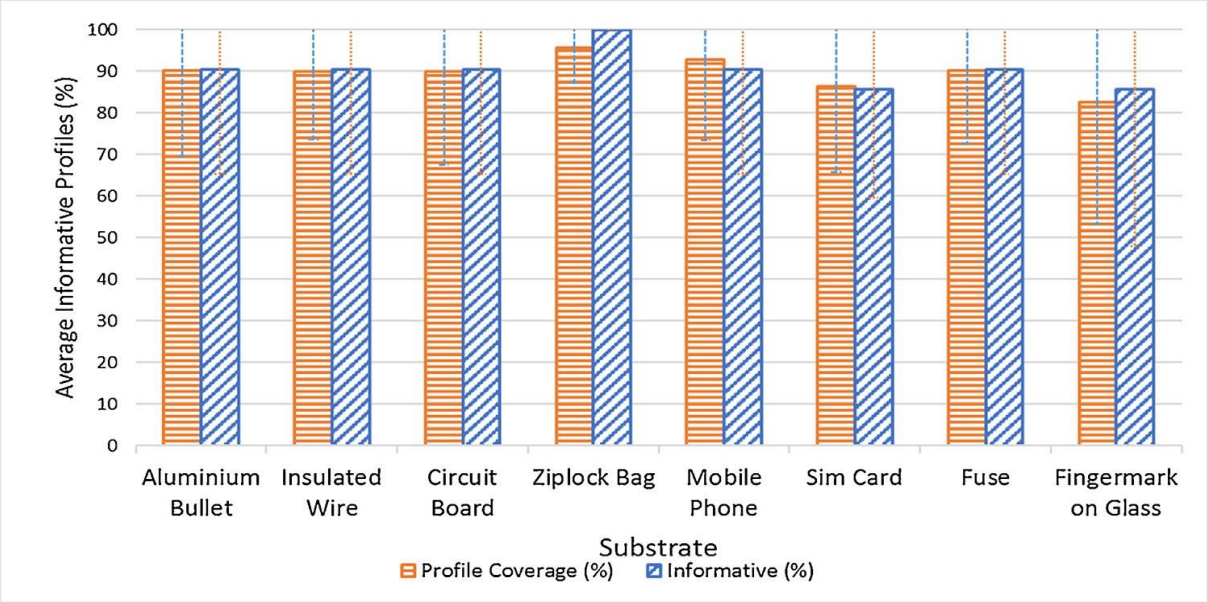


Figure 3.5. Difference in the average percentage of profile coverage (out of a total number of 32 alleles) of all profiles from each volunteer between the exhibits. Each volunteer touched each exhibit in triplicate. Amplification was performed using Identifiler® Plus.

3.3.5 Conclusion

Touch DNA data can be crucial to a criminal case or for intelligence purposes to determine who may have made contact with an exhibit. In this study a range of substrates were chosen that are typical of such items: ziplock bags may be used to store controlled substances, wires and fuses are found in timing devices, communication uses mobile phones and SIM cards, and generating data from cartridge cases has obvious implications. Direct PCR has niche application for exhibits that may be unlikely to produce informative DNA profiles if swabs taken from them are subjected to extraction-based STR typing methods; results post-extraction are particularly unlikely if an individual had made brief contact with an item. Although only 4% of samples processed after extraction, using Identifiler® Plus, produced informative profiles when direct PCR methods were employed 77% of those processed using GlobalFiler® and an overall average of 90% of those processed using Identifiler® Plus produced informative profiles.

A previous study DNA typing touch DNA used only marks after thumbs or fingers were placed on glass [264]. Successful STR DNA typing was performed using NGM SElect™ and highlighted the potential of direct PCR use to obtain STR DNA data from these trace sources. A fingermark on a glass slide was included in this study and data from these samples, amplified using Identifiler® Plus resulted in 86% informative profiles compared with 71% in previous research amplified using NGM SElect™ [264]. This is comparable when considering the differences in the kits used, with respect to total alleles

amplified within the kit and the buffer systems used. Although unfired cartridge cases were tested in this study, testing fired cartridges would represent a more commonly found evidence type.

Mixed DNA profiles were obtained from a number of samples, this was expected as volunteer activities between washing and DNA deposition were not controlled. This would also be expected in casework. These were typically able to be deconvoluted easily without the use of software as there were only a few low-level alleles or a clear major or minor was present. There is potential that the few DNA profiles with ratios closer to 1:1 could be analysed using software such as STRmix™.

Both Identifiler® Plus and GlobalFiler® generated informative STR profiles from touch DNA samples when no, or very few, alleles were obtained if the same touched items were subjected to an extraction process. The extraction process used was the DNA IQ™ System, which uses magnetic beads. Other extraction processes are available, such as Chelex® or solid phase silica-based methods, although previous studies have also shown that these methods result in a high percentage loss of DNA [2, 193, 253].

The evidence presented in this study indicates that the STR kit employed for amplification impacts the quality of the DNA profile obtained. There are three variables within these kits that may be responsible for the advantage Identifiler® Plus displays over GlobalFiler®; this advantage may be due to the inherent benefit that hot start enzymes give, the length of the 95 °C pre-PCR hold or the buffer supplied with Identifiler® Plus having an increased ability to overcome inhibitors and facilitate amplification. Considering the data presented in this study, with the stated restriction in ascertaining the variable of advantage, the recommendation remains that Identifiler® Plus be used in preference of GlobalFiler® for the amplification of touch DNA samples.

3.3.6 Conflict of Interest

The authors declare no conflict of interest.

3.3.7 Acknowledgements

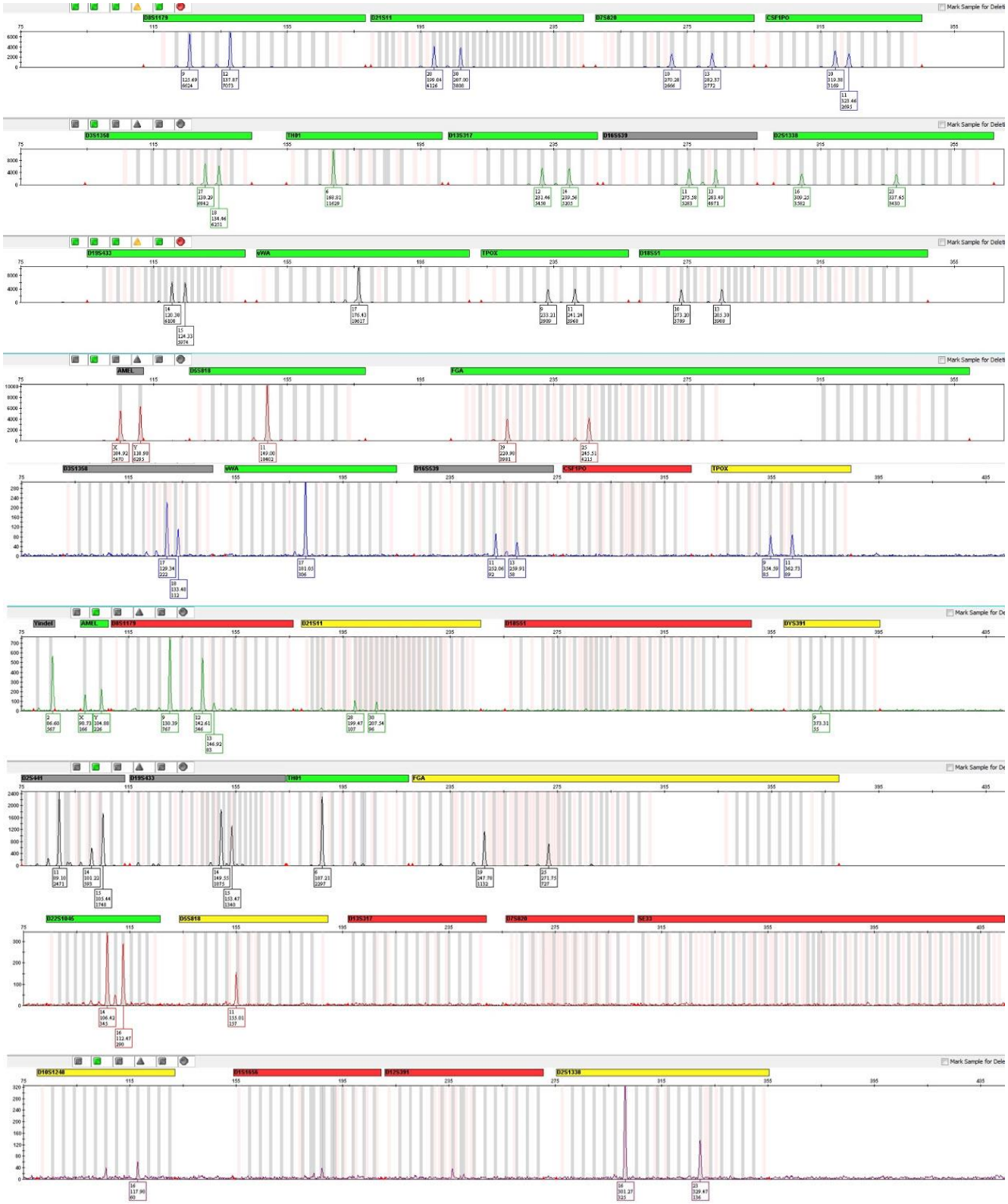
This research is supported by the Defence Science and Technology Group (DSTG) of the Australia Department of Defence and Combating Terrorism Technical Support Office (CTTSO) of the USA Department of Defence. Financial support by DSTG and CTTSO does not constitute an express implied endorsement of the results or conclusions of the research by either DSTG or CTTSO or their respective Departments. We would also like to thank all volunteers that participated in this study.

3.3.8 Supplementary Material

Supp. Table 3.1: Qubit quantification values of extracted samples. Quantification values below the level of detection, 20 pg, are labelled as '-'.

Sample Number	Contents		DNA Quant (ng/µL)	Used for PCR
1	PRI 01 Replicate 1	Aluminium Bullet	-	-
2		Insulated wire	-	-
3		Circuit Board	-	-
4		Ziplock Bag	-	-
5	PRI 02 Replicate 1	Aluminium Bullet	0.023	Y
6		Insulated wire	0.02	Y
7		Circuit Board	-	-
8		Ziplock Bag	0.023	Y
9	PRI 04 Replicate 1	Aluminium Bullet	-	-
10		Insulated wire	-	-
11		Circuit Board	0.025	Y
12		Ziplock Bag	0.025	Y
13	PRI 05 Replicate 1	Aluminium Bullet	-	-
14		Insulated wire	-	-
15		Circuit Board	0.023	Y
16		Ziplock Bag	-	-
17	PRI 01 Replicate 2	Aluminium Bullet	-	-
18		Insulated wire	0.021	Y
19		Circuit Board	-	-
20		Ziplock Bag	-	-
21	PRI 02 Replicate 2	Aluminium Bullet	-	-
22		Insulated wire	-	-
23		Circuit Board	0.022	Y
24		Ziplock Bag	-	-
25	PRI 04 Replicate 2	Aluminium Bullet	-	-
26		Insulated wire	0.030	Y
27		Circuit Board	0.024	Y
28		Ziplock Bag	0.030	Y
29	PRI 05 Replicate 2	Aluminium Bullet	-	-
30		Insulated wire	0.022	Y
31		Circuit Board	-	-
32		Ziplock Bag	0.02	Y
33	PRI 01 Replicate 3	Aluminium Bullet	-	-
34		Insulated wire	-	-
35		Circuit Board	-	-
36		Ziplock Bag	-	-
37	PRI 02 Replicate 3	Aluminium Bullet	-	-
38		Insulated wire	-	-

39		Circuit Board	-	-
40		Ziplock Bag	-	-
41	PRI 04 Replicate 3	Aluminium Bullet	0.021	Y
42		Insulated wire	0.021	Y
43		Circuit Board	0.024	Y
44		Ziplock Bag	-	-
45	PRI 05 Replicate 3	Aluminium Bullet	0.021	Y
46		Insulated wire	-	-
47		Circuit Board	-	-
48		Ziplock Bag	-	-



Supp. Figure 3.1. Comparison of the EPGs obtained using Identifiler® Plus (A) and GlobalFiler® (B) from a ziplock bag touched by volunteer 1.

3.4 PHASE 2 ANALYSIS OF SHEDDER STATUS ASSOCIATION WITH STR PROFILING SUCCESS

3.4 Manuscript: Shedding light on shedders

Manuscript published: *Forensic Science International: Genetics*, 2018

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
PK	65	80	70	80	50		04.08.21
BM	**10**	**20**	**20**	**10**	**20**		03.08.21
KPK	5		5		10		03.08.21
AL	20	-	5	10	20		05.08.21

3.4.1 Abstract

All previous examinations of the shedder status of individuals have been based on conclusions inferred from the amount of DNA deposited by donors after they have held an object for a fixed period of time. In all interpretations of shedder status experiments have involved a range of uncertainties, especially in regards to results arising from studies carried out in different laboratories. These apply to the efficiency of the swab collecting DNA from the item touched, the amount of DNA left on the swab after attempts to recover it, and the percentage loss of DNA during the lysis and extraction processes. No previous study has attempted to mitigate these uncertainties or verify how much of the DNA deposited was collected through swabbing, how much DNA present on the swab was recovered or how much DNA is lost during the extraction process.

We present a study that accurately measures the deposition, collection and amplification of DNA deposited by a range of donors allowing for an accurate determination of the shedder status of individuals. Eleven donors were asked to wash their hands and then deposit a thumbprint onto glass slides by making pressure for 15 s 0, 15, 60 and 180 mins after handwashing. Both left and right thumbs were used, and all testing was performed in triplicate. Measurement of the quantity of cellular material deposited on the slides was carried out using Diamond™ Nucleic Acid Dye (DD) and

fluorescence microscopy on each of 264 thumbprints. Fluorescence microscopy was then used to demonstrate that all the DNA present on the slides was recovered by the swabbing operations and then direct PCR, using the Identifiler™ Plus kit, was used to ensure that none of the DNA present on swabs was lost during DNA profiling.

The combination of using a DNA binding dye and direct PCR allowed an accurate means of measuring the extent to which individuals exhibit different extents of shedding. This small study, 11 donors, showed that individuals fell into one of three distinct groups: heavy, intermediate, and light shedders, regardless of the hand used.

3.4.2 Introduction

There continues to be much research into the transfer of DNA. Central to the means by which DNA transfers during direct contact between skin and a substrate is the shedder status of the person touching an object. The concept of a shedder or non-shedder came from the now well-cited paper by Lowe *et al.* [149]. By holding plastic tubes at a controlled time after handwashing, it was found that some donors had a higher propensity to transfer their DNA than other donors; and so came about the concept of a 'shedder' or 'non-shedder'. There ensued a number of follow-up examinations with initially varying results [150, 282] but comprehensive studies have more recently shown that there is a difference between peoples' propensity to transfer DNA, regardless of gender or hand-dominance [153, 281, 312, 346].

In all previous studies, donors were asked to hold or make direct contact with an object for a defined period of time. This was either after known time intervals post-handwashing or without this knowledge to simulate real-world scenarios [305]. Following the direct contact, an area was sampled with a swab and the process of DNA extraction performed. The method of DNA extraction varied from Chelex® to automated liquid handling systems using either solid phase extraction or magnetic beads. Based on the amount of DNA recovered, and any subsequent STR DNA profile, a conclusion could be made as to the shedder status of the donor. All previous studies started by collecting transferred biological material to a swab, and as any swabbing process is unlikely to collect 100% of the cellular material deposited on contact this process needs to be uniform in collection and the same relative amount of cellular material was removed from every sample collected. During the DNA extraction process the same relative amount of the DNA on the swab needs to be released into the initial buffer used for the DNA extraction process and that the amount of DNA isolated during the extraction process is uniform for all samples tested and that the percentage loss of DNA during this DNA extraction process is the same for trace amounts of DNA compared to large amounts of initial starting DNA.

We present a study where the amount of DNA transferred by contact at specific time intervals post-handwashing, and through subsequent collection using a swab, can be estimated and recorded as a series of images collected using fluorescence microscopy. DNA can be detected by DD as this is a molecule that binds to an external groove in DNA. Importantly, DD cannot bind effectively to microbial DNA and it has recently been shown to detect DNA with no subsequent effect on the amplification process [347, 348]. The dye has no known mutagenic effect at the concentrations used, is very inexpensive, and as it has an excitation of 494 nm and emission at 558 nm such that it can be visualised using a fluorescence microscope with a filter at 510 nm even under ambient lighting. DD has only recently been applied to hair, saliva, skin flake [347, 349] and here it is used specifically to determine shedder status.

Direct PCR gained much prominence when first shown to generate DNA profiles from biological deposits on fibres [259], and has more recently through its use to effectively amplify DNA from fingermarks [134, 264], single hairs [262] nails [260] and items of forensic interest [142]. This current study combines the use of a DNA staining dye with direct PCR to examine the shedder status of volunteers in a simple and easy to perform method. The study was designed to show whether all the DNA transferred at the point of contact was collected by the swabbing method with this process viewed in real-time. Further, by using direct PCR we circumvent the issue regarding unknown loss during the DNA extraction process and can make a direct correlation with the amount of DNA that was collected on the swab and the subsequent DNA profile.

3.4.3 Methods

Approval from the Social and Behavioural Research Committee (reference 7569) was obtained prior to initiating this project.

Deposition of DNA

A total of 11 individuals comprising 5 males (designated M1 – M5) and 6 females (designated F1 – F6) washed their hands using water. Glass slides were used for contact. These were cleaned with 3% bleach, followed by wiping with absolute ethanol, and were then irradiated with ultraviolet (UV) light, by being placed approximately 3 cm below from UV lamp for 15 min, before use to ensure no DNA was present. Control samples were collected from areas of the glass prior to any deposition of a thumbprint. The volunteers made contact with these clean DNA-free glass slides with their left and right thumb for 15 s with medium pressure. The time intervals post-handwashing were: 0 min, 15 min, 60 min and 180 min. All tests were performed in triplicate creating 264 data-sets.

Staining of DNA

The slides were stained with 20-fold dilutions of the stock (10,000 x) solution of DD (Promega, Madison, WI, USA). A Dino-Lite fluorescent digital microscope (AnMo Electronics Corporation, New Taipei City, Taiwan) equipped with an emission of filter of 510 nm and a blue LED excitation light source (480 nm) was used to visualise the presence of dyed material, referred to as cellular material hereafter. Scoring of cellular material abundance was performed by counting the number of bright spots in three frames (each 1 mm^2) under the microscope at 220 × magnification.

Collection of DNA

A micro-applicator (ultra-fine) swab (City Dental, Adelaide, Australia) was used to collect material from the slides. The swabs were moistened with 2 µL of 0.1% Triton™ X-100 (Sigma, Victoria, Australia) solution and applied to the entire thumbprint on each slide.

Amplification of DNA

Direct PCR was performed using the Identifiler Plus™ kit (Thermo Fisher Scientific, Melbourne, Australia) by removing the swab head directly into a 0.2 mL thin-walled PCR tube. Amplification was performed in 25 µL, with exceptions to the validated protocol being 2 µL of Prep-n-Go™ (Thermo Fisher Scientific, Melbourne, Australia) and Low TE Buffer (Thermo Fisher Scientific, Melbourne, Australia) in place of water. All amplifications were performed using a ProFlex thermal-cycler (Thermo Fisher Scientific). The total number of cycles was the validated 29 cycles. PCR product (1 µL) was added to 8.7 µL Hi-Di formamide and 0.3 µL 600 LIZ™ (Thermo Fisher Scientific, Melbourne, Australia) and separated on a 3500 Genetic Analyser (Thermo Fisher Scientific, Melbourne, Australia). Data were analysed using GeneMapper ID-X (version 1.4).

3.4.4 Results and Discussion

An image of the fingermark deposited on a glass slide and stained with DD is shown in Figure 3.6 A. An area not touched by the donors, but stained with DD, is shown in Supp. Figure 3.2 to illustrate that no background fluorescence was recorded from the glass substrate. This allowed the verification that all the cellular material visualised within the fingermark samples were deposited by the donor. As indicated previously, DD cannot bind effectively with microbial DNA therefore it can reasonably be assumed that the dots represent the location of human DNA deposits on the slides. The dye used binds with cell-free DNA as well as DNA within a nucleus, however, it is possible that dyed, cell-free DNA would be too small to observe as discrete spots using fluorescence microscopy and may contribute to the background fluorescence. DD does not bind efficiently to single-stranded mRNA therefore although there may be some fluorescence from mRNA, therefore it has been assumed that the majority of the material visualised with the dye is from cellular DNA. It has been reported that cell-

free DNA is deposited by touch [112] and both this cell-free DNA and DNA within nuclei will be deposited for potential collection. Figure 3.6 B shows a swab in the act of removing cellular material. A 'clean' area on the slide is visible where cellular material has been removed from the slide and transferred onto the swab. Figure 3.6 C shows the same area after swabbing the entire area to confirm that all the stained cellular material that was deposited had been effectively removed. Figure 3.6 D shows the same swab with stained material now placed directly into the PCR tube, confirming that the cellular material collected was now a template for direct PCR. A sterile swab and a sterile swab stained with DD were placed into PCR tubes and visualised, as shown in Supp. Figure 3.3, to demonstrate the lack of background fluorescence when compared to the swab that collected cellular material.

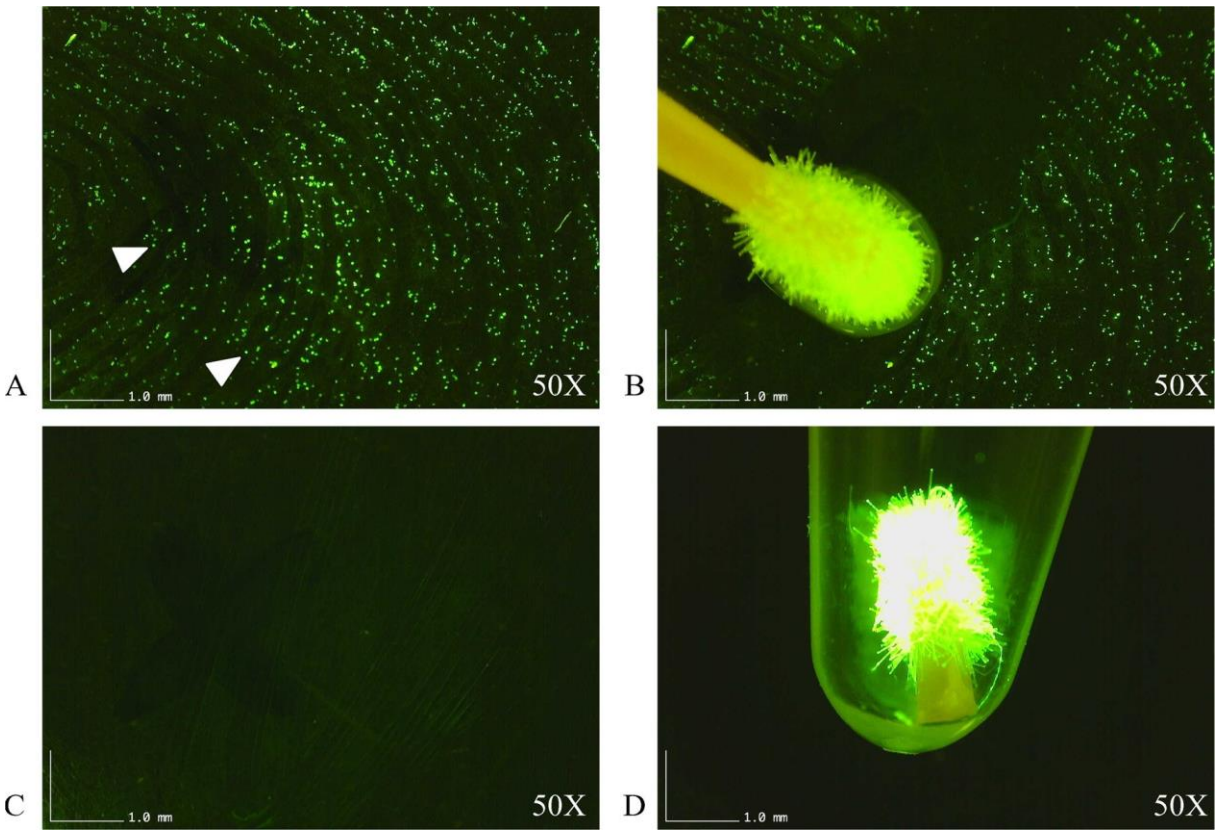


Figure 3.6. Illustrating four steps in the collection of DNA from touched glass slides. Image A shows the fingermark stained with DD. Individual ridges are visible with numerous nuclei associated with the pores (examples indicated by the blocked arrows). B shows a micro-swab in the act of removing cellular material. The swab had been moved back and forth diagonally to collect the cellular material, leaving a clean area either side of the swab head. C shows the sample area post swabbing with a now non-stained surface. D shows the material that was on the surface has now been transferred to the swab and is in a PCR tube for downstream processing. All images were taken at 50 x magnification.

The amount of cellular material deposited on each glass slide was recorded, in triplicate, for each of the 11 volunteers' thumbprints at the four time points (time point 0, 15 min, 60 min and 180 min post-handwashing). These data are shown in Figure 3.7. It is evident that between time point 0 and 15 min there is a large increase in the amount of cellular material available for deposition within the thumbprint. The accumulation plateaus by 1 h post-handwashing.

The 11 donors appear to fall into one of three categories, as evident in Figure 3.7. Two male donors fall into the heavy shedder status. Four female donors fall into the light shedder status. The remaining 5 donors fall into an intermediate shedder status and comprise 3 males and 2 females. These three categories have been proposed previously [155] and although supported by this study there is evidence of a gradation of shedder status from heavy to light.

Direct PCR was performed on samples taken 60 min post-handwashing using the Identifiler Plus™ kit. This amplifies 15 STR loci plus amelogenin. A full DNA profile would therefore include 32 alleles, including amelogenin. In concordance with the DD staining data, there are three broad trends present in the STR data when considering the correlation between the number of cells (cell/mm^2) and the total RFU across the DNA profiles (Figure 3.8). A correlation is demonstrated by an R^2 of 0.92175 when calculated based on data from 9 donors of the 11 donors, with outliers being F5 and F6. These data support a correspondence with the shedder designation outlined in Figure 3.7, with only minor variations. Figure 3.8 indicates that amplifiable DNA is only detected when dyed deposits are visible using fluorescence microscopy. At this initial stage of research, it is not clear whether or not cell-free DNA can be detected using the techniques described here.

The donors that fell in the heavy shedder status based on cellular deposition, M1 and M3, produced full profiles with large RFU values about 50,000 in total RFU value, as expected (as see in Figure 3.8). The donors that fell in the light shedder status based on cellular deposition, F1, F2, F3, and F4, did not produce full profiles, as expected. Their resulting partial profiles averaged 47% profile coverage. There is some variation seen in the intermediate shedder profiles with respect to profile coverage and profile RFU. Three of the donors that fell in the intermediate shedder status based on cellular deposition, M2, M4 and M5, produced full profiles, although with reduced total RFU values approximately 20,000 RFU as see in Figure 3.8. The remaining two intermediate shedder donors, F5 and F6, generated partial profiles with 77% of alleles recorded and lower total RFU value when compared with male intermediate shedders. The total RFU value obtained from the fingermarks of F5 and F6 exhibited, on average, higher RFU values compared to light shedders, which are about 3500 and 1000 RFU respectively; this was not predicted by their cellular material count (Figure 3.7).

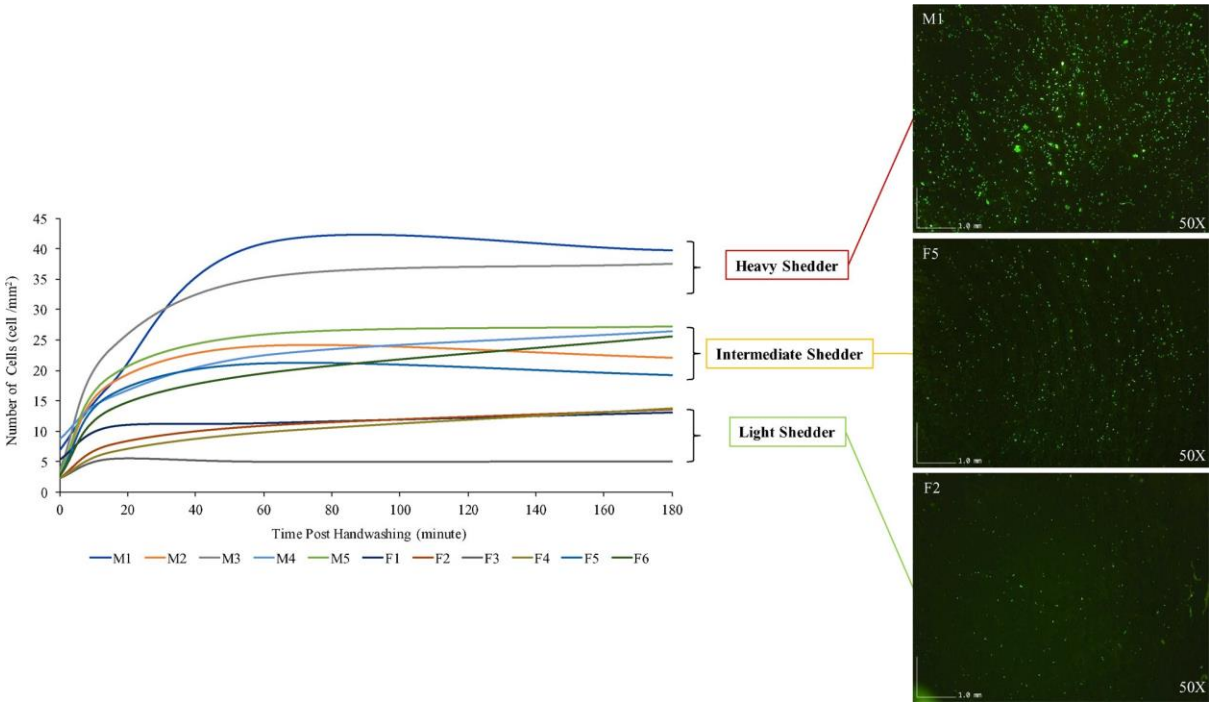


Figure 3.7. Illustrating the amount of cell nuclei deposited at four different time points. In all cases the 11 donors washed their hands and then made contact with a clean glass slide for 15 s with medium pressure. The times post-handwashing were 0, 15, 60 and 180min. The tests were performed in triplicate and an average shown. Data points were joined using 'scatter with smooth lines' options using Excel. Stained fingermarks post-handwashing for 60 min in each category is demonstrated to the right.

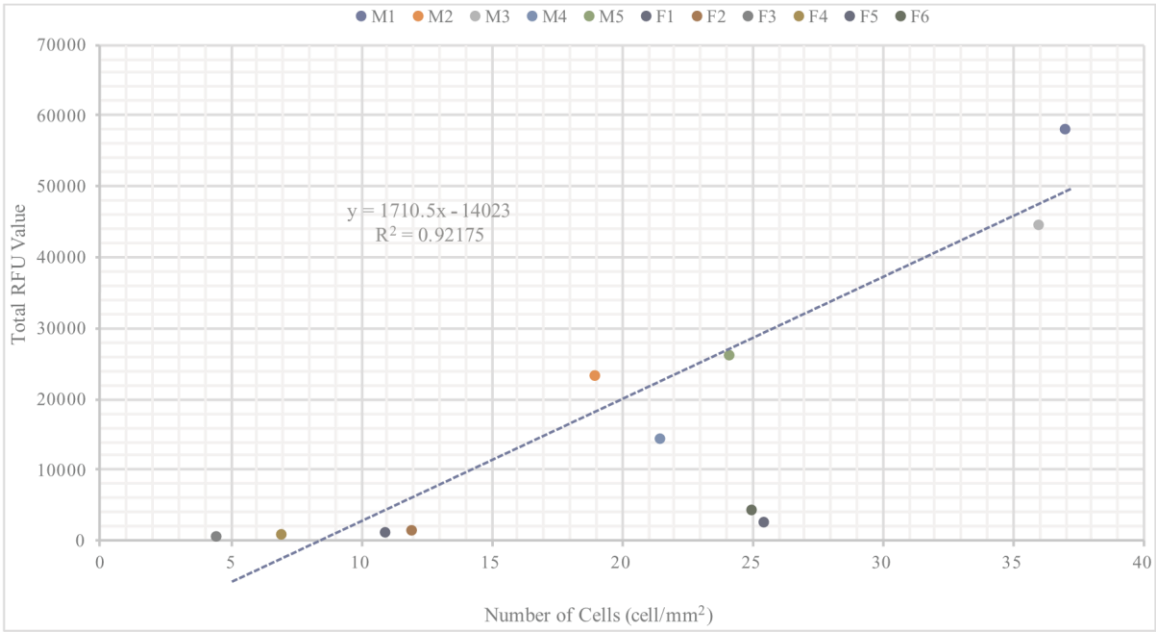


Figure 3.8. Illustrating the correlation between the total RFU value (y-axis) and number of cells (cell/mm²) (x-axis) both left and right thumbprints deposited 60 min post-handwashing. The coloured dots indicate the data for the 11 volunteers (5 males and 6 females) noted above.

Based on these DNA data there is a distinction between the four light shedders and the intermediate shedders when considering the quality of the profiles produced, with the exception of F5 and F6. The distinction between the heavy shedders and the intermediate shedders is less obvious, indicating more of a continuum in shedder status. As the intermediate shedders, who fall in the middle STR RFU group, produced full profiles, or near complete partial profiles, there is no difference in allele coverage when compared to the heavy shedders (Figure 3.9). There is an increase in the RFU in the high shedder compared with the intermediate shedder samples at each locus, an average of 3256 compared with 1312 RFU, as would be expected due to the increased cellular material available as a template. The order in which the participants were scored, in comparison to one another, in terms of shedder status (Figure 3.7) and average RFU across the profile was consistent. This displays a correlation between the amount of cellular material observed, using the staining technique, and the resulting STR profile.

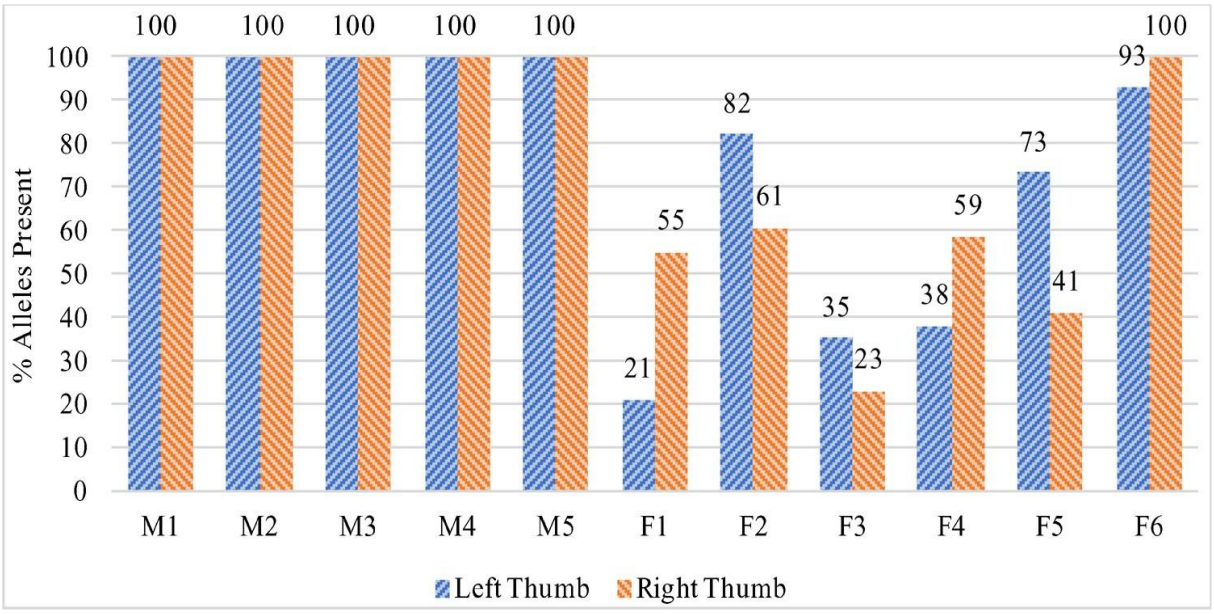


Figure 3.9. Illustrating the resulting Identifiler Plus™ profile coverage, in percentage, of the left and right thumbprints from each participant. This is based on 30 STR alleles, plus the two amelogenin markers from each profile.

There are two considerations regarding the STR profiles: one being the component of cell free DNA and the other the size of the fingermark. It is noted that with the DNA collected that there will be not only DNA within cellular material but also a quantifiable amount of cell free DNA. In forensic applications, the entire fingermark would be targeted to maximise profiling success, therefore the entire thumbprint was sampled within this study. The shedder status is based on the amount of cellular material transferred (as shown in Figure 3.7) and this will vary with natural variation in

thumbprint sizes among the 11 donors. It is noted that F5 and F6 were classified as intermediate shedders based on cellular deposition, however full DNA profiles were not obtained. As well as thumbprint size, the miscorrelation observed may be due to a difference in the ratio between cellular and cell-free DNA present on the participant's hand. The profiles obtained, although poorer quality than the other intermediate shedders, were still informative and would be able to be uploaded to many criminal justice DNA databases for comparison.

Triplicate samples from each person showed little variation in the amount of cellular material deposited for each independent time point. There was also little variation between the left and right thumbprints with respect to the amount of cellular material deposited. An example of this intra-personal variation is shown in Figure 3.10. The deposition of cellular material shows the same trend regardless of which thumb is used. This is contrary to the findings of Allen *et al.* [155].

Supp. Table 3.2 provides the data for all the time points for the left and right thumbprints. Although the amount of cellular material was of little difference between thumbs, there was variance observed in the quality of the DNA profiles when considering the RFU values, for all participants, and the number of alleles generated, for 6 of the donors. The number of cells (cell/mm^2) of all donors is presented in supplemental Supp. Figure 3.5 at 60 min time interval after hand washing. The reproducibility of this test was further demonstrated using a left and right hand dominant volunteers and a high, intermediate and light shedder (M1, M3, F3, F4 and F5) at 0, 15, 60 and 180 min post-handwashing data shown in Supp. Figure 3.5).

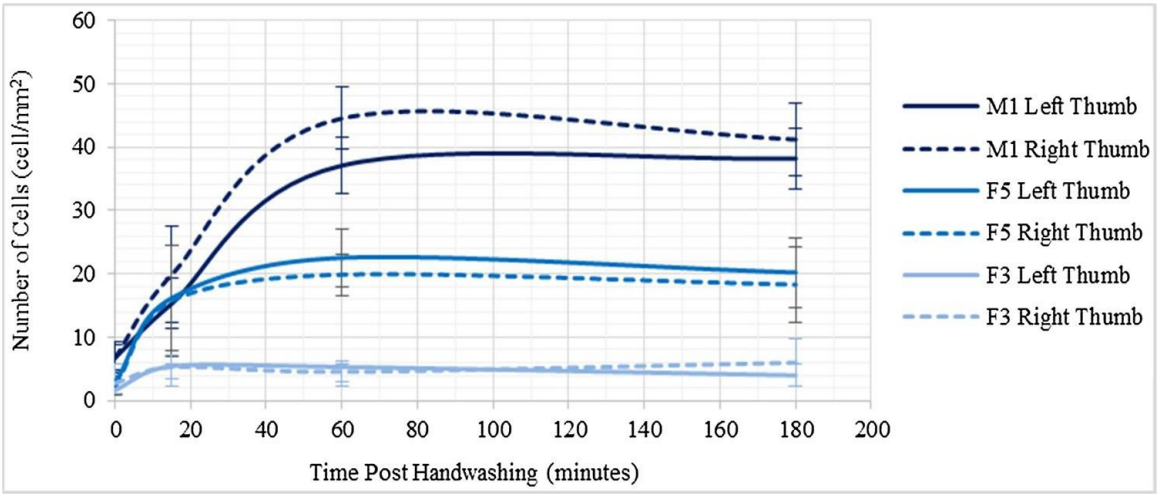


Figure 3.10. Showing the variation in the number of cells deposited at each of the four time points post-handwashing for 3 of the donors. Testing was performed in triplicate for both thumbs. M1 and F3 are left-hand dominant and F5 is right-hand dominant to ensure sampling of both handiness and M1 is a heavy shedder, F3 a light shedder and F5 an intermediate shedder to represent the extremes and intermediate shedder types.

The concept of this study was to demonstrate that individuals do have a reproducible shedder status. As a by-product, it is possible to rapidly determine the shedder status of a specific person of interest as this may be relevant in determining the likelihood of whether a major contributor in a mixed DNA profile was the last person to make contact with an item [311]. This question can be readily addressed if required.

3.4.5 Conclusion

We have shown that it is possible to determine the shedder status of an individual. The application of a DNA binding dye allowed the number of cells deposited on a substrate to be counted and an accurate estimate recorded. The process described allows the transference of cellular material to a swab head to be visualised and therefore the effective removal of the cellular material from the slide to be verified. All the cellular material on the swab is then available as a substrate for PCR and the vagaries in both the collection phase and DNA extraction process are effectively circumvented. The data presented illustrates the use of DD to visualise latent DNA and could be used to indicate the potential, or otherwise, of generating an informative DNA profile. DD has no known mutagenic effects at the dilutions used, is relatively inexpensive, and has the benefit of being able to be visualised under the microscope in ambient light.

Combining the ability to observe the presence of cellular material transferred by touch and its recovery with the process of direct PCR minimizes uncertainties that otherwise are significant when determining the shedder status of an individual. Our data support the previous reports that males shed more DNA than females. This may be affected by the size of the area of fingermark where a larger mark will inevitably contain more cellular material. In the study of generating DNA profiles from fingermarks [264] a thumb print was found to generate more alleles than a pinkie, again reflecting the size of mark created. The size of mark and therefore the effective area sampled needs to be factored when considering the propensity of an individual to transfer their DNA.

The dominance of hand had been examined previously to see if this affected the amount of DNA transferred [4, 194, 281, 282, 305]. For each individual, the data obtained from right and left thumbs, which should have approximately the same area and shedder status, indicate that effectively there is no difference if contact is made by the dominant or non-dominant hand.

Our data also support the proposition that some persons were heavy shedders and others light. Five of the eleven volunteers were intermediate to these extremes, which indicates that there may be a continuum of shedder status with many falling between these two extremes of heavy and light shedders. These results have been seen in previous studies [4, 150, 166, 194, 281, 282, 305] and are supported by this new study.

3.4.6 Conflict of Interest

None

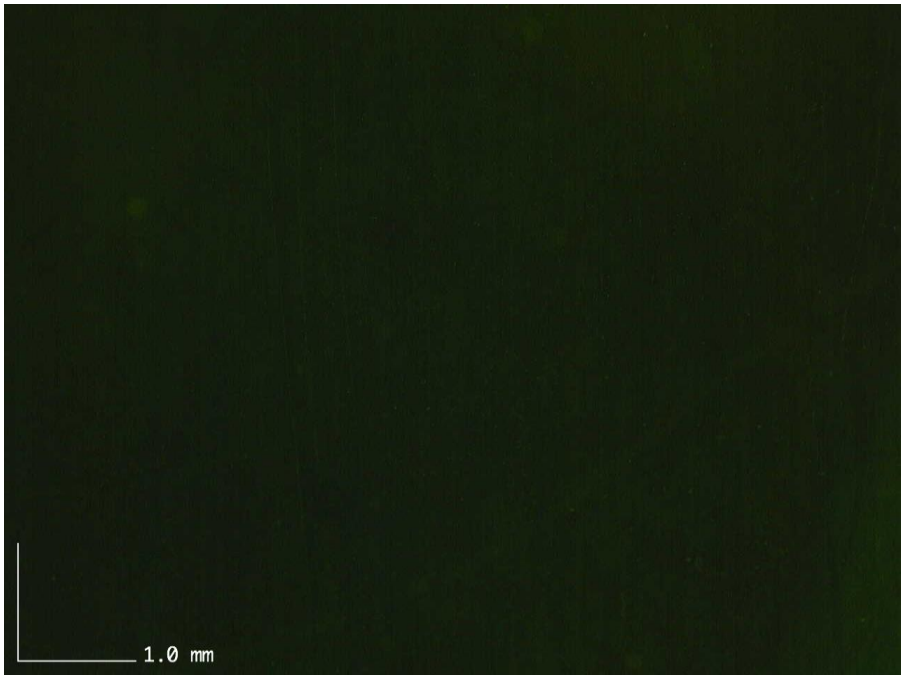
3.4.7 Funding and Acknowledgments

Funding was for this research was provided by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund. The Development and Promotion of Science and Technology Talent Project (DPST), Royal Thai Government Scholarship supported Piyamas Kanokwongnuwut.

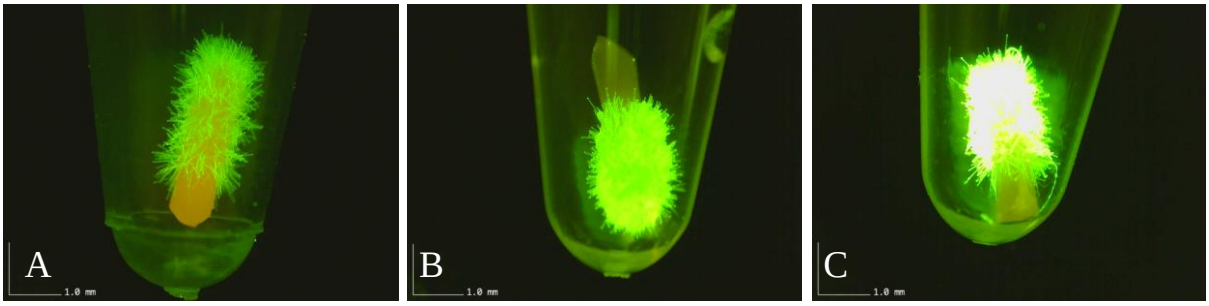
3.4.8 Supplementary Material

Supp. Table 3.2: The number of cells within left and right thumbs of respective donor in each category in four time points post handwashing.

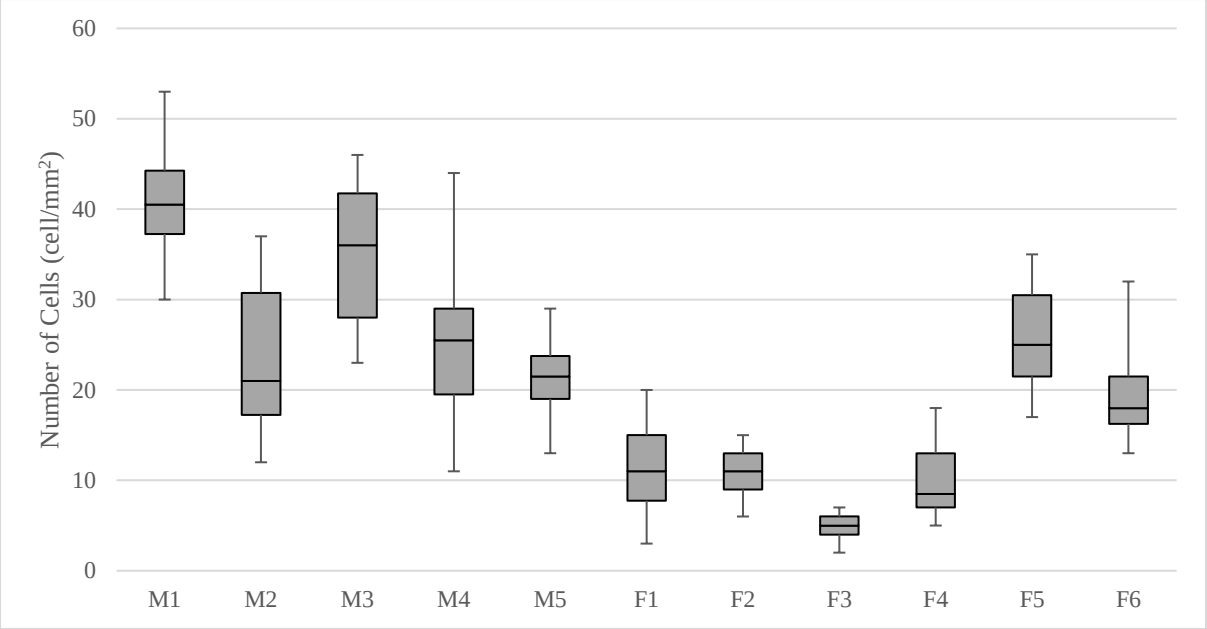
Left Thumb (LT)							Right Thumb (RT)					
Replicate	Name	Frame	Number of cell post handwashing (min)				Name	Frame	Number of cell post handwashing (min)			
			0	15	60	180			0	15	60	180
1	M1	1	8	19	35	36	M1	1	9	27	40	38
		2	6	20	45	43		2	9	34	41	40
		3	10	21	40	42		3	10	26	41	32
2	M1	1	5	11	37	37	M1	1	5	15	42	47
		2	5	13	41	47		2	3	13	45	51
		3	4	11	38	38		3	4	10	40	42
3	M1	1	7	12	34	33	M1	1	8	19	53	38
		2	7	15	34	34		2	7	18	50	45
		3	9	16	30	34		3	6	17	49	38
	Average		**6.78**	**15.33**	**37.11**	**38.22**	**Average**		**6.78**	**19.89**	**44.56**	**41.22**
	SD		**1.99**	**3.91**	**4.48**	**4.79**	**SD**		**2.44**	**7.66**	**4.93**	**5.72**
1	F5	1	2	26	29	15	F5	1	1	22	21	17
		2	4	29	27	19		2	4	30	23	18
		3	4	25	24	16		3	2	25	21	11
2	F5	1	1	11	13	16	F5	1	1	16	21	12
		2	1	13	24	18		2	1	18	25	14
		3	1	16	19	16		3	1	12	14	16
3	F5	1	6	8	22	27	F5	1	4	5	18	25
		2	4	10	23	26		2	5	7	19	26
		3	3	8	22	29		3	2	7	17	26
	Average		**2.89**	**16.22**	**22.56**	**20.22**	**Average**		**2.33**	**15.78**	**19.89**	**18.33**
	SD		**1.76**	**8.27**	**4.61**	**5.52**	**SD**		**1.58**	**8.77**	**3.30**	**5.94**
1	F3	1	2	7	5	3	F3	1	1	6	4	11
		2	4	10	7	5		2	1	5	7	9
		3	2	4	4	3		3	1	7	4	12
2	F3	1	1	3	4	6	F3	1	4	2	6	6
		2	2	3	6	6		2	2	6	6	5
		3	1	2	5	4		3	2	3	4	4
3	F3	1	1	5	6	1	F3	1	4	5	5	1
		2	1	6	5	5		2	4	8	3	3
		3	1	9	6	3		3	6	6	2	3
	Average		1.67	5.44	5.33	4.00	**Average**		2.78	5.33	4.56	6.00
	SD		**1.00**	**2.79**	**1.00**	**1.66**	**SD**		**1.79**	**1.87**	**1.59**	**3.84**



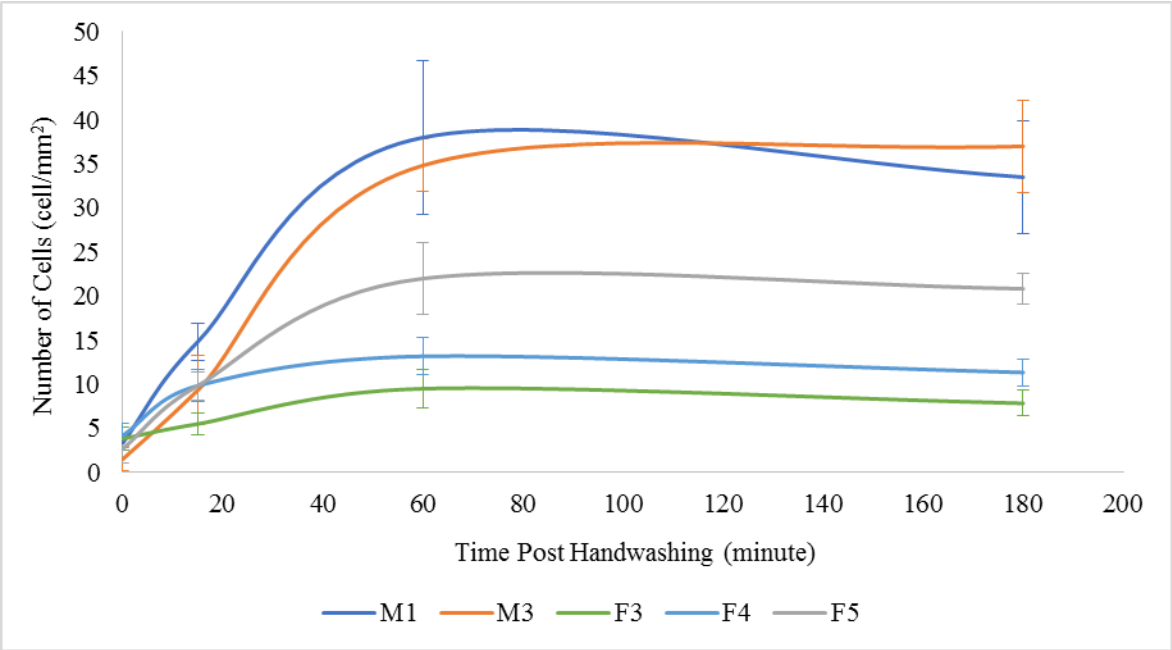
Supp. Figure 3.2. An area of a cleaned glass slide not touched by the donors but stained with DD illustrating that the substrate does not auto-fluoresce and no background material will be detected.



Supp. Figure 3.3. Illustrating (A) a non-stained swab, (B) a swab stained with DD before collecting any cellular material and (C) a swab stained with DD after the collection of cellular material.



Supp. Figure 3.4. Illustrating the number of cells (cell/mm²) within a thumbprint of 11 donors deposited after handwashing 60 mins. Some of the males may have larger prints than the female donors, however the scoring of cells/mm² was designed to provide a uniform scoring system.



Supp. Figure 3.5. Illustrating reproducibility of this technique at a further time point. The number of cells (cell/mm²) within a thumbprint of 5 donors deposited after handwashing 0, 15, 60, and 180 mins. These were performed in duplicates with little variation, as can be seen. Data from these five persons are provided as representative of a heavy (M1 & M3), light (F3 & F4) and intermediate shedder (F5).

3.5 PHASE 3 EMPIRICAL ANALYSIS OF DNA TEMPLATE SOURCE QUANTITY AND STR PROFILING THROUGH TWO WORKFLOWS

Following the identification of the parameters for shedder status determination, a deeper analysis of STR typing success for each shedder status classification was required. An understanding as to the STR profile quality obtained due to donors of different shedder status' works to increase the translational strength of this classification and provide clarity as to the benefit and limitations of this as a technique. In addition to understanding how shedder status impacts STR profile results, identifying and defining the relationship between the number of cells put into a workflow to obtain full DNA profiles is also of vital interest.

This study has been separated into two sections; section one includes a preliminary study performed with FASS to provide them with data to make assertions as to appropriate workflows within their laboratory, section two includes a large-scale study as to the number of cells required for amplification of touch DNA given a broad range of variables.

3.5.1 Section 1: Industry partnership with FASS

3.5.1.1 Methods and Materials

Deposition of cellular material

One volunteer was asked to touch five glass slides sequentially with both the left and right thumb, equalling ten in total, 15 mins post-handwashing with water. This was performed enough times to allow 20 samples from each shedder designation to be sourced. These samples were stained, visualised, and categorised into heavy, intermediate, and light deposition as in Kanokwongnuwut *et al.* (2018) [163]. Fifteen samples from each shedder designation were selected for sampling with a nylon ultra-fine microapplicator (City Dental, Adelaide) moistened with 2 µL of 0.1% Triton™ X-100 (Sigma). The swab heads were removed, using a sterile scalpel blade, into either a 0.2 mL PCR tube for direct PCR or an LHP tube for automated extraction. The remaining five samples from each shedder designation were swabbed using a Rayon swab and placed into an LHP tube for extraction. Amplification for the latent DNA samples were performed with either PowerPlex® 21 or Identifiler® Plus; all rayon samples underwent extraction and PowerPlex® 21 amplification, five ultrafine samples from each shedder designation underwent extraction and PowerPlex® 21 amplification, the remaining ten ultrafine samples from each shedder designation were split into two groups and were processed by direct PCR with either Identifiler® Plus or PowerPlex® 21.

Extraction and Quantification

Extractions were performed using the DNA IQ™ System (Promega). Automated extractions were performed at FASS for all samples undergoing PowerPlex® 21 amplification. Manual extractions were performed at Flinders University for all samples undergoing amplification by Identifiler® Plus. Quantification was performed using the Investigator Quantiplex pro RGO kit (QIAGEN).

DNA STR Amplification and Data Analysis

PowerPlex® 21 amplifications for post-extraction samples were performed following manufacturer's protocol. For rayon swab samples which underwent extraction, 15 µL was added to the PCR as all samples returned low quant values. No samples swabbed with the nylon ultrafine microapplicators were processes through STR amplification due to returning low or no quantification data post-extraction. PowerPlex® 21 amplification following a direct PCR workflow were performed following manufacturer's protocol with the exception of 2 µL Prep-n-Go™ and Low TE Buffer replacing water. Identifiler® Plus direct PCR amplifications were performed following manufacturers protocol with the exception of 2 µL Prep-n-Go™ and Low TE Buffer replacing water. PCR product (1 µL) was added to 9.6 µL Hi-Di formamide and 0.4 µL 600 LIZ® and separated on a 3500 Genetic Analyser. Profiles were considered true if they were ≥80 RFU and heterozygosity was accepted if ≥800 RFU; these values are different to other studies within this thesis but were used for these analyses as they are the standard allelic thresholds used at FASS.

3.5.1.2 Results and Discussion

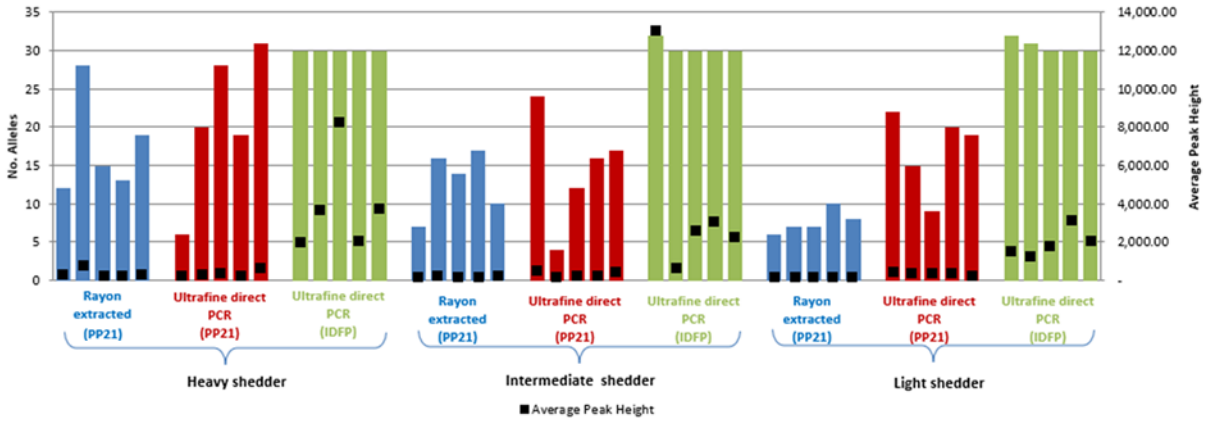


Figure 3.11. Comparison between the standard FASS workflow (rayon swab collection, extraction, PowerPlex® 21 (PP21) amplification), and direct PCR amplification using ultrafine microapplicators for DNA collection with both PowerPlex® 21 and Identifiler® Plus (IDFP) amplification. Due to no DNA recovery from the ultrafine microapplicator extracted samples these have no STR data and are not shown. Peaks were considered true alleles when ≥80 RFU and heterozygosity was considered when ≥800 RFU. The number of alleles for each of the five replicates is shown with the average peak height per profile shown as a black square.

Comparison of direct PCR techniques against FASS standard processing workflow was performed to inform as to the potential value of the workflow within their facility. Standard processing within FASS involves swabbing a sample with a single rayon swab, moistened with water, LHP extraction using DNA IQ™ System, and amplification with PowerPlex® 21 (RP). This yielded an average of 17.4 alleles (ave peaks 324 RFU) for a simulated high shedder, 12.8 alleles (184 RFU) for a simulated intermediate shedder, and 7.6 alleles (141 RFU) for a simulated low shedder (Figure 3.11). Although shedder status determinations were simulated using one donor, they will be referred to a high/intermediate/low for the remainder of this study. Direct amplification of the ultrafine swabs produced more alleles and greater peak heights, on average, than from amplification post extraction. PowerPlex® 21 amplification (UP) resulted in 20.8 alleles (338 RFU), 14.6 alleles (302 RFU), and 17 alleles (333 RFU) for high, intermediate, and low shedders respectively. Direct PCR amplification with Identifiler® Plus (UD) produced the most alleles with highest RFUs, on average, compared with the other workflows. High, intermediate, and low shedders produced 30 alleles (3,912 RFU), 30.4 alleles (4,284 RFU), and 30.6 alleles (1,917 RFU) respectively (Figure 3.11).

When considering the average number of alleles per profile, there is a significant difference between the standard FASS workflow (RP) and both direct PCR methods (UD $p= 5.21x10^{-11}$, UP $p= 0.029$). The difference in the average number of alleles between processing methods became more apparent with lower DNA template; low shedder 7.6:17:30.6 alleles per profile for RP:UP:UD. This supports the introduction of direct PCR with either PowerPlex® 21 amplification or Identifiler® Plus amplification for the processing of low-level latent DNA samples.

The difference observed in the profiles between shedder groups for Identifiler® Plus profiles was in the RFU values, these increased with increasing cellular deposition and collection. For the direct PCR PowerPlex® 21 samples there was a drop observed in the number of alleles between the high and intermediate depositions, but this was not observed between the intermediate and low categories with respect to RFU or number of alleles. For the post-extraction samples the number of donor alleles was seen to drop as cellular deposition reduced.

FASS noted that the baseline was cleaner for the extracted samples, however they exhibited a loss of DNA data when compared to the direct PCR samples. FASS also noted that quantification data showed a correlation to shedder status classification, with high shedder depositions producing the highest quantification values, and this data also corresponded to the allelic return within subsequent profiles (data not shown).

3.5.1.3 Study Conclusion

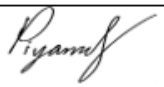
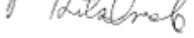
There was an increase in the amount of genetic data obtained in the STR profiles of direct PCR samples. This was true for both Identifiler® Plus and PowerPlex® 21; Identifiler® Plus was superior to PowerPlex® 21. These data suggest a correlation between the number of cells collected from a substrate and the resultant profile with respect to number of alleles and RFU. This supports the need for a more quantitative observation of how a discrete number of cells influences DNA processing workflows.

3.5.2 Section 2: Analysis of sampling and workflow variations on STR amplification success

3.5.2 Manuscript: How many cells are required for successful DNA profiling?

Manuscript published: *Forensic Science International: Genetics*, 2021

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
PK	45	50	40	75	35		04.08.21
BM	**45**	**50**	**35**	**25**	**35**		03.08.21
DT	-	-	25	-	10		03.08.21
KPK	5	-	-	-	10		03.08.21
AL	5	-	-	-	10		05.08.21

3.5.2.1 Abstract

Through advances in fluorescent nucleic acid dye staining and visualisation, targeted collection of cellular material deposited, for example by touch or within a saliva deposit, is possible. In regard to the potential evidentiary value of the deposit the questions remain: 'How many cells are required to generate an informative DNA profile?'; 'How many visualised corneocytes within a touch deposit compared to typical nucleated cells are required in order to achieve successful DNA profiling?'.

DD staining of cellular material, and subsequent visualisation utilising portable fluorescence microscopy, was performed for touch and saliva samples to target defined numbers of cells for collection, by swab and tapelift, and subsequent processing via direct PCR and PCR post-extraction. The resulting DNA quantification data and alleles generated within subsequent DNA profiles could be correlated to the number of cells initially collected to determine cellular threshold requirements for DNA profile generation for each workflow.

Full profiles were consistently generated using direct PCR when the template was ≥40 buccal cells collected by either a swab or tapelift. By contrast ≥800 corneocytes collected by swabbing or

≥4,000 corneocytes collected by a tapelift were required to generate same number of STR alleles from touch samples. When samples were processed through a DNA extraction workflow, ≥80 buccal cells were required to generate full profiles from both swab and tapelift, while touch samples required ≥4,000 corneocytes collected by a swab and >8,000 corneocytes collected by a tapelift. The data presented within this study allow for informative sample triage and workflow decisions to be made to optimise STR amplification based on the presence and visual quantification of stained cellular material.

3.5.2.2 Introduction

Currently, the optimum DNA input to the PCR for STR profiling is around 500 pg [255, 334, 350], which equates to approximately 80 diploid cells (~6 pg/cell [351]). If the potential of DNA loss through workflow processing is not considered, any item from which 80 cells are collected should generate a full DNA profile. Due to inefficient collection of the DNA template present by swabbing or tapelifting techniques and the loss of DNA through standard extraction methods, reported to range between 20% - 80% [2, 201], far more than 80 diploid cells would need to be collected to ensure a full profile downstream given the optimum template stated [255, 334, 350]. Recently, England *et al.* reported the number of spermatozoa and epithelial cells required to generate informative DNA profiles using a laser microdissection one-tube DNA extraction method, and processed through massively parallel sequencing (MPS) [352]. The number, or range, of cells from touch DNA required for full profile generation has not been previously accurately reported for multiple workflows.

The use of DD has allowed the recording of cellular material *in situ* [141, 192, 340, 347, 353]. When visualised under ~200x magnification, using fluorescence microscopy, cell size, cell shape and the intensity of their fluorescence emission are known to vary depending on the cell type and whether they are nucleated or anucleated cells [161, 339]. With the advent of staining latent DNA [162, 163, 192, 347, 353], it is possible to visualise the cells present on an item, which may be used as a cue regarding where to sample it or even if it is worthy of sampling if cellular material thresholds can be found.

One source of DNA template commonly encountered during casework is 'touch DNA' [133, 341]. Touch DNA has been reported as a potential template source for DNA profiling for decades [108], however compared to other sample types touch DNA analysis routinely results in poor STR profiling success [106, 131, 299]. Handled items represent a large percentage of exhibits analysed in forensic laboratories [133], therefore the low success can adversely impact a forensic investigation [106].

Previous studies have reported a potential relationship between the number of visualised cells within fingermarks and STR profiling success have scored/counted the number of cells in one square millimetre at 220x magnification and related the STR profiling success to the donor's shedder status

[162, 163]. The limitation to their relationship observation is that the number of cells that were collected from the entire fingermark, which acts as DNA template for PCR, was not determined.

Understanding the components of the touch DNA sample and how best to analyse this sample type is therefore pivotal. Touch DNA deposits contain anucleate cells (i.e. corneocytes), nucleated cells and cell-free DNA, which is extracellular nucleic acids [111, 113, 115]. The amount of cell-free DNA within fingermarks is not known but is speculated to be a considerable component of the sample [113]. The majority of cells in touch DNA deposits are corneocytes, which are anucleated terminally differentiated skin cells [354]. During cornification much of the DNA within the cell is broken down to its constituent nucleotides, greatly affecting the DNA quality and quantity available for amplification [118, 354, 355].

Loss of DNA from touched items, once submitted to the forensic laboratory, starts with the choice of collection method from the substrate. A tapelift is commonly used for the collection of cellular material from porous substrates [198, 200, 341]; the adhesive facilitates the collection and retention of cellular material. A swab is a sampling medium routinely used for collection from non-porous surfaces; an improvement in the collection of cellular material is observed if the swab is moistened and friction is applied, with absorption properties likely responsible for increased collection of cells when cotton swabs are used and electrostatic attraction when a nylon-based swab is used [200, 205, 206]. Whilst these collection processes are widely and routinely used in operational forensic laboratories, the amount of DNA left uncollected on the item after their use has not been quantified.

Further loss of DNA experienced during the extraction process is also widely reported [7, 8, 143, 193, 202]. Direct PCR is an alternate workflow for trace material examination, the omission of an extraction process means that the entire sample is added directly to the PCR [136, 193, 259, 313], however due to the lack of an extraction, any inhibitors within the sample are also added to the PCR. In addition to these considerations the STR kit used influences the relationship between cellular material on an exhibit and the resulting DNA profile [289]; different kits utilise different buffer systems, primer mixes and enzyme concentrations that can influence a PCRs robustness against inhibition and the efficiency of amplification.

We report on a study to examine how many visualised stained cells are needed to generate informative STR DNA profiles from both saliva samples (buccal cells) and touch samples (corneocytes) processed through various workflows. The collection and processing workflows from sample to profile are as follows: 1) collection with a swab followed by a DNA extraction; 2) collection with a tapelift followed by a DNA extraction; 3) collection with a swab followed by a direct PCR amplification; and 4) collection with a tapelift followed by a direct PCR amplification. Through the comparison of swab and

tapelift results, the relative amounts of DNA associated with corneocytes and the amount of cell-free DNA within touch samples was also assessed. The data presented in this study may allow the number of stained cells observed in trace biological deposits to be used as a basis for sample triage and workflow decision-making.

3.5.2.3 Methods

Ethical approval

Approval from the SBREC at Flinders University, Australia (reference 8109) was obtained prior to initiating this project.

Sample preparation and collection

Table 3.1 shows a summary of all samples processed under each workflow within the study with respect to the numbers of collected buccal cells in saliva (ranging from 1-600) and corneocytes in touch deposits (ranging from very low (VL 500-1) up to high (H ≥3,501)). The number of replicates of each cell category for all four workflows are shown. There are no data for direct PCR when more than 80 buccal cells were used as full profiles were obtained from 80 cells. There are no data for less than 50 buccal cells for those samples processed by an extraction, as quantification data indicated that the samples had insufficient DNA for DNA profiling. Only the '50' and '80' cell counts in the saliva samples were processed in all four ways. There are also no data for 'VL' from touch samples for those samples processed by extraction as quantification data indicated that the samples had insufficient DNA.

Table 3.1: Summary of all samples processed under each workflow in this study.

Cell types	Cells number	The number of replicates for each workflow				Total
		Isohelix Swabs	Packing Tape	Ultrafine Swabs	Packing Tape	
		Extraction	Extraction	Direct PCR	Direct PCR	
Buccal cells	600	5	5	-	-	
	400	5	5	-	-	
	200	5	5	-	-	
	80	5	5	5	5	
	50	5	5	5	5	
	40	-	-	5	5	
	20	-	-	5	5	
	10	-	-	5	5	
	1	-	-	5	5	
	Total	25	25	30	30	110
Corneocytes	H (≥3,501)	5	5	5	5	
	I (3,500-1,001)	5	5	5	5	
	L (1,000-501)	5	5	5	5	
	VL (500-1)	-	-	5	5	
	Total	15	15	20	20	70

Preparation and collection of saliva samples

One volunteer (male) was asked to provide a saliva sample. The number of buccal cells per 1 μL was determined by the following method. An aliquot (1 μL) of saliva sample was spread onto a plastic slide using a clean pipette tip and allowed to air dry. The dried saliva was stained with 1 μL of 20x DD (Promega Corporation, Madison, WI, USA) diluted in 0.01% Triton™ X-100 (Sigma, VIC, AUS) and allowed to air dry. Visualisation of cells was performed using a Dino-Lite EDGE AM4115T-GFBW digital fluorescence microscope (AnMo Electronics Corporation, New Taipei City, Taiwan) with a 480 nm LED light source and a 510 nm emission filter at 220x magnification. Cells within each deposit (1 μL) were counted manually and recorded; buccal cells were only counted when a visible cytoplasm and nucleus was present. This was performed using three replicates. Dilutions of this stock saliva sample, with DNA-free water, were made to approximately 600, 400, 80, 50, 40, 20 and 10 cells per μL. An aliquot (1 μL) of each dilution was spread onto a plastic slide using a clean pipette tip and allowed to air dry. The dried saliva aliquots were each stained with 1 μL of 20x DD, visualised, and their number of cells counted. Samples were categorised based on their cell number, as described in Table 3.1.

Sampling of saliva samples with swabs

Swabbing was performed under the microscope to visually ensure the appropriate number of stained cells were collected from the slides by the swabs. For samples undergoing extraction, Isohelix™ MS-02 (Cell Projects Ltd, Kent, UK) swabs, moistened with 0.1% Triton™ X-100 by dipping the swab into the solution, were used for collection and their heads removed into 1.5 mL tubes. For

direct PCR samples, each sample was double-swabbed using nylon ultrafine microapplicator swabs (City Dental Supplies, ADL, AUS). The heads of ultrafine swabs were moistened with 2 µL of 0.1% Triton™ X-100. After collection, the swab heads were removed using a sterile scalpel blade and placed directly into 0.2 mL PCR tubes.

Sampling of saliva samples with tapelift

For all tapelift samples, 1 µL of the stock saliva sample was pipetted on a plastic slide and allowed to air dry. The dried deposit was stained with 1 µL 20x DD in 0.01% Triton™ X-100 and allowed to dry. Packing tape (Packmate™, ADL, AUS), measuring approximately 10 mm x 20 mm, was used to tapelift the plastic slide due to the tape's high recovery efficiency and facilitation of clear visualisation of cells [317]. The tapelift was visualised at both 50x and 220x magnifications to allow for a defined number of cells to be targeted for downstream processing. A portion of the tapelift with an appropriate number of cells present, as seen in Table 3.1, with a ±10% tolerance with regards to cell numbers, was cut out with a sterile scalpel blade and placed into either a 0.2 mL PCR tube for direct PCR amplification or a 1.5 mL tube for extraction.

Preparation and collection of touch samples

One volunteer (male) was asked to touch each glass slide for 15 s performing three sequential depositions with each thumb (six glass slides in total). Contact was made at an undefined time post-handwashing (≥30 mins), during which regular activities were performed (e.g. office work). This was conducted to produce thumbprints with variable numbers of cells available for downstream collection. The volunteer deposited cells during twelve separate events until the dataset, as described in Table 3.1, was complete. Thumbprints on glass slides were stained with 5 µL 20x DD solution in 75% ethanol, which was spread over the mark using a pipette tip, and images of the entire thumbprint were taken at 50x magnification for cell scoring (≥16 images per thumbprint). An aliquot of 5 µL 20x DD solution in 75% ethanol was applied to the thumbprint because it was a larger area to cover compared to the 1 µL of saliva dilution. The stained cells were scored using an in-house "cell counting" software program both before and after sampling to record the number of collected cells (Figure 3.12) [356]. Five parameters were used following the values 1) hSize = 9, 2) Sigma1 = 1, 3) Sigma2 = 3, 4) RBG filter threshold = 6-10, and 5) Size threshold = 10-12. For an explanation of these parameters see [356], but in brief the method used to process slide images and count cells uses a Difference of Gaussians (DoG) method [357], where 'hSize' is the kernel size of the Gaussian filter, 'Sigma1' and 'Sigma2' are sigma values for the first and second Gaussian filter, 'RBG filter threshold' is the threshold at which the DoG image blacks out the pixel and 'Size threshold' is used to remove connections between objects that are below the user-defined size. This produces a black and white image, and the number of cells is then counted as the number of unconnected objects. Each thumbprint was

additionally visualised at 220x magnification to ensure there were fewer than 10 nucleated cells present; those with greater than 10 nucleated cells were excluded from the study to ensure that effectively only corneocytes were collected.

Sampling of touch samples with swabs

For samples undergoing extraction, entire thumbprints were sampled with a single Isohelix swab moistened with 0.1% Triton™ X-100, by dipping the swab into solution, and the head was removed into a 1.5 mL tube. For samples undergoing direct PCR amplification, double-swabbing with nylon ultrafine swabs, moistened with 2 µL 0.1% Triton™ X-100, was performed and their heads were removed using a sterile scalpel into a 0.2 mL PCR tube. After collection, the glass slides were re-stained with 5 µL 20x DD solution in 75% ethanol and re-visualised under 50x magnification, images were taken and any cells remaining were counted. To ensure the remaining cells were accurately counted, the samples were also visualised under 220x magnification. As previously stated, the number of collected cells were calculated by comparing the number of cells before and after swabbing (Figure 3.12) [356]. Each collected sample was categorised as containing high (H: ≥3,501), intermediate (I: 1,001-3,500), low (L: 501-1,000), or very low (VL: 1-500) cell numbers with five replicates of each condition existing in each category (Table 3.1).

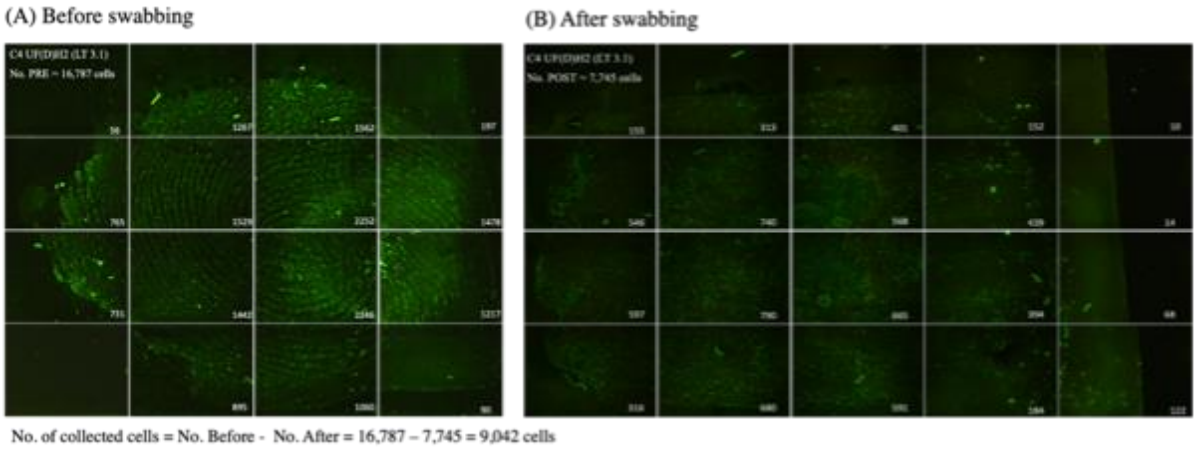


Figure 3.12. Illustrating an example of a thumbprint stained with DD and visualised before (A) and after (B) sampling with a nylon ultrafine swab. Numbers in the bottom right of each frame are the cell scores for that frame at 50x magnification; the total number within the thumbprint is shown at the top left of each thumbprint. A total of 9,042 cells were collected.

Sampling of touch samples with tapelifts

Thumbprints were collected with packing tape (Packmate™) measuring approximately 20 mm x 25 mm. The tapelift post-sampling was examined and the number of cells present was counted using the previously mentioned software program. A portion of tape, 10 mm x 20 mm for samples that were to be extracted or a 5 mm square for direct PCR samples, with a known number of cells present was categorised into H, I, L and VL, as described in Table 3.1, was placed into either a 1.5 mL or 0.2 mL tube respectively.

Confirmation of capability of tape for touch DNA collection

To assess the capability of tape as a medium for touch DNA collection (i.e. cellular material and cell-free DNA) three pairs of left and right thumbprints were deposited onto glass slides, visualised and counted as in Section 2.2.2 to ensure all thumbprints contained a similar number of visualised cells (about 6,000 cells per print).

As performance control samples, the left thumbprints were doubled-swabbed using ultrafine swabs each moistened with 2 µL 0.1% Triton™ X-100. The heads of ultrafine swabs were removed with a sterile scalpel blade and placed into 0.2 mL PCR tubes for direct PCR amplification. These samples act as control samples as swabs were presumed to collect both cellular material and cell-free DNA.

To compare the tape as a collection medium, the right thumbprints were lifted using packing tape (Packmate™), as previously described in Section 2.2.2.2, to remove biological material. The glass slide was visualised under 50x magnification to ensure the visualised cells were removed by the tape. Two ultrafine swabs, each moistened with 2 µL 0.1% Triton™ X-100, were used to remove any residual and invisible biological material (i.e. cell-free DNA) from the same area as the tapelift was taken from. A portion of the tape (approximately a 5 mm square) was cut and placed directly into 0.2 mL PCR tubes for direct PCR amplification. Three replicates were performed for all the described processes.

DNA extraction and quantification

DNA extractions were performed manually using the DNA IQ™ System (Promega, Madison, WI, USA) following the manufacturer's protocol, with a final DNA extract volume of 30 µL. DNA yields were quantified using the Investigator® Quantiplex Pro RGQ kit (QIAGEN, VIC, AUS) following the manufacturer's protocol. Samples were amplified on a Rotor-Gene Q (QIAGEN) and analysed using the QIAGEN Quantification Assay Data Handling Tool (QIAGEN).

DNA loss, for saliva samples, was calculated based on the assumption that diploid cells contain approximately 6 pg of DNA, and the staining, visualisation and cell counting before and after collection was accurate.

Short tandem repeat profiling

Direct PCR and PCR post-extraction was performed on each sample using Identifiler® Plus kit (ThermoFisher Scientific), comprising 15 STR loci and the amelogenin marker. Direct PCR amplifications were performed in 25 µL following the manufacturer's protocol, with exception of 2 µL of Prep-n-Go™ (ThermoFisher Scientific) and Low TE Buffer (ThermoFisher Scientific) replacing water. Amplifications following extraction were performed following the manufacturer's protocol with 500 pg of DNA template added to the PCR, or 10 µL if the DNA concentration of the eluate did not yield 500 pg. All amplifications were performed on a ProFlex™ thermal-cycler (ThermoFisher Scientific). PCR product (1 µL) was added to 8.5 µL Hi-Di formamide and 0.5 µL 600 LIZ® (ThermoFisher Scientific) and separated on a 3500 Genetic Analyser (ThermoFisher Scientific).

Data analysis

Number of alleles and relative fluorescent units

Data were analysed using GeneMapper® ID-X (version 1.4). The number of autosomal donor alleles were counted, with a maximum of 30 with peak thresholds (i.e. a full DNA profile comprises 30 alleles). Heterozygous peaks were considered true and recorded if they were ≥50 RFU and homozygosity was considered if ≥150 RFU. Profiles were considered informative, or unloadable to the NCIDD for comparison, if ≥12 autosomal donor alleles were present; subsequently referred to as 'an informative profile'.

Statistical analysis

Dataset information was recorded for each sample on the following dependant variables:

- The collection device (swab or tapelift, note that both swabs were considered together as one group),
- The cellular source (saliva or thumbprint),
- The processing of the sample (direct PCR or extraction),
- The number of cells sampled for PCR,
- The total amount of DNA obtained from extraction (only applies to extracted samples),
- The amount of DNA added to the PCR (only applies to extracted samples),
- The degradation index from quantification (only applies to extracted samples), and
- The inhibition index from quantification (only applies to extracted samples)

There are two sets of response variables measured:

- The number of alleles from the DNA donor observed in the profile, and
- The total summed peak height for the profile

To assess how many cells are required to obtain a good quality profile, the parameters listed above that might affect the profile quality were tested using multiple regression in software R (version 3.6.0) [358]. Inherent in this analysis is the fact that what makes a profile 'good quality' can be defined in different ways, specifically the number of alleles detected, or the strength of the profile. These two aspects are related, however the number of alleles is a truncated form of response variable, i.e. as the amount of DNA template present in the PCR increases, so too does the strength of the profile (which has been shown to be approximately linear [89]). This is true of the case where no increased inhibitor presence is recorded by increased sample presence, such as that which can be observed with direct PCR. However, this may not necessarily translate to increasing the number of alleles i.e. if the profile is already complete. It is reasonable to state that if a technique produced full profiles (e.g. 30 alleles) with an average peak height of 200 RFU and another technique produced full profiles with an average peak height of 5,000 RFU then the second technique is considered to be producing better quality profiles. It is therefore reasonable to consider profile strength as the response variable when assessing the effect of variables on profile quality.

However, many forensic laboratories have the primary goal of interpreting or obtaining a DNA profile for comparison to a reference and/or uploading it to a searchable DNA database. In these instances, the number of alleles (or more specifically when that number crosses some threshold) will be the aspect of most interest. In this paper, investigating the effect of the variables on the profile quality for both measures is important.

Statistical analysis using number of alleles as response variable

The number of cells used in this study ranges from 1 to 9,510 with many small discrete categories tested at the lower range due to the fact that the sensitivity of current DNA profiling systems only requires a few cells to produce a DNA profile. Also, it is expected that the effects of the variables will work in a relative way on the number of alleles, rather than affecting their absolute number. For these reasons, a $\log_{10}$ transformation of the number of cells was used in regressions. Additionally, from the type of data analysed, and the properties of the response variable, it is known that as the number of cells reduces the number of alleles will plateau at 0 and as the number of cells increases the number of alleles plateaus at 30. Such a relationship is described well by logistic regression. In order to carry out logistic regression, the number of alleles observed were transformed by division of the value by 30 so that all values used in the regression fell within the range of 0 and 1. To carry out the analysis, a generalised linear model (GLM) in R with a quasibinomial link function was used, as values exist between 0 and 1 for partial profiles.

Statistical analysis using fluorescence as response variable

The use of fluorescence has a similar property to the number of alleles, in that there is an upper and lower bound on values, although the upper bound is at a much higher level and relates to the saturation of the CE instrument. The DNA profiles were run on an ABI 3500xl instrument, which has an approximate saturation of 30,000 RFU. Given a full profile is 30 alleles then an upper bound on total profile fluorescence would be approximately 900,000 RFU; the highest value recorded in our dataset was 389,021 RFU, which is less than the maximum. Given the broad range of total RFU values, and again an expectation of a relative effect on fluorescence, a $\log_{10}$ transformation of the variable for regression was used. The distribution of the total RFU value in this study is also closer to normal on a $\log_{10}$ scale than a natural scale (data not shown).

The difference between direct PCR and extracted samples

There are a few differences in the data collected for the two laboratory processes trialled (i.e. direct PCR vs extraction) that need to be handled prior to regression analysis. The first difference is the fact that only a portion of the DNA extract is able to be added to the PCR. When samples are taken for direct PCR processing, the entire sample is used in the PCR. In contrast, when the extraction process is carried out, the entire sampling device is used in the extraction to produce a DNA extract, however due to volume limitations only a portion of this may be used in the PCR. The elution volume is a choice made in the extraction process that has a direct effect on profile quality and needs to be accounted for in order to be put on comparable levels with direct PCR i.e. we want to compare the processes of direct PCR, where the whole sampling device is used, with extraction results that have been multiplied by a factor that compensates for the fraction used of the whole sampling device. In order to make an adjustment for the proportion of total extracted DNA used in the PCR, either the response variable values (number of alleles or fluorescence) can be adjusted, or the main input value (number of cells) can be adjusted. The main input was chosen to be adjusted as this was intuitive (i.e. if X cells were extracted and half the extraction is used in PCR then it is as though X/2 cells were extracted and all used in the PCR). This does not result in situations (i.e. as it would be if we chose to adjust the response variable) where the adjusted number of alleles exceeds the theoretical maximum.

The second aspect to consider is that the extracted samples have inhibition and degradation values associated with them due to the fact that they have been through a quantification step, whereas direct PCR samples do not. Some initial analyses (data not shown) showed that the inhibition index had an effect on the total fluorescence of a profile in the extracted samples. Most samples that displayed possible inhibition (according to the Quantifiler™ Trio assignment suggestions) resulted in profiles that presented no alleles, even when they had cell numbers or quantification values that would otherwise suggest a profile should have been seen. In some instances halving or adding a third

volume of the extract pool to the PCR, for samples that display inhibition in the quantification data, reasonable profiles can be obtained [359], however this was not performed for these samples as their inclusion was not crucial to statistical analyses. There are STR kits that can indicate inhibition within a PCR using synthetic internal lane controls such as VeriFiler™ Plus and the Investigator® 24plex QS kit; these were not utilised within this study. Using the Identifiler® Plus kit, it is not possible to obtain inhibition values for direct PCR samples, however complete PCR inhibition was not observed in any sample, with reasonable and comparable cell numbers, following direct PCR amplification. Due to the effect of inhibition noted in some extraction samples, these have been removed from the analysis (n = 2 out of 15 swabbed touch samples and n = 3 out of 25 swabbed saliva samples).

3.5.2.4 Results and Discussion

Quantification of DNA post-extraction

Regression was carried out by comparing the total DNA yield obtained from the DNA extraction process and input cell numbers. Cell count, cell type, collection method and the interaction between cell type and collection method were all included in the model.

Figure 3.13 shows the DNA yield post-extraction of two sample types (saliva and touch samples) collected either by a swab or a tapelift, along with the results of the regression (predicted value plus or minus two standard errors). Of significance in the modelling of DNA yield was cell count ($p= 9x10^{-11}$), and cell type ($p= 8x10^{-14}$), but not the collection device ($p= 0.536$).

The average DNA yield for all the buccal cell samples (n = 50) when the input ranged between 50 to 600 cells, was 1.01 ng. The minimum was 0.07 ng when 50 cells were added, and maximum was 3.08 ng when 600 cells were added (Figure 3.13). It should be noted that there was variation between the resulting DNA yield post-extraction when the same number of buccal cells were collected. For example, the average DNA yield was 2.07 ng in a range of 1.08 - 3.08 ng when 600 buccal cells were added to the extraction (Figure 3.13).

The average DNA yield of touch samples (n = 30) when the input ranged between 500 cells to 10,000 cells was 0.22 ng. The minimum was 0.013 ng when approximately 800 cells were added, and maximum was 0.85 ng when approximately 8,000 cells were added. Similar to the results from saliva samples, the DNA yield obtained from touch samples also showed variation post-extraction. The expectation is that buccal cells will contain intact chromosomal DNA, while touch samples contain fragmented DNA template within cells processed through cell cornification, along with cell-free DNA [118, 354].

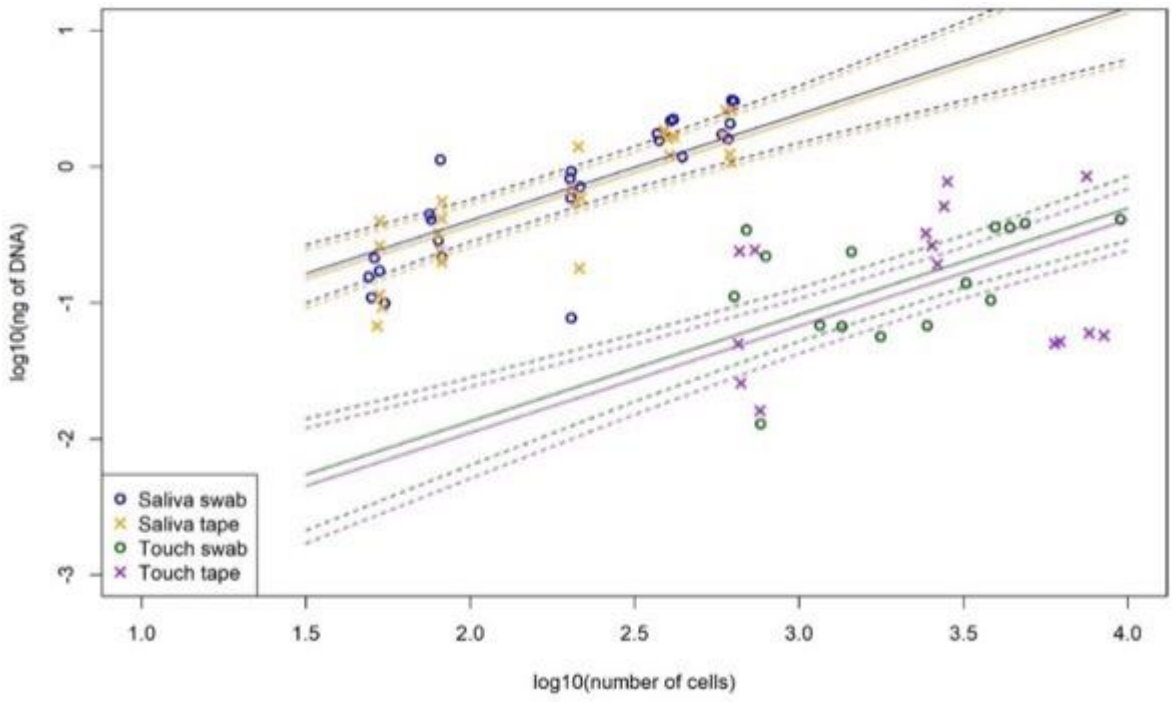


Figure 3.13. Illustrating DNA yield extracted from saliva and touch samples; x-axis shows the log10 of the number of collected cells and y-axis shows DNA yield log10(ng) quantified using the Investigator® Quantiplex Pro RGQ kit. Dashed lines show plus or minus two standard errors.

DNA loss during DNA extraction

DNA loss during extraction, calculated from the saliva samples only, can be determined based on the known number of buccal cells initially added (assuming 1 buccal cell = 6 pg) and the final DNA yield. As the result in Section 3.1 showed, there was no significant difference between using a swab or taking a tapelift to the quantity of DNA obtained post-extraction. The average percentage of DNA loss during extraction were 38.6% ± 22.2% (collected by a swab) and 47.2% ± 20.8% (collected by a tapelift), as seen in Figure 3.14.

There was a massive variation of % DNA loss (1% - 94%) post-extraction for the two sampling methods. This supports previous studies that have shown the variability and vast amounts of DNA template that is lost during extraction (20% - 80%) [2, 201]. The template quality from visualised corneocytes and cell-free DNA components of touch deposits does not allow the same loss percentage to be calculated as with the saliva samples previously discussed.

Although the quantification data for touch DNA samples showed a linear relationship overall with respect to cellular input and concentration post-extraction (Figure 3.13), the DNA loss observed was not accurately predictable; the same amount of DNA (approximately 0.1 ng) was obtained from both ~4,000 cells and ~700 cells following swab collection and extraction.

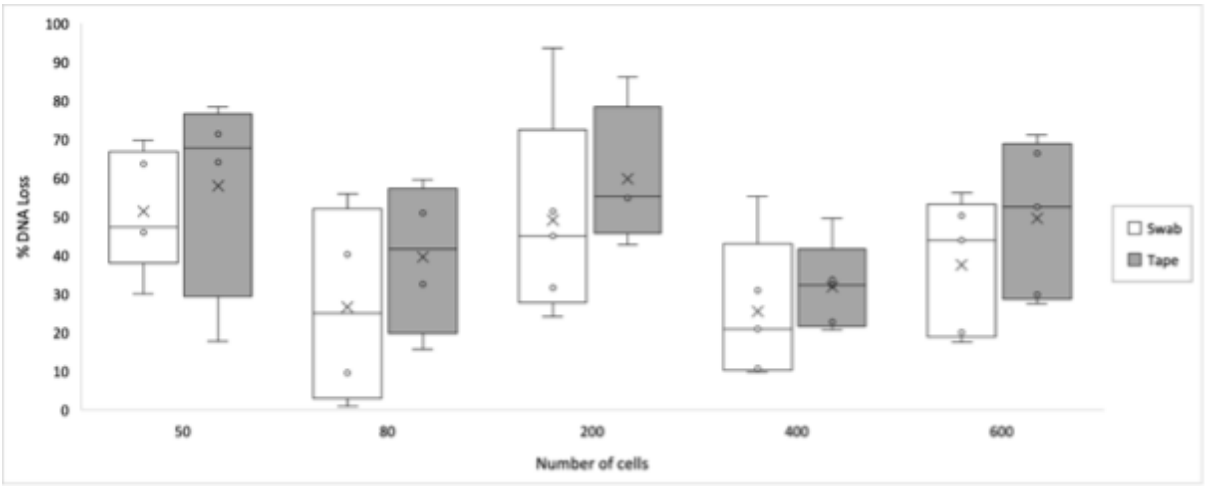


Figure 3.14. Illustrating the percentage of DNA loss post-extraction, following both swabbing and tapelifting, in each cell number category of saliva samples. X-axis is the number of cells in each category, y-axis is % DNA loss during extraction, (x) shows mean markers and (o) shows inner points.

Success of STR profiling

Determining the variables that affect STR profile quality

Initially, the response variable was considered as the total fluorescence detected in the profile. The 'adjusted cell count' was arrived at by taking account of the proportion of total DNA collected that was used in the PCR for samples that were processed using the extraction method (as described in Section 2.5.2.3). Significant in the description of total RFU were the cell count ($p < 2x10^{-16}$), cell type ($p< 2x10^{-16}$), collection method ($p= 4.5x10^{-12}$), laboratory process ($p= 0.00195$), and the interaction between cell type and collection method ($p= 3.29x10^{-8}$). The model that was fit using the regression was:

$$log_{10}(rfu) = -0.6508 + 1.68256\, log_{10}(cells) - 0.33944i + 2.19098j - 1.2516k + 1.24408jk$$

where:

$$i = \begin{cases} 1 & extraction \\ 0 & direct \end{cases} \quad \text{and} \quad j = \begin{cases} 1 & saliva \\ 0 & fingermark \end{cases} \quad \text{and} \quad k = \begin{cases} 1 & tape \\ 0 & swab \end{cases}$$

On the natural scale this becomes:

$$\widehat{rfu} = 10^{-0.65082} \times cells^{1.68256} \times 10^{-0.33944i} \times 10^{2.19098j} \times 10^{-1.2516k} \times 10^{1.24408jk}$$

This modelling suggests that the effect of following an extraction approach compared to a direct PCR approach is to reduce the total fluorescence by approximately 46%, and the effect of processing buccal cells compared to those from thumbprint is an increase in the total fluorescence by approximately 100-fold. Note also the parameter values that consider sampling device and the

interaction term. They are very close in value and opposite in sign (-1.2518 and 1.24408 respectively), which suggests that there is very little effect of the sampling device for saliva samples but a significant effect for touch samples. This finding supports the previously stated result when quantified extracted DNA concentration was being examined alone. Supp. Figure 3.6 shows the predicted total fluorescence, using the model above, for the different datasets within this study. Given the results of this regression it is clear that each variable tested in this study has some effect on the profile quality, in terms of the profile fluorescence. The effect of these variables in profile quality when considering the number of alleles in a DNA profile was considered as the next response variable. There are likely other factors (such as inhibition and degradation) that will also have an effect, which have not been explored here.

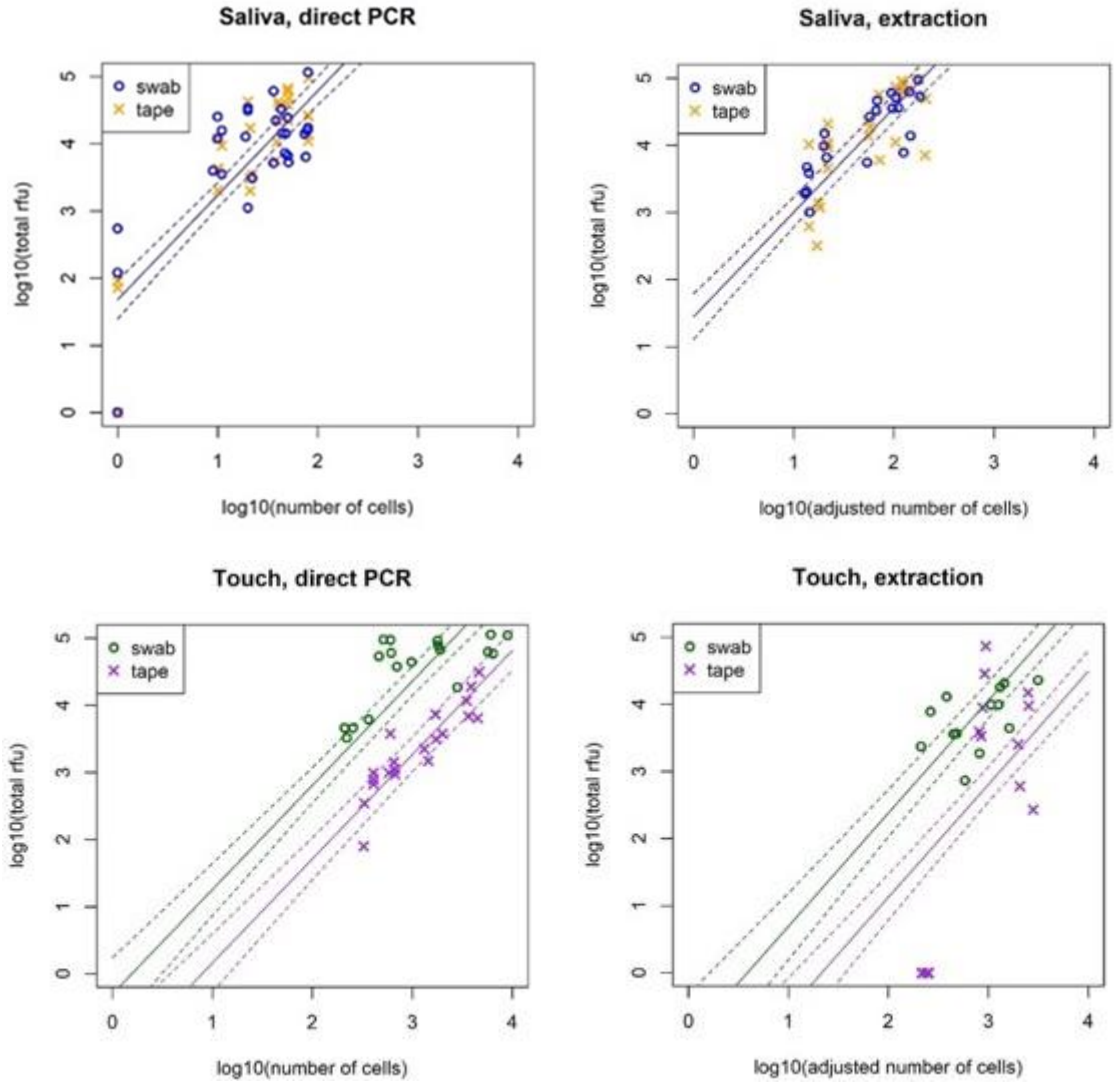


Figure 3.15. Observed and predicted values (solid line is prediction and dashed lines are plus or minus two standard errors) for total fluorescence for each category of sample used in this study.

Considering allele count as the response variable

Figure 3.16 is a violin plot showing the quality of profiles produced from each data category. The model to the data using allele count used the same variables as when total fluorescence was considered the response variable but used a logistic transformation of the allele count as follows:

$$ln\left(\frac{a/30}{1-a/30}\right) = -9.8367 + [4.3855 - 1.2235k]\, log_{10}(cells) - 0.7822i + 6.5745j + 1.0484k$$

where 'a' is the allele count and:

$$i = \begin{cases} 1 & extraction \\ 0 & direct \end{cases} \quad \text{and} \quad j = \begin{cases} 1 & saliva \\ 0 & fingermark \end{cases} \quad \text{and} \quad k = \begin{cases} 1 & tape \\ 0 & swab \end{cases}$$

Significant in the description of the number of alleles were the cell count (p= 4.56×10^{-11}), cell type (p= 1.24×10^{-12}), and collection-laboratory process (p= 0.0317). The interaction between cell type and collection method was not significant in this model (p= 0.5869), and other interaction terms were trialled and found to be insignificant. An interaction between the cell count and the collection device was found to be significant (p= 0.00647) and hence was included in the model. The collection device was not significant (p= 0.257); however it was retained in the model given the results of Section 3.3.1. Supp. Figure 3.6 shows the predicted total RFU, using the model above, for the different datasets in this study.

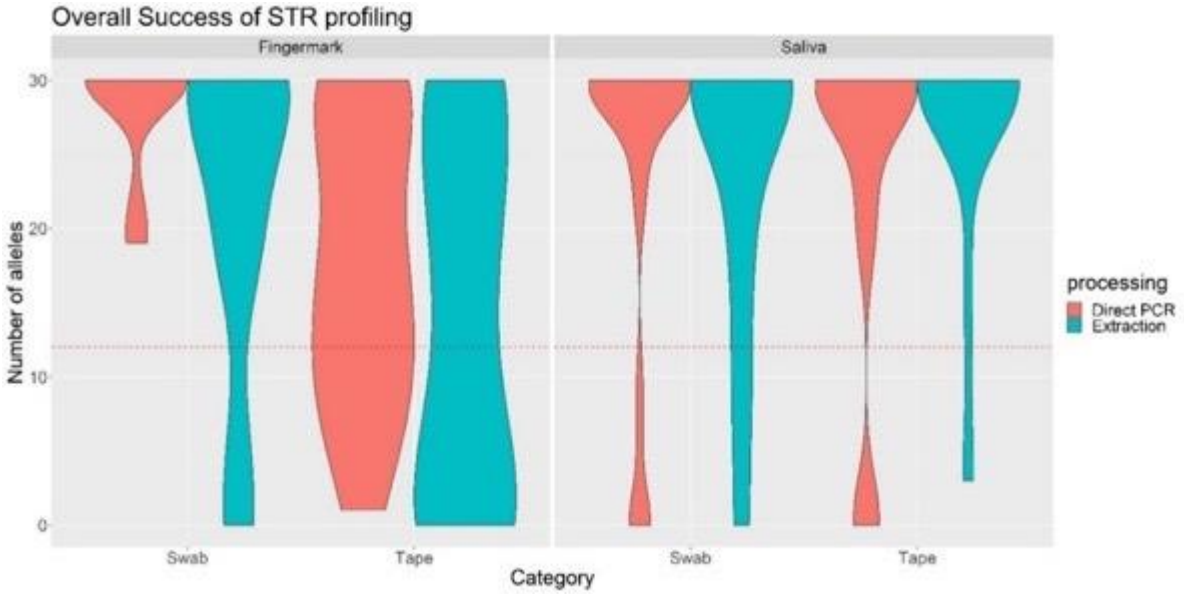


Figure 3.16. Shows the overall success of STR profiling from saliva and touch samples with four different workflows. The width of the violins represents the number of samples observed at that point. The red line shows the threshold considered to be informative (≥12 autosomal alleles).

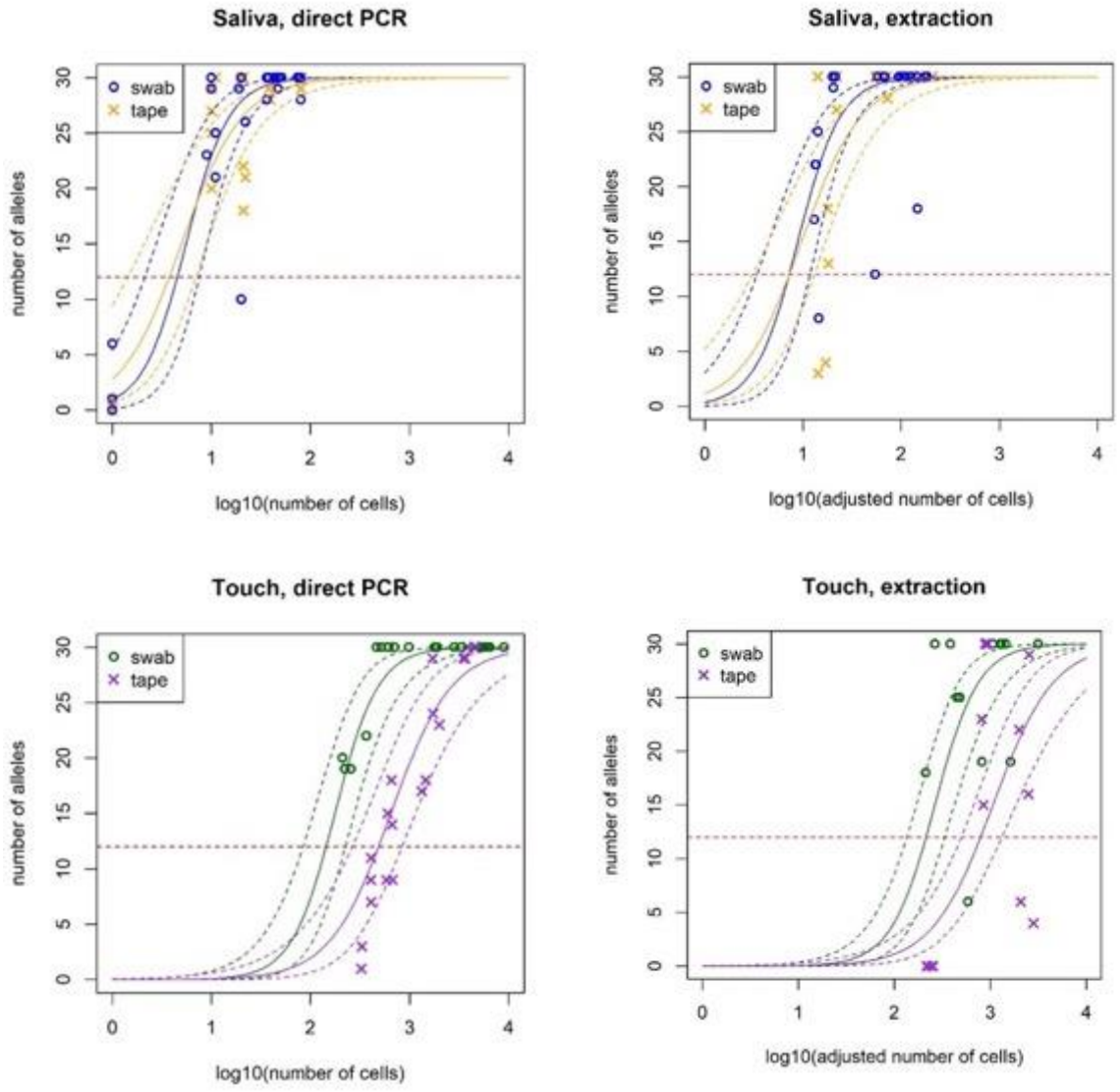


Figure 3.17. Observed and predicted values (solid line is the prediction and dashed lines are plus or minus two standard errors) for donor allele count for each category of sample used in this study. Red line indicates a threshold for an informative DNA profile (≥12 autosomal alleles).

Saliva samples

As can be seen visually in Figure 3.17, there is a lack of significant difference in terms of the number of donor alleles amplified between the saliva samples collected with a swab or tape using PCR post-extraction or direct PCR.

Saliva samples (n = 50) with input ranging between 50 – 600 buccal cells and processed through an extraction generated informative profiles from 44 of the 50 samples; of these 44 samples, 33 gave full profiles. Six of these 44 samples returned uninformative profiles with eight or fewer alleles recorded. Of the 40 samples where the input was ≥80 cells and processed through extraction,

32 generated full profiles, a further three resulted in nearly full profiles (27-29 alleles), one 18 alleles, one 12 alleles and three yielded uninformative profiles. For the three samples that returned <12 alleles), there was an indication of 'possible inhibition' from the quantification data (inhibition index ranged -1.19 to -1.49).

Of the 50 saliva samples amplified by direct PCR with an input of 10 or more cells, 30 generated full profiles and a further 19 resulted in informative profiles. Only one sample (20 cells input) resulted in an uninformative profile (10 autosomal alleles). Of all the samples where ≥40 cells were added, the minimum number of alleles detected was 28/30. All samples with 1-cell input returned uninformative profiles with a maximum of six alleles recovered. Informative profiles generated from saliva samples, collected by either swab or tapelift, could be consistently obtained with PCR amplification post-extraction when samples contained ≥80 cells; however, approximately half (≥40 cells) were required when a direct PCR process was used (Table 3.2).

Of the dataset pertaining to buccal cells, only two cell input numbers (50 and 80 cells) were processed by both direct PCR and PCR post-extraction using each collection method (Table 3.1). When considering the 20 samples for each amplification workflow (both swab and tapelift collection), 17 samples generated full DNA profiles when amplified directly while only 7 samples resulted in full DNA profiles following post-extraction amplification.

The results from this study showed a saliva sample required ≥80 buccal cells when processed through the extraction or ≥40 buccal cells when processed by direct PCR. England *et al.* showed that, following a laser microdissection one-tube extraction and MPS, a full DNA profile (27 loci) can be generated from approximately 50 epithelial cells [352]. This difference may be due to the different sensitivities inherent within the different amplification methods, when considering direct PCR (≥40 cells) and laser microdissection results (≥50 cells), or due to a higher amount of DNA lost during the manual extraction performed when considering extraction-based PCR (≥80 cells) and laser microdissection results.

Touch samples

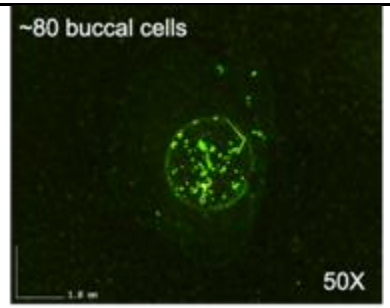
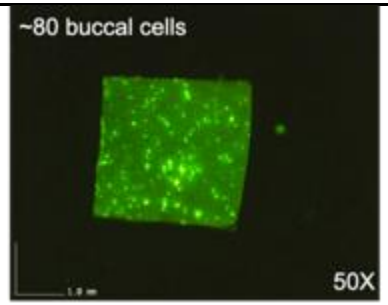
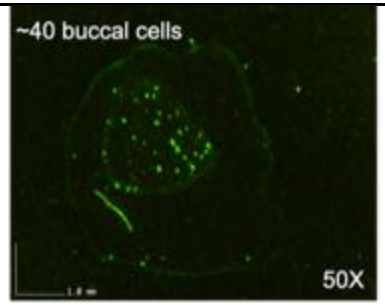
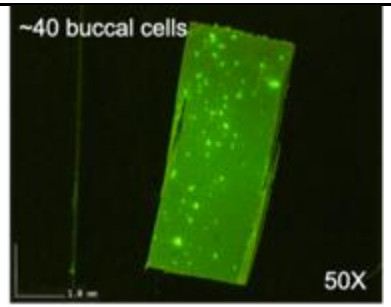
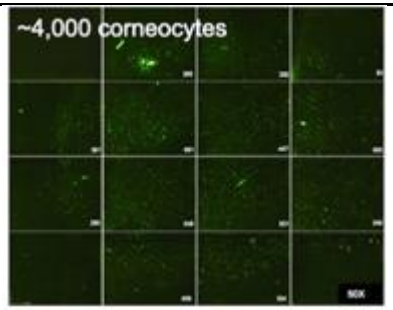
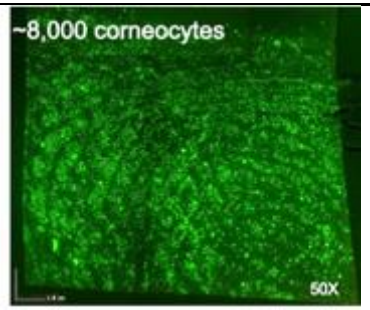
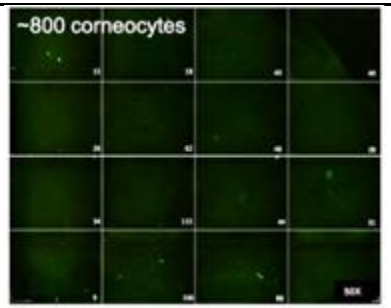
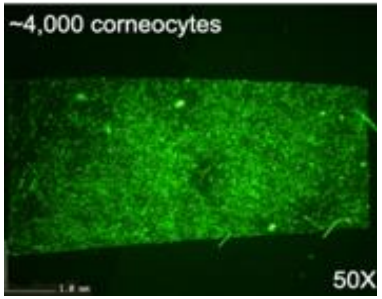
In contrast with the results from saliva samples, the difference in the amount of data within the DNA profiles generated from touch samples was significant between the two sampling methods for samples processed by either post-extraction or direct PCR amplification (Table 3.2). There was a reduction in the number of alleles observed comparing touch samples collected by a tapelift with those collected by a swab (Figure 3.17).

When considering touch samples that underwent tapelift and extraction (n = 15), eight returned informative profiles and seven returned <12 alleles. Of these seven samples, two returned profiles with six and four alleles and the remaining five generated no profiles. These five samples (from ~600-800 cells) returned very low quantification data (typically 0.00038 ng/μL). Whereas, of 15 touch samples which underwent swabbing and extraction, 12 returned informative profiles, one returned a profile with six alleles, and the remaining two generated no profiles. Of the two samples that amplified no alleles, one (from ~700 cells) had an indication of 'possible inhibition' (inhibition index = -18.53) and the other (from ~800 cells) returned a quantification concentration of 0.00043 ng/μL.

Conversely, of the 20 tapelift samples that underwent direct PCR amplification, 13 generated informative profiles, which included only three full profiles (from ≥4,000 cells). Of the 20 touch samples collected by a swab, all 20 returned informative profiles following direct PCR amplification, including 15 full profiles. Touch samples consistently generated full profiles when ≥800 cells were collected by swabbing and amplified directly (Table 3.2). There was a clear contrast in the data obtained within the DNA profiles, following direct PCR, from a similar number of visualised cells within touch samples between collection methods.

These results showed there was a vast difference in the required number of cells between saliva and touch samples to generate full DNA profiles processed either through direct PCR or an extraction. The number of cells required for four different workflows are summarised in Table 3.2 along with the number of collected cells observed at 50x magnification.

Table 3.2: Summary the number of cells required for full DNA profile generation and the visualisation of the required number of cells at 50x magnification for four different workflows.

	Collection and Processing			
	Swab – Extraction	Tapelift – Extraction	Swab – Direct PCR	Tapelift – Direct PCR
Saliva sample				
	≥80 cells	≥80 cells	≥40 cells	≥40 cells
Touch sample				
	≥4,000 cells*	>8,000 cells	≥800 cells*	≥4,000 cells

* The number of cells within an entire thumbprint counted from 16 frames of image recorded at 50x magnification. Scale bars is 1 mm.

Relationship between STR results and quantities of buccal cell, corneocyte and cell-free DNA

Only samples processed through direct PCR were included in any evaluation of the relationship between STR results and quantities of buccal cell, corneocyte and cell-free DNA, due to the vast variation of DNA loss during extraction. The data presented indicate that 40 buccal cells contained sufficient DNA template to generate full DNA profiles regardless of the collection method employed. In contrast, the number of corneocytes needed to generate the same data were 800 if collected by a swab, and 4,000 if collected by a tape. This corresponds to a 20-fold difference between the number of buccal cells and corneocytes needed to generate the same amount of genetic data if collected by a swab; this difference becomes 100-fold when cellular material is collected by tapelifting.

This supports the assumption that corneocytes do not contain the full complement of DNA [118, 354, 355], like buccal cells do, and exist on a degradation continuum. The amount of chromosomal DNA will vary depending on a state of cornification [118, 354, 355], and therefore each stained cell may no longer contain the loci targeted. This would explain why 'touch DNA samples' have such a poor success as either 20- or 100-fold more are needed in order to generate the same amount of STR data. These data also raise a question around collection medium regarding touch DNA being 'what accounts for this loss of DNA using a tape compared to a swab?'.

This reduced amplification is unlikely to be due to inhibition caused by tape, as the results from saliva samples showed no difference between the two sampling methods. One possible explanation for the difference in the data obtained between collection using a swab or tapelift is due to the template sources collected compared with the visualised cells targeted. We propose that swabs collected all the DNA template sources available within the touch deposit, including corneocyte and cell-free components, whereas tapelifts predominantly only collected corneocytes. Additionally, although approximately the same number of visualised cells were isolated when collected by both tape and swab, the surface area targeted area was different. A swab was used to sample an entire thumbprint, whereas only a portion of the tape used to tapelift the thumbprint (approximately 10 mm x 20 mm) that contained the required number of visualised cells that was used downstream. As a result, the portion of cell-free template available within the PCR, or extraction protocol, is clearly different between swab and tapelift samples due to this difference in surface targeted.

The difference in STR data obtained between the two sampling methods is therefore most likely to be due to cell-free DNA presence in the samples collected by the swab but its absence in those collected by the tape. To confirm this hypothesis, three pairs of left and right thumbprints were deposited, where a similar number of visualised cells were recorded between left and right prints (section 2.2.3); the assumption that similar concentrations of cell-free DNA would be present between

left and right thumb pairs was made [154, 163]. Full DNA profiles were obtained from left thumbprints, collected by ultrafine swabs and processed through direct PCR as baseline samples (Figure 3.18 and Supp. Figure 3.7 A).

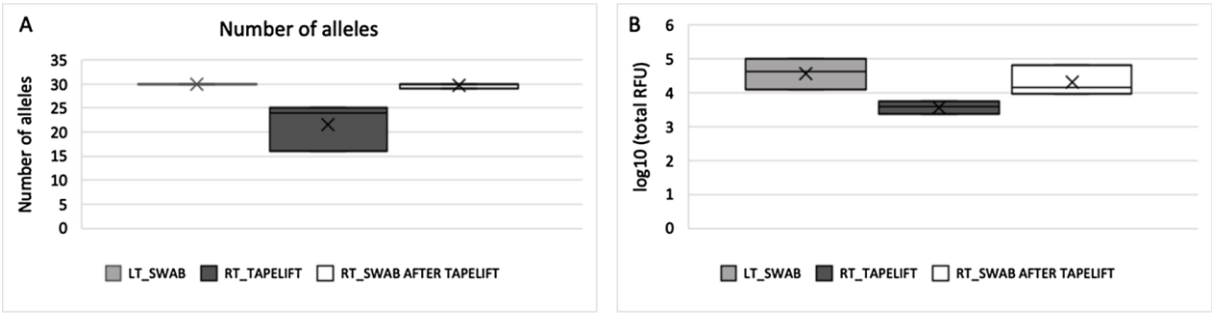


Figure 3.18. Illustrating the collection capability of a swab and a tapelift for touch DNA and subsequent STR amplification success by direct PCR. Figure A shows the number of obtained donor alleles and Figure B shows total RFU. (x) shows mean markers.

When the visualised cells within the right thumbprint were collected by tapelifting until no visualised cells remained, informative DNA profiles (16, 24 and 25 alleles detected) were obtained from a portion of the tape (Figure 3.18 and Supp. Figure 3.7 B). This same area was sampled using ultrafine swabs and processed through direct PCR; full/nearly full DNA profiles could be generated from the swabs applied to the thumbprint area post-tapelifting (Figure 3.18 and Supp. Figure 3.7 C). Total RFU obtained from these swabs was about 60% of the total RFU obtained from baseline samples, however the total RFU from the tapelift amplification was approximately 10% (Figure 3.18).

A reasonable inference from these data is that the swabbing method collects more components of touch deposits, containing amplifiable but not visualisable DNA (i.e. cell-free DNA), than collection by tapelifting facilitates. These data support the finding of previous studies that indicate the vast majority of DNA (80% - 100%) from touch samples originated from cell-free DNA [115].

The data presented in this study were obtained from fresh samples deposited on substrates where collection and processing occurred within 72 hours to demonstrate the best-case scenario. The number of cells in the aged samples or those that contain inhibitors are likely to require more cells to be collected and available for extraction or direct PCR template than the reported number. In addition, mixed DNA contributions to a sample will result in the requirement of larger cell number to be collected to ensure effective allelic data generation. Where corneocytes are visible in the ridge pattern

of a fingermark, it is likely that the concern regarding mixed contribution is lower than if the visualised cells do not appear in a defined pattern.

3.5.2.5 Conclusion

Cell-free DNA appears to play a major role in the STR profiling success of sampled touch deposits, contributing approximately 60% of total DNA template responsible for RFU. From these data, it is proposed that tapelifting is unsuitable for cell-free DNA collection from non-porous surfaces (i.e. glass slide) and only facilitates the collection of corneocytes, which carry a lower amount of DNA than nucleated cells. Therefore, collection of touch deposits from non-porous surfaces by tapelift, especially when extractions are required, is not recommended. Where no alternative to tapelifting exists, it is recommended processing the samples through direct PCR; this approach requires ≥4,000 visualised corneocytes for the generation of a full DNA profile.

Even though cell-free DNA cannot be visualised at 50x or 220x magnification, which is the highest magnification of the microscope we used, this study found that there was a relationship between STR amplification and the number of visualised corneocytes targeted and collected. Therefore, the visualisation of corneocytes, achieved through DD staining, is a proxy for the visualisation of the richer template source of cell-free DNA within fingermarks.

The precise number of visualised and collected buccal cells required to generate full DNA profiles is significantly less than the required number of visualised corneocytes targeted for collection from touch deposits. Saliva samples processed through extraction or direct PCR (collected using either swab or tapelift) required ≥80 and ≥40 buccal cells respectively, for the consistent generation of full DNA profiles. Touch samples processed through 1) swabbing with direct PCR required ≥800 corneocytes within a thumbprint, 2) tapelifting with direct PCR required ≥4,000 corneocytes, and 3) swabbing with PCR amplification post-extraction required ≥4,000 corneocytes for consistent generation of full DNA profiles. The number of cells required for informative DNA profiling reported in this study is based on the best-case scenario; aged, inhibited, and/or mixed samples are likely to require the collection of more visualised cellular material than the reported numbers.

The regressions we have described can provide an indication of the type of profile that is expected given an observed cell count. For example, if a saliva sample is swabbed that had a cell count of 10 and is then processed via an extraction in the laboratory, we might expect to see a DNA profile with between approximately 9 and 25 alleles (obtained from the standard error curves in Figure 3.15) with a total fluorescence of approximately 1,000 RFU (Supp. Figure 3.6), which is approximately 40 to 100 RFU for each peak.

The methods described here, with considerations of the stated limitations, indicate that the visual cues presented by stained corneocytes or buccal cells could aid exhibit/sample triage, evidence recovery efforts, and direct subsequent workflow choices to optimise the generation of informative STR profiles.

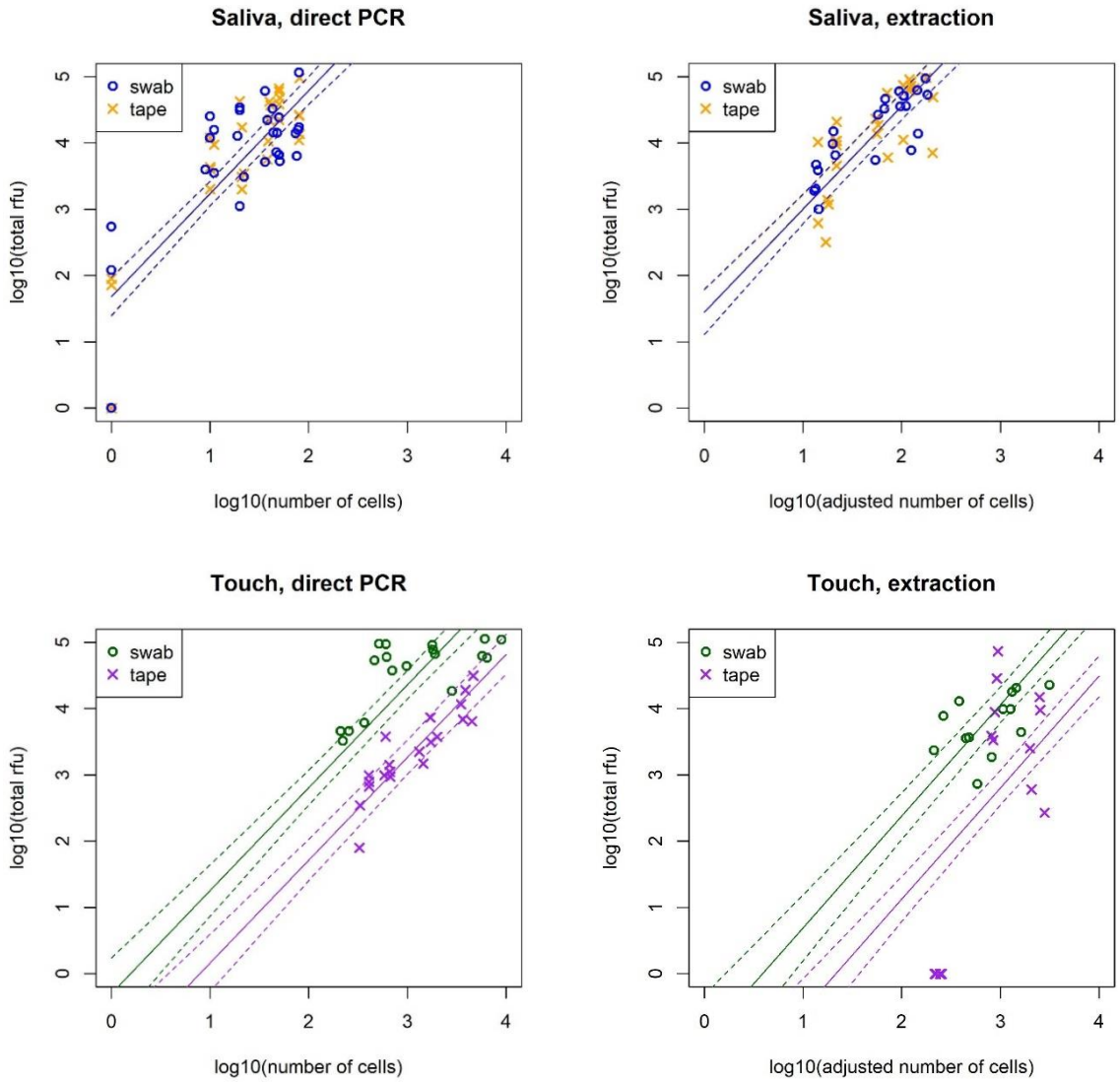
3.5.2.6 Conflict of Interest

None.

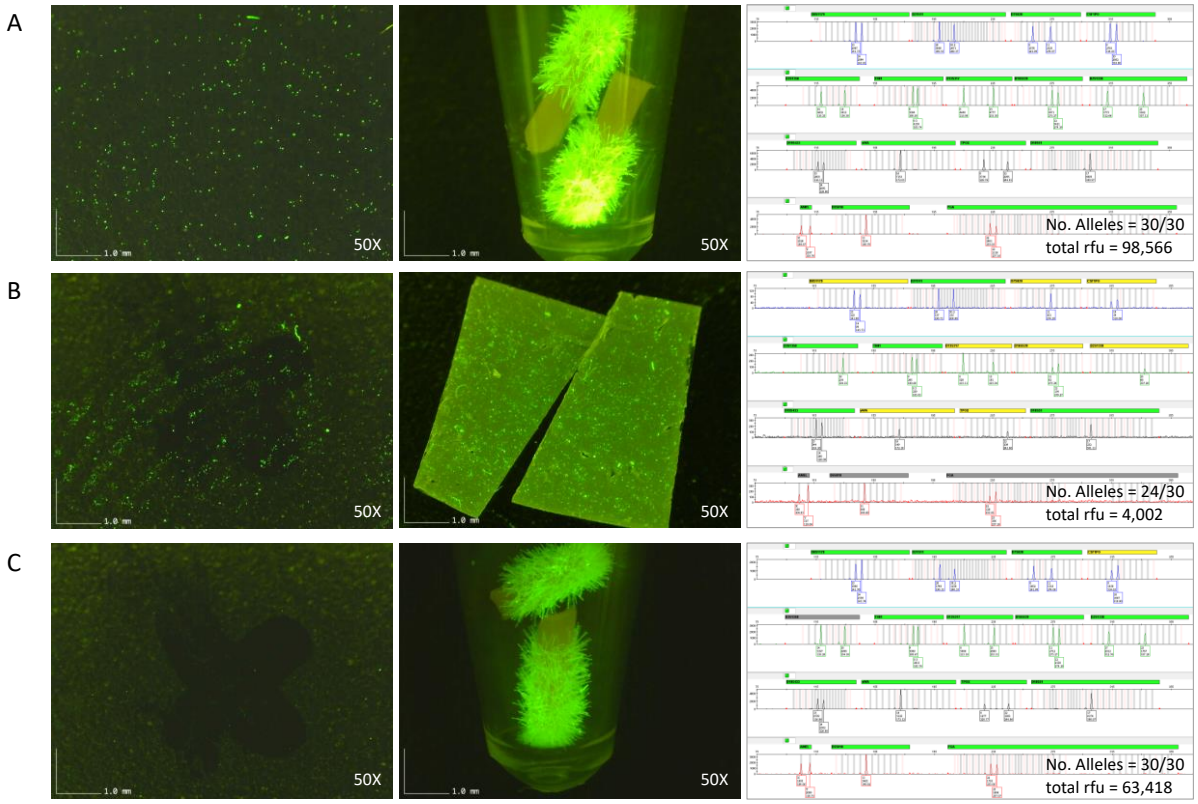
3.5.2.7 Funding and Acknowledgments

Funding for this research was provided by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund. We would like to thank the DPST, Royal Thai Government Scholarship for supporting Piyamas Kanokwongnuwut. We would like to acknowledge Dr. Catherine Hitchcock from the New South Wales Health Pathology, FASS, Australia for conception of this study and proofreading the manuscript. We would like to thank Dr. Jennifer Young for proofreading the manuscript. We also would like to thank Todd Kaesler and Amy Griffin for their contribution to this work.

3.5.2.8 Supplementary Material



Supp. Figure 3.6. Observed and predicted values (solid line is prediction and dashed lines are plus or minus two standard errors) for total fluorescence for each category of sample used in this study.



Supp. Figure 3.7. Illustrating the collection capability of a swab and a tapelift for touch DNA and subsequent STR amplification success by direct PCR. Row A shows the following: a DD stained left thumbprint (LT) on a glass slide before sampling occurred, by two ultrafine swabs; the swabs within the PCR tube; and the subsequent DNA profile. Row B shows the following: a DD stained right thumbprint (RT) on a glass slide before tapelifting; the tapelift section placed within the PCR; and the subsequent DNA profile. Row C shows the following: the RT after tapelifting (the same area row B) and before the collection of invisible DNA (i.e. cell-free DNA) using ultrafine swabs the swabs within the PCR tube; and the subsequent DNA profile.

3.6 PHASE 4 RAPIDHIT TECHNOLOGY ASSESMENT FOR TOUCH DNA TEMPLATE

3.6 Manuscript: Analysis of RapidHIT application to touch DNA samples

Manuscript published: *Journal of Forensic Sciences*, 2022

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**75**	**40**	**70**	**80**	**65**		03.08.21
TK	20	60	30	20	15		05.08.21
AL	5	-	-	-	15		05.08.21

3.6.1 Abstract

Rapid DNA technology is being utilised for reference profiles worldwide. There is also strong data in the literature to support its use for high template DNA sources, the same is not true for low template sources such as touch DNA; this is a requirement before wider implementation to forensic casework is considered. We report on the RapidHIT Intel cartridge's ability to facilitate successful amplification of touch DNA to obtain profiles from template deposited on items commonly encountered in forensic casework. Eight items were touched in ten replicates, two were tapelifted, three swabbed and three directly inserted.

Significance was observed in the alleles amplified and RFU with respect to sample type. Three samples performed well: cable tie, fabric, and matchstick. As two of these were directly inserted, this should be considered for any sample small enough. Placement of highly absorbent substrates into the cartridge is not advised as it can cause a lysate-pull error. Heterozygote loci often presented as homozygous (32%-78% loci per profile); this was influenced by substrate type and profile RFU. Loci with larger masses exhibited higher false homozygosity also.

Comparison of the donor's profile analysed was performed against previous datasets analysing touch DNA through standard workflow, including manual DNA extraction, PCR and CE separation. These data show that for all substrates, except for a fabric swatch, standard processing is preferential to RapidHIT analysis. In its current form, rapid DNA technology is not fit for the routine analysis of touch DNA samples in forensic casework.

3.6.2 Introduction

Rapid DNA technology offers the potential to obtain a DNA profile within 90 mins from a forensic sample [360]. Validation studies for the technology's use have centred around reference samples and have showed good success with respect to heterozygous balance and/or the number of samples that could be uploaded to a database [361]. Due to its limited bench space requirements, ease of transportation, and minimal training requirement [59], RapidHIT has enormous value for rural communities separated from centralised forensic institutes [360, 362], at border control locations [363, 364], at DVI sites [60, 364], and in warzones during IED campaigns [59]. The self-contained nature of the cartridge and capillary system mean that risk and occurrence of contamination is low [365] , but has been observed [366]; all fluidics and reagents are within this sealed, self-contained system [365, 367]. The technology has streamlined sample processing by performing all the required protocols, usually performed in highly controlled and specialised laboratories, including DNA extraction, the PCR, and the CE separation and detection. Under normal workflow, these procedures require manual preparation and tube changes between processes increasing labour costs and the chance for contamination [365].

To date, this technology is only permitted for the processing of single-sources reference samples in a forensic capacity [55]. In a research capacity successful application of rapid DNA technology has been applied to bone samples [56], blood samples [57], tissue samples [58], mock sexual assault kit (SAK) samples [368], and some casework samples including drinking items, handled items and clothing [369]. The literature is sparse regarding DNA template and sample sources excluding reference buccal swabs, however results have indicated that this system performs more poorly than the highly specialised standard manual workflow [54, 55, 369] . SWGDAM and the Swedish National Forensic Centre have released reports stating that, in its current capacity, rapid-DNA processing is unsuitable for crime-scene samples [366, 370]; although the Swedish study utilised a previous model, the RapidHIT 200, the SWGDAM position was based on this model and the current RapidHIT instrument. This is also reiterated by Hares *et al.* (2020), where five key areas that need to be addressed before forensic evidence is processed using rapid technology are outlined [55]. There are concerns about its potential to process low-template DNA sources and adequately analyse mixed DNA samples. A study by Hinton *et al.* (2021) is the first to apply the technology to touch DNA [59]; this study tested the ANDE™ for its suitability to IED sample analysis and was very limited in sample size.

Here we present data on touch DNA samples, typical of those found in forensic casework [106, 133], processed with Rapid-DNA technology by placing the sample within the cartridge directly or a swab or tapelift of the DNA template targeted. This study adds to the limited resources available in

the literature for the assessment of rapid-DNA processing, in its current capacity, to touch DNA template.

3.6.3 Methods

One donor was asked to touch one of each set of sample types; these individuals had been previously identified as an intermediate or high shedder following the protocol explained in Kanokwongnuwut *et al.* (2018). The substrates were as follows: electrical tape wound around a 5 mL tube; 50 x 90 mm ziplock bags; standard 40 mm matchsticks; sections (40 mm) of 370 mm cable ties; 40 mm pieces of insulated wire; 12G casings, now referred to as 12G; 80 x 40 mm cotton fabric swatch, now referred to as 'fabric'; and 100 mm piece of twine. Ten replicates of each substrate type were handled for 15 s, an undefined time post-handwashing (≥30 mins); contact was made by at least the forefinger and thumb for all samples. Alterations to the handling conditions stated above were as follows: the ziplock bag was opened and closed once to mimic normal use; the matchstick was struck and allowed to be extinguished passively; and the cable tie was looped over and pulled through. All samples underwent processing via the RapidHIT Intel cartridge (Thermo Fisher Scientific, VIC, Australia). The cable ties and insulated wire were placed directly into the RapidHIT Intel cartridge, while the matchstick samples were mounted to the back end of a cotton swab (Puratin, ME, USA), as seen in Figure 3.19, before being placed into the cartridge; this was also performed for two twine replicates. The electrical tape, ziplock bag, and 12G samples were swabbed using the Copan 4N6 (Interpath, VIC, AUS) swabs, premoistened with 20 µL 0.01% Triton™ X-100 (molecular grade, Sigma-Aldrich MO, USA), and the swab-head was placed into the RapidHIT Intel cartridge for processing. Tapelifting was performed on the fabric swatches and twine samples (8/10) as outlined in Martin *et al.,* 2019 [315]. Possible cartridge loading scenarios are shown in Figure 3.19.

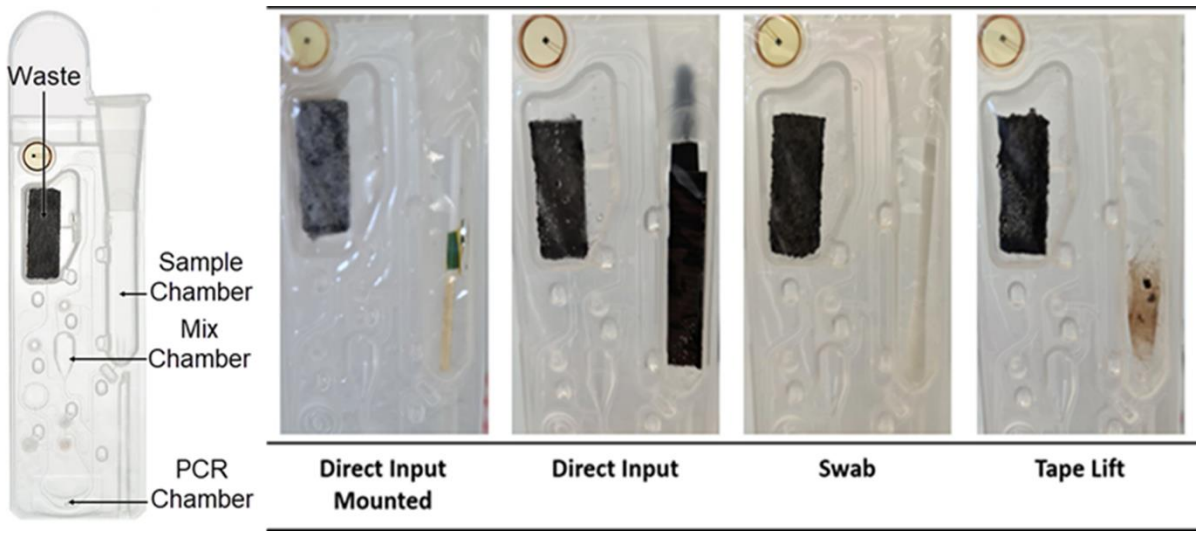


Figure 3.19. Different Rapid Intel cartridge loading scenarios based on the surface type and sampling method analysed. Left to right is exemplars of the cartridge design, direct insertion of a matchstick mounted on the back end of a cotton swab, direct input of a cable tie, swab, and tapelift.

All profiles were initially recorded using GeneMarker® along-side the RapidHIT instrument. For alterations to peak height thresholds and exportation of data, GeneMapper IDX v1.4 was used. The .fsa files were exported from the RapidHIT instrument and imported into GeneMapper IDX v1.4. The analysis methods and parameters required for this software were supplied by Thermo Fisher Scientific. Given the baseline of resultant profiles a threshold of 30 RFU was selected for alleles to be considered as real, with homozygous consideration threshold at 150; only autosomal alleles were considered. Samples were considered 'informative' if they contained 12 or more amplified autosomal alleles [3, 70, 193]; this meets the requirement for a profile to be considered up-loadable to the Australian NCIDD.

Comparison against existing datasets, from previously published data or in-house sources, was conducted also. As the raw files of these data were not available the previously used RFU thresholds of 50 RFU and 150 RFU were retained. Extractions were performed using DNA-IQ (Promega Corporation, VIC, Australia), and data for ziplock bags, matchsticks, 12G samples was performed with GlobalFiler® amplification while data from electrical tape, fabric and twine was performed with VeriFiler™ Plus amplification following manufacturers protocol with the exception of 2 µL Prep-n-Go™ (Thermo Fisher Scientific) and Low TE in place of water.

3.6.4 Results and Discussion

Following preliminary results, it was determined that a single fingermark was not sufficient template for amplification of STR data through the RapidHIT system (data not shown). This template

has been shown to previously result in amplification of STRs through standard extraction and amplification methods [326], the inference from these data is that RapidHIT lacks the same sensitivity for touch DNA amplification. This may be due to the nature of the extraction, as no mechanical disruption to the cells present on the swab can be performed, alterations in lysis parameters or other protocols, or the use of GlobalFiler™ Express in place of an STR kit more suited to low template amplification [261, 331].

Two replicates of twine were performed using an attachment to a cotton swab, however an error occurred due to an inability for the lysate to pull through the cartridge appropriately. Tapelifting of this substrate was performed for the remaining 8 replicates. Due to this, these data suggest that heavily absorbent substrates should not be placed into the cartridge directly, they need to be sampled first. The baseline noise was never observed ≥10 RFU, therefore 30 RFU was chosen as a value three times this amplitude to ensure confidence in allele calls. There was no difference in peak presence between the expert software systems, but an increase in allele call was observed in GeneMapper IDX v1.4 when this threshold was chosen.

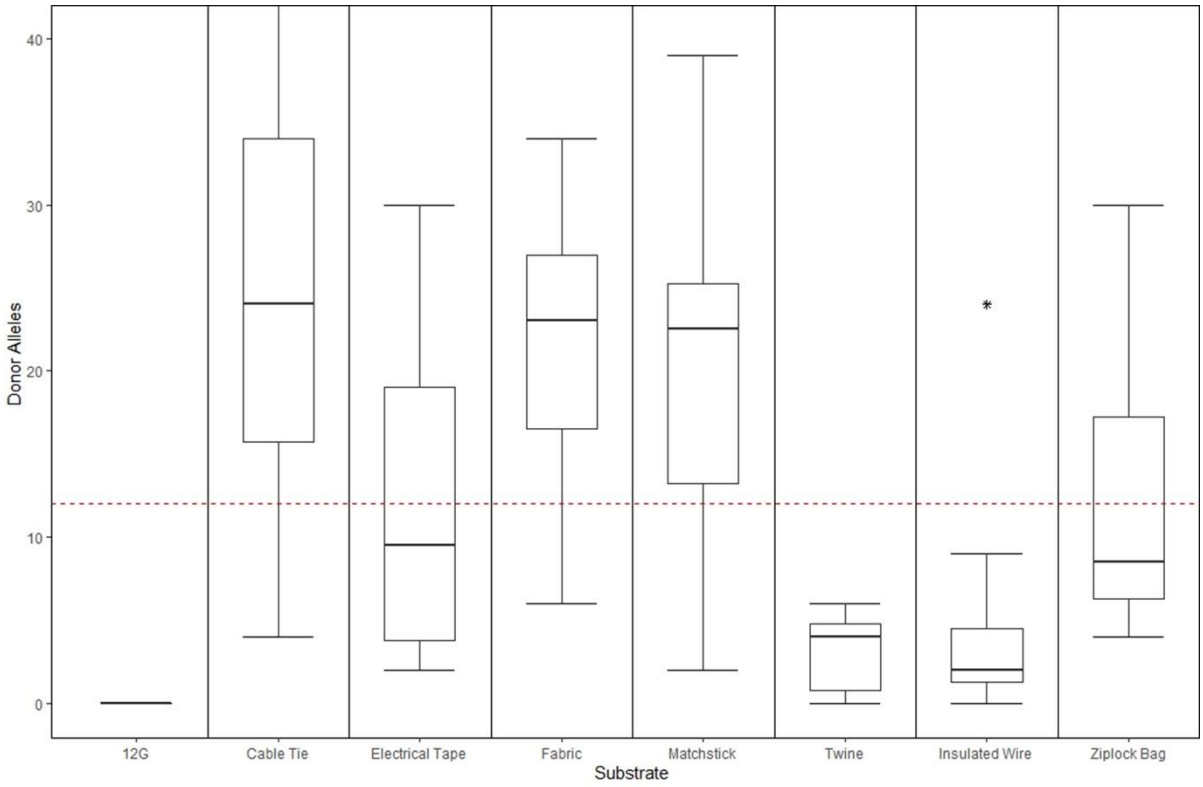


Figure 3.20. Number of donor alleles observed following RapidHIT amplification. 12G casings, electrical tape and ziplock bags were swabbed, the fabric and twine were tapelifted, and the cable tie, matchstick and wire were inserted directly (N=10 per substrate). Horizontal dashed line represents 12 alleles, the threshold considered up-loadable to a DNA database in South Australia [70].

Due to the 12G samples returning no allelic data for all replicates these samples were removed from the dataset for statistical analyses. There was significant difference observed between the substrates, with respect to number of alleles ($p= 5.50x10^{-6}$) and total profile RFU ($p= 4.11x10^{-5}$) (Figure 20, Supp. Figure 3.8). Only one sample, a cable tie, produced a full DNA profile with 41916 as the total autosomal allele RFU.

The low success in generating STR data from the ziplock bags and electrical tape has also been seen previously, for example: Martin *et al.* (2018) found that post-extract ziplock bag yielded no informative DNA profiles [289]; Krosch *et al.* (2020) showed a 12.7% suspect identification rate with ziplock bags and 5.4% of profiles being full for electrical tape samples [133]; Wong *et al.* (2019) reported 45% of ziplock profiles were informative (informative consideration threshold not given) with 16% of tape samples being full DNA profiles [371]; and Mapes *et al.* (2016) described 9% profile success with tape samples [299]. Martin *et al.* (2019) reported that following extraction, touch DNA on matchstick samples resulted in 62% of profiles that were informative, containing ≥12 autosomal alleles, with an average of 17 of 32 alleles; this is however, based on laboratory experiments rather than a report of exhibit success rates as these are not available in the literature. Within our dataset, 80% of matchstick profiles were considered informative, containing ≥12 autosomal alleles, with an average of 19 of 46 alleles. When data were considered against the cable tie set (average 24 alleles), there was no significant difference between the fabric (average 21 alleles) or matchstick (average 19 alleles); allelic yield was significantly lower for all other sample types (Figure 3.20). With the exception of the insulated wire, the samples that underwent direct application into the cartridge performed well; both the matchstick and cable tie substrates resulted in 8/10 samples producing informative profiles. The tapelifted fabric also produced informative profiles in 8/10 samples while the electrical tape and ziplock bag samples resulted in 4/10 and 3/10 samples respectively; the wire produced only one informative profile (24 alleles) and the twine produced none. The reduction in alleles observed in DNA profiles for the other sample types, excluding fabric, may be due to ineffective recovery of cellular and cell-free DNA by the swab [205, 372], or a stunted release of collected material into the extraction solution [373]. Additional sample types that are either swabbed, tapelifted or placed directly into the cartridge would be worth investigating to explore whether there is any relationship between sample treatment prior to cartridge use and allelic yield.

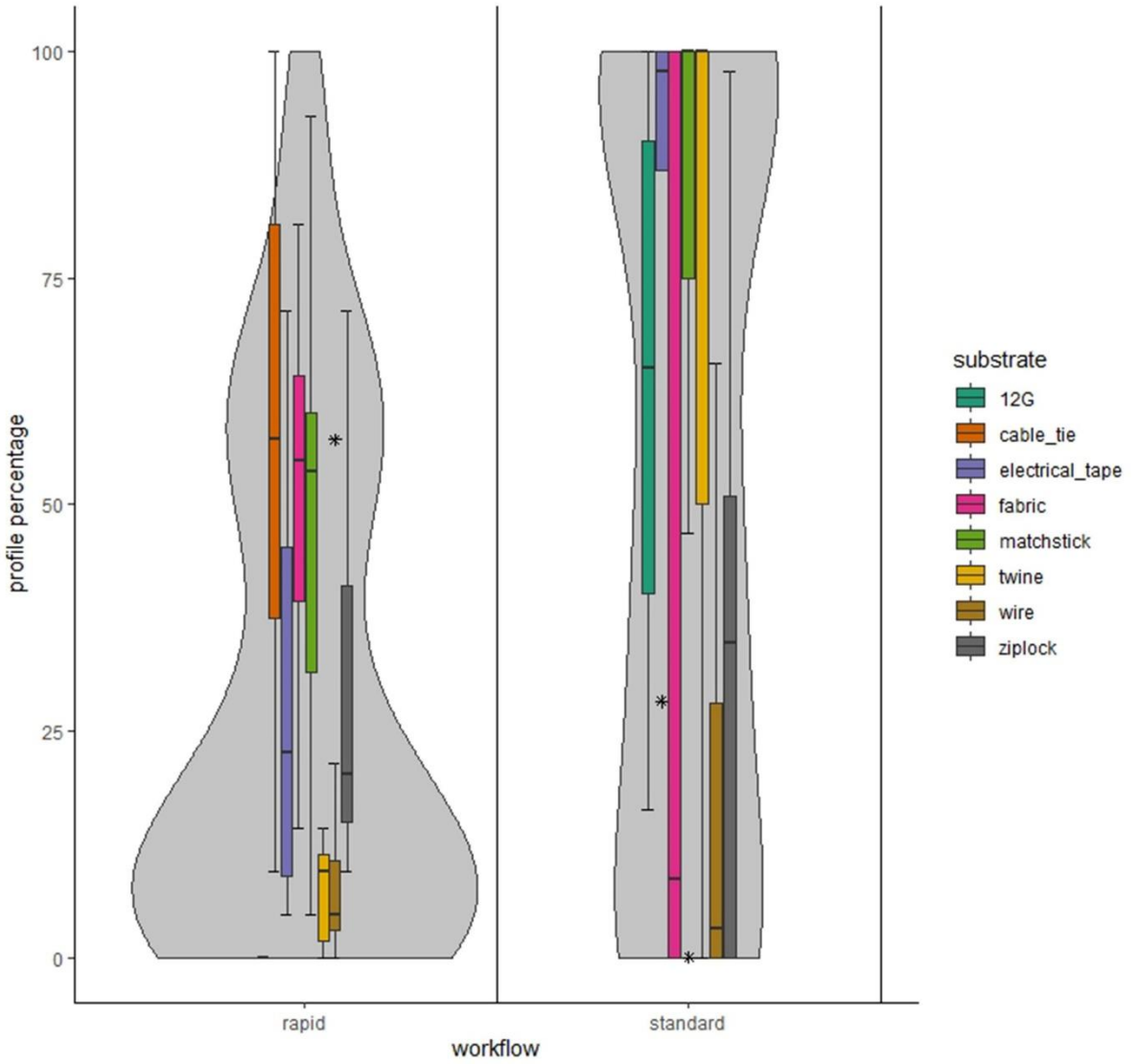


Figure 3.21. Violin plot showing the profile coverage observed following RapidHIT sample processing (left) or standard practice processing (right) for all substrates. Overlayed are box and whisker plots of the individual substrates profile coverage, which collectively forms the overall distribution (violin).

All samples, except cable ties, have been previously been part of published or in-house datasets for standard extraction workflows with the same deposition methods and similar known donor shedder status [289, 315]; only autosomal allele counts were considered from these works. As different kits were used for some of these sub-sets, the percentage of the profile amplified was considered rather than the number of alleles or RFU present. The proportion of the dataset present at each 'profile percentage', shown as a violin distribution, shows that a larger portion of the RapidHIT dataset exists lower than the 'standard workflow' set indicating a larger proportion of these samples resulted in lower STR amplification (Figure 3.21). This is also shown statistically as the comparison between the percentage of the profile amplified and the workflow utilised, being RapidHIT system

processing or extraction by DNA-IQ (Promega) followed by standard PCR, showed significant difference between the workflows (p= 0.009722). Overlayed are the data for individual sample types, displayed in standard box and whisker plots, which allows visual comparison of the individual substrate types between processing methods (Figure 3.21). The standard workflow resulted in higher profile coverage, the percent of the profile present, for all substrates except the fabric; significance was seen for electrical tape (p= 0.00323), matchsticks (p= 0.030325) and twine (p= 0.0005871).

Through the incorporation of lower allelic thresholds to 30 RFU, possible due to the low baseline noise observed, the number of alleles called may be increased for low level samples; analytical thresholds are usually set to 50 RFU [374, 375]. Identification of baseline noise observations across users worldwide, and standardisation of RFU thresholds for allele calls would be valuable to maximise allelic data recovery and increase the ease of research result comparisons; this desire has also been shared by Hares *et al.* (2020) [55]. This standardisation is missing in current workflows as individual laboratories set their own allele call threshold guidelines [70, 376-378].

A concerning observation made through the analysis of the subsequent profiles was the homozygous appearance of many loci that were meant to exhibit heterozygosity. To analyse this, the number of loci that were expected to be heterozygous and its alleles were above 150 RFU were counted as either 'true heterozygous' or 'false homozygous'. The proportion of these counted loci that were 'false homozygous' was then calculated; the twine and insulated wire samples were removed due to the lack of relevant data present for these substrate types. LR tests of the logistic regressions involving the log10 (total profile RFU), the proportion of the profile amplified per sample, and the substrate type against the false homozygosity proportion were conducted; allele number ($p= 1.4x10^{-7}$), substrate (p= 0.0023) and RFU ($p= 2.6x10^{-8}$) were all found to influence the instance of false homozygosity; as allele or RFU value increased, the instance of false homozygosity decreased (Supp. Figure 3.9) Wire and twine samples resulted in the highest average proportion of heterozygous loci exhibiting a false homozygous peak in 62% and 78% heterozygote loci respectively, with the remaining substrates falling between an average of 32% - 49% heterozygote loci (Figure 3.22). Loci were also affected differently with the general trend being with increasing mass the rate of false homozygote classification also increased (Supp. Table 3.3); D21 was the exception to this and exhibited the highest rate with 63% occurrence.

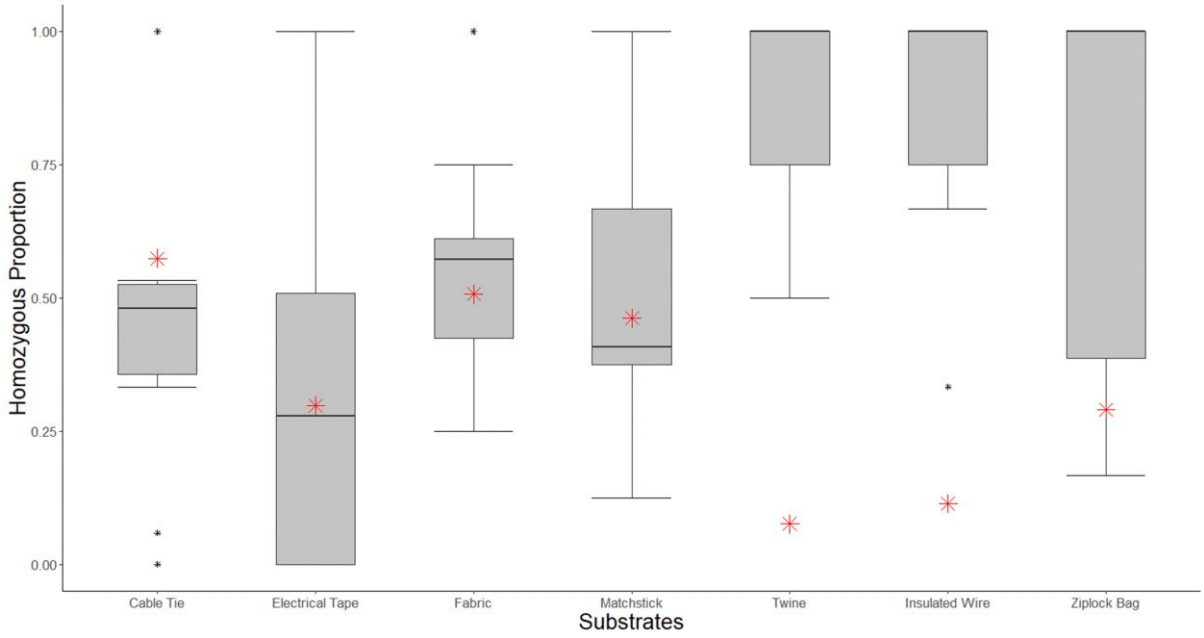


Figure 3.22. Box and whisker plot showing the proportion of loci exhibiting false homozygous peaks per profile; these are separated by substrate. The asterisk represents the average allele percentage of the profiles obtained from each substrate with y=1 representing 42 alleles.

This phenomenon of false-homozygosity could lead to complications with peak imbalance ratios, currently expected and accounted for in statistical modelling software, and therefore lead to inaccurate comparisons to reference data. Peak imbalance can be exaggerated with low level samples [379, 380], however in many profiles there were loci with no indication of heterozygosity where this was the expected result (Figure 3.23). Peak RFUs where this occurred were low in comparison to those often observed in traditional extraction-based methods, therefore this apparent homozygosity may simply be due to a preferential amplification of one allele over the other in the first few rounds of PCR. This could lead to complications with peak imbalance ratios, currently expected and accounted for in statistical modelling software, and therefore lead to inaccurate comparisons to reference data. Differences in profile interpretation, compared with standard methods, may need to be considered before the introduction of rapid DNA analyses from trace sources into criminal casework. In addition to this, due to the peak imbalance observed, and distinct homozygosity, mixture presence is expected to complicate profile interpretation and analyses. Mixture presence will also complicate the issues of homo/heterozygosity assessment and contributor number designation in profile interpretation when such low RFUs are observed in subsequent profiles. This work supports the requirements for readiness outlined by Hares *et al.* (2020) with respect to peak height ratio balance and mixture interpretation [55].

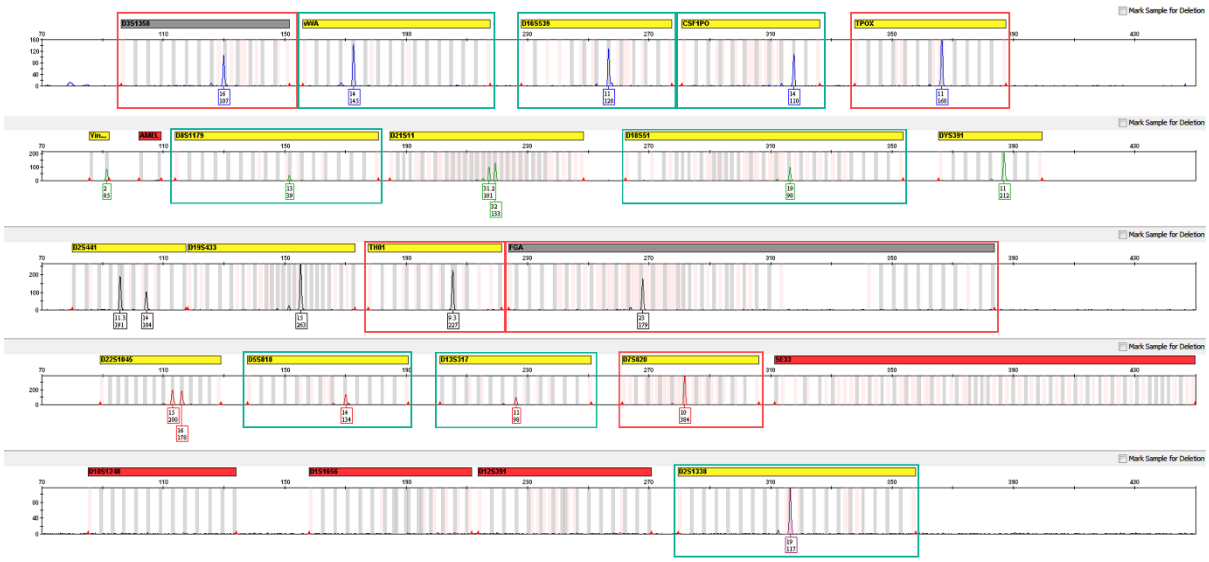


Figure 3.23. Example of a profile showing high unexpected homozygosity. The green boxes are around loci that are expected to be heterozygous but are below the 150 RFU threshold to be considered. The red boxes are around loci that are above 150 RFU and are therefore called as false homozygotes.

Sensitivity studies that explore the cellular input thresholds of touch DNA for amplification success should be performed to allow a robust comparison of the amplification of the appropriate alleles by RapidHIT compared to standard processing. It has been reported that >4,000 corneocytes are required for the generation of a full DNA profile following standard profiling methods [326]. Determining the guidelines for RapidHIT amplification success would be valuable to enable its incorporation into workflow when it is suitable to do so. This may also indicate the applicability of the RapidHIT system to rural communities already utilising rapid technology for casework samples.

This work identifies touch DNA samples that are suitable and unsuitable for rapid DNA technology application [55]. With the technology as it currently stands, RapidHIT processing should not be incorporated into touch DNA analysis, unless the sample itself can be placed into the cartridge; porous substrates are the exception to this as they need to be sampled first. Processing touch DNA samples for intelligence purposes, where standard laboratory facilities are not available or swift results are required, has been shown to be possible with the limitations and caveats discussed. Investigation into alternate rapid DNA processing, for example with the ANDE [55, 59], and an in-depth analysis as to the cellular input thresholds required for success [326], should be conducted before remote processing of touch DNA samples is performed using rapid amplification.

3.6.5 Conclusion

It was found that rapid DNA technology was not suitable for 12G cartridge, insulated wire, or twine sampling in its current state. Cable ties and matchsticks were able to be placed directly into the cartridge and facilitate STR amplification, while tapelifting of fabric also resulted in informative profiles in the majority of samples. Swabbed samples did not result in the same amplification success and optimisation of this technique should be considered; the porosity of the swab may cause eluate drawing issues similar, but with reduced intensity, to that observed with the twine.

To provide a more robust comparison against standard processes sensitivity studies, similar to those undertaken by Kanokwongnuwut *et al.* (2021), should be performed and compared against existing datasets. Understanding the instances of artefacts or profile interpretation complications such as drop-in/out, imbalance assessments, and stutter will be crucial before implementation into casework or adequate research studies can be performed with sub-optimal template sources. These considerations in profile interpretation may not be as necessary for intelligence purposes.

The benefits of analysis speed are vast, however its application to compromised or low template sources is not appropriate in its current iteration. Improvements or alterations in cartridge design to allow the user to input an eluate easily or to perform amplification using a different kit, specifically a kit with a buffer system more robust against inhibitors, would increase the suitability of the technologies application to a wider range of template sources. Although alternate kit use may incur increased PCR times the benefits of 'ease of use' and the lack of specialised training required would still make this a valuable tool for the forensic and law enforcement communities.

3.6.6 Conflict of Interest

None declared

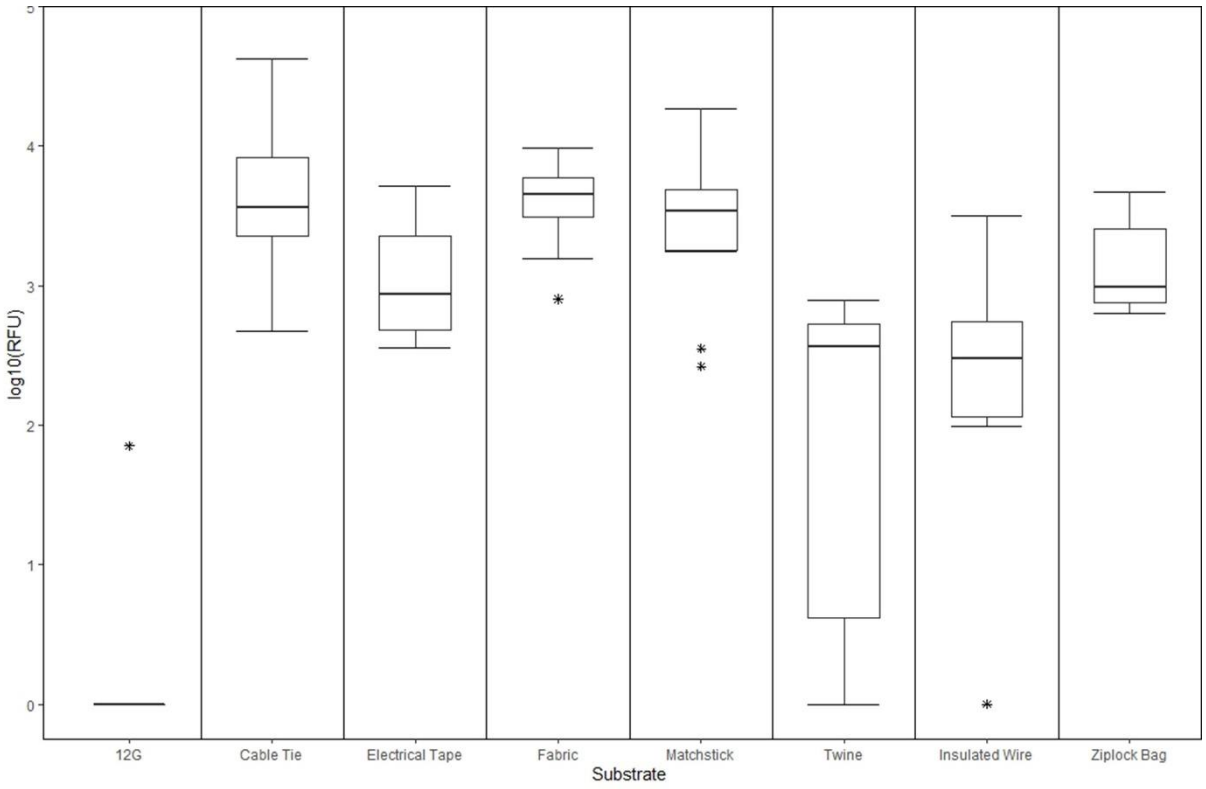
3.6.7 Funding and Acknowledgements

This research was supported by Ross Vining Research Fund provided by the Attorney General's Department of South Australia operated by Forensic Science SA We would like to acknowledge the DNA donors for their contribution to this work.

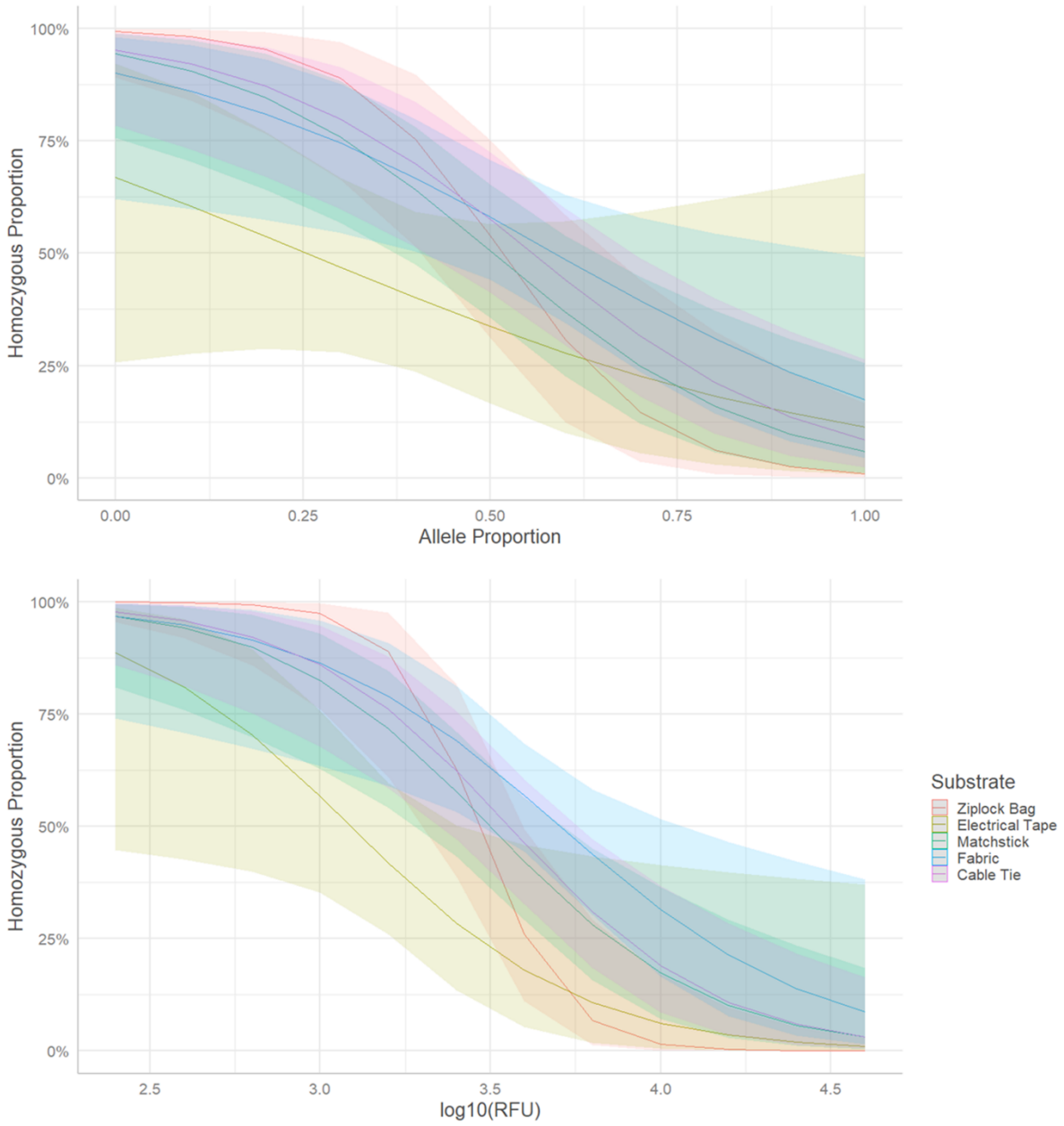
3.6.8 Supplementary Material

Supp. Table 3.3: False homozygous call occurrence by loci for all samples within the dataset. Loci are coloured by dye lane and the relative locus location left to right is the descending order of loci within each dye lane in the table. 'Occurrence' denoted the percentage of profiles in which the corresponding locus exhibits false homozygosity.

Lane	Locus	Occurrence
6-FAM	D3	31%
	VWA	17%
	D16	40%
	CSF	56%
	TPOX	54%
VIC	D8	25%
	D21	63%
	D18	33%
TED	D2S441	19%
	D19	28%
	THO1	47%
	FGA	47%
TAZ	D22	34%
	D5	44%
	D13	52%
	D7	54%
	SE33	60%
SID	D10	21%
	D1	43%
	D12	33%
	D2S1338	58%



Supp. Figure 3.8. Log10(RFU) of profiles observed following RapidHIT amplification. 12G casings, electrical tape and ziplock bags were swabbed, the fabric and twine were tapelifted, and the cable tie, matchstick and wire were inserted directly; N=10 per substrate.



Supp. Figure 3.9. Plot of the logistic model observing the interaction between the number of alleles within a profile and the substrate (upper) and the total RFU within a profile and the substrate (lower) for their combined influence for their combined influence over the proportion of loci exhibiting false heterozygous peaks.

3.7 CONCLUDING COMMENTS

The data presented within 3.3, Phase 1, highlights the capabilities of direct PCR to amplify up-loadable profiles from trace amounts of latent DNA from a range of surface types that have previously remained unanalysed. DNA profiles generated from eight non-porous substrate types with different surface properties showed no statistical difference in the number of alleles present per profile when amplified by direct PCR; direct PCR produced profiles with significantly more data compared with the extraction workflow. The aim "*to investigate further the viability of the direct PCR amplification workflow to a wider range of exhibit surface and sample types*" has been addressed within this work. This body of work indicates that care must be taken when analysing latent DNA with direct PCR as mixtures, and major contributor assessment, can be misleading when forming conclusions surrounding item handling.

The data presented within 3.4, Phase 2, provides a simplified approach to determine an individuals' shedder status and indicates a relationship between the number of cells visualised and the quality of the resultant STR profile post direct PCR amplification. It has been determined that the following cell counts constitute these corresponding shedder category classifications: low 0-15 cells/mm^2; intermediate 16-30 cells/mm^2; high 30+ cells/mm^2. The aim "*to identify whether a relationship exists between the shedder status of an individual and the expected quality of an STR profile once a fingermark is processed*" has been addressed within this work. This work identified that additional research into the relationship between visualised cells and donor peaks amplified would provide invaluable guidance to forensic evidence recovery personnel and other research groups as to collection requirements if a nucleic acid staining method is integrated into current workflows.

The data presented within 3.5 Phase 3 highlights the value of the direct PCR workflow with respect to low template samples through the visualisation and targeted collection of distinct amounts of cellular material. It has supported the introduction of direct PCR into casework settings where the number of cells available for collection and subsequent amplification are too low for standard processing methods. These thresholds are now known for direct PCR and PCR post-extraction workflows for latent DNA on simple substrates. A requirement of 80+ buccal cells for the extraction workflow and 40+ buccal cells for the direct PCR workflow exists regardless of sampling tool. For touch DNA, sampling device affects the cellular requirements for profile generation; for the extraction workflow there is a cellular requirement of 4,000+ when utilising a swab and 8,000+ when a tapelift is used while for the direct PCR workflow 800+ cells are required when utilising a swab and 4,000+ cells are required when tapelifting. The aim "*to discover the visualised cellular material thresholds at which an informative and full DNA profile are able to be obtained given different cell types and workflow*

choices" has been addressed within this work. This work identifies the importance of exhibit triage, selection of sampling devices, and amplification workflow choice.

The data presented within 3.6, Phase, 4 highlights that the RapidHIT system is not currently suitable for the robust amplification of latent DNA as template. Much improvement to the cartridge design is required to optimise this system for low level analysis, and care must be taken as to what items are placed within the cartridge before loading. The aim "*to analyse the potential use of the RapidHIT system for the amplification of latent DNA samples*" has been addressed within this work. It has been concluded. It would be valuable to assess the RapidHIT's performance following the same methods as presented in 3.5; as improvements are made within the construction of the cartridges, the systems use should be re-assessed for latent DNA application.

3.8 APPENDICES

3.8.1 Poster presentation at "The Australian and New Zealand Forensic Science Society" conference, Perth, Australia, 2018. 'Shedding Light on Shedder'.

Shedding Light on Shedder

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Presented at the ANZFSS 24th International Symposium on Forensic Sciences
Perth Convention and Exhibition Centre (PCEC), Perth, Australia

POSTER NUMBER: 206

Abstract

We present a study that accurately measures the deposition, collection and amplification of DNA by a range of donors allowing for an accurate determination of the shedder status of individuals. Eleven donors were asked to wash their hands and then deposit a thumbprint onto glass slides for 15 seconds 0, 15, 60 and 180 minutes after hand-washing. Both left and right thumbs were used and all testing was performed in triplicate. Measurement of the quantity of cellular material deposited on the slides was carried out using Diamond™ Nucleic Acid Dye (DD) and fluorescence microscopy on each of 264 thumbprints. Real-time monitoring of the collection and transfer of cellular material from the substrate to the PCR substrate ensure that none of the DNA present on swabs was lost during direct PCR DNA profiling. The combination of using a DNA binding dye and direct PCR allowed an accurate means of measuring individual's differences in their propensity to deposit DNA. The 11 donors fell into one of three groups: heavy, intermediate, and light shedders, regardless of the hand used.

Introduction

The concept of a shedder or non-shedder came from the now well-cited paper by Lowe *et al.* [1] when it was reported that some individuals had a higher propensity to transfer their DNA than other donors; and so came about the concept of a 'shedder' or 'non-shedder'. There ensued a number of follow-up examinations with initially varying results [2,3] but comprehensive studies have more recently shown that there is a difference between peoples' propensity to transfer DNA, regardless of gender or hand-dominance [4-7]. All previous studies started by collecting transferred biological material to a swab and as then the DNA extracted by a stand process. Both the swabbing step and DNA extraction can lead to uncertainties in the uniformity of collection and retention of DNA.

We present a study where the amount of DNA transferred by contact at specific time intervals post-handwashing, can be estimated and recorded as a series of images collected using fluorescence microscopy. DNA can be detected by DD as this is a molecule that binds to an external groove in DNA and importantly DD does not bind effectively to microbial DNA and has little effect on the amplification process [8,9]. The dye has an excitation of 494 nm and emission at 558 nm such that it can be visualised using a digital fluorescence microscope equipped with an emission of filter of 510 nm and a blue LED excitation light (480 nm) even under ambient lighting. DD has only recently been applied to hair, saliva, skin flake [9,10] and here it is used specifically to determine shedder status. Further, by using direct PCR we circumvent the issue regarding unknown loss during the DNA extraction process and can make a direct correlation with the amount of DNA that was collected on the swab and the subsequent DNA profile.

Materials and Methods

2.1 Deposition of DNA

A total of 11 individuals comprising 5 males (M1 – M5) and 6 females (F1 – F6) washed their hands. Control samples were collected from areas of the clean glass prior to any deposition of a thumbprint. The volunteers made contact with these clean DNA-free glass slides with their left and right thumb for 15 seconds with medium pressure. The time intervals post-handwashing were: 0, 15, 60 and 180 minutes. All tests were performed in triplicate creating 264 data-sets.

2.2 Staining of DNA

The slides were stained with 20 fold DD diluted with 75% (v/v) ethanol. A Dino-Lite fluorescent digital microscope was used to visualise the presence of dyed material, referred to as cellular material after as shown in Fig. 1. Scoring of cellular material abundance was performed by counting the number of bright spots in three frames (each 1 mm^2) under the microscope at 220 x magnification.

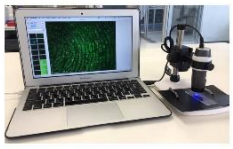

Fig. 1 showing a DD stained fingermark on a glass slide visualising under a digital microscope connected with MacBook Air laptop (11").

2.3 Collection of DNA

An ultra-fine swab was used to collect material from the slides. The swabs were moistened with 2 µL of 0.1 % Triton-X solution and applied to the entire thumbprint on each slide.

2.4 Amplification of DNA

Direct PCR was performed using the Identifiler Plus™ kit by removing the swab head directly into a PCR tube. Amplification was performed in 25 µL, with exceptions to the validated protocol being 2 µL of Prep-n-Go™ and Low TE Buffer in place of water. All amplifications were performed using a ProFlex thermal-cycler. The total number of cycles was the validated 29 cycles. PCR product (1 µL) was added to 8.7 µL Hi-Di formamide and 0.3 µL 600 LIZ™ and separated on a 3500 Genetic Analyser. Data were analysed using GeneMapper ID-X (version 1.4).

Results and Discussion

- The use of a DD DNA staining dye can visualise the presence of cellular material and allow real-time collection of the cellular material to a swab head.

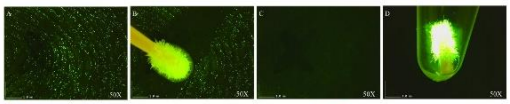

Fig. 2 illustrating four steps in the collection of DNA from touched glass slides. Fig. 2A shows the fingermark stained with DD. Fig. 2B shows a micro-swab in the act of removing cellular material. Fig. 2C shows the sample area post swabbing with a now non-stained surface. Fig. 2D shows the material that was on the surface has now been transferred to the swab and is in a 0.2 mL PCR tube for downstream processing.

- Correlations can be drawn between the amount of cellular material and the resulting DNA profiles.

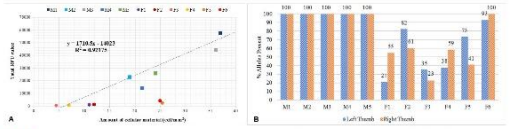

Fig. 3A illustrating the correlation between the total RFU value (y-axis) and amount of cellular material (cell/mm²) (x-axis) both left and right thumbprints deposited 60 minutes post-handwashing. Fig. 3B illustrating the resulting Identifiler Plus™ profile coverage (%) of the left and right thumbprints from each participant. This is based on 15 loci, plus the two amelogenin markers from each profile

- The shedder status is based on the amount of cellular material transferred as shown in Fig. 4. The 11 donors fell into one of three distinct groups: heavy, intermediate, and light shedders, regardless of the hand used.

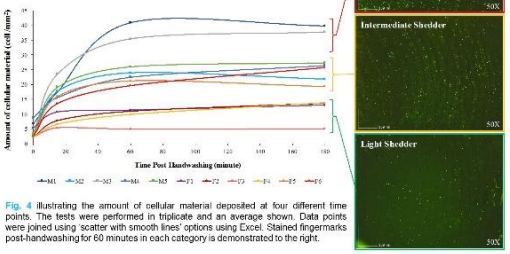

Fig. 4 illustrating the amount of cellular material deposited at four different time points. The tests were performed in triplicate and an average shown. Data points were joined using 'scatter with smooth lines' options using Excel. Stained fingermarks post-handwashing for 60 minutes in each category is demonstrated to the right.

Concluding Remarks

- A new shedder status determining method is presented that is simple to perform.
- Heavy and light shedders were observed, with others being intermediate between these two shedder types, and with little variation between marks deposited by an individual's left and right thumbs.
- By recording the amount of cellular material deposited in a thumbprint and considering the resulting DNA profile, variations in the shedder status of individuals can be more accurately than was previously possible.
- Triplicate samples from each person showed little variation in the amount of cellular material deposited for each independent time point. There was also little variation between the left and right thumbprints with respect to the amount of cellular material deposited.

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3.8.2 Poster presentation at "The Australian and New Zealand Forensic Science Society" conference, Perth, Australia, 2018. 'DNA Profiles from Touched Samples'

CHAPTER IV:

REPRODUCIBILITY IN DIRECT PCR

Manuscripts enclosed:

Martin, B., Taylor, D., Linacre, A., (2022). "*Exploring tapelifts as a method for dual workflow STR amplification*"

Forensic Science International: Genetics

4.1 PREFACE

One of the major drawbacks to the direct PCR workflow that restricts its uptake for the examination of forensic casework samples is the inability to re-test an exhibitory sample. As many operational laboratories require both quantification and the ability to re-test a sample for either prosecution or defence purposes, due to accreditation or internal guidelines, direct PCR has not been integrated into many workflows for the analysis of latent DNA samples. The applicability of this concept can be addressed by identifying whether:

- multiple direct PCRs performed on sub-samples from a single sample can be considered technical replicates of one another;
- both direct PCR and PCR post-extraction can be performed off a single sample.

This chapter is split into two phases, each of which examines aspects relating to the reproducibility of direct PCR utilising tapelifts as a collection medium. Although previous results (see 3.5.2) have shown that swabs have a higher collection efficiency than tapelifts for touch DNA deposits utilising direct PCR, tapelifts were selected due to their ability to be easily sectioned. It is also understood from previous work that tapelifts are a viable collection tool for the amplification of STRs from latent touch DNA samples (see Chapter II and Chapter III Phase 1 previously, and Chapter V Phase 1 subsequently; Chapter V Phase 1 was performed prior to this work chronologically). Results from both phases are reported in a manuscript under review at *Forensic Science International: Genetics*.

The first phase of the study compares triplicate STR profiles obtained from one tapelift sample utilising the direct PCR workflow. Allelic concordance and profile quality, with respect to RFU values, are analysed to determine the viability of performing multiple PCRs from a single tapelift sample.

The second phase of the study investigates the possibility to obtain a direct PCR profile from a portion of a tapelift while using the remainder of the tapelift through the standard extraction-based workflow. This study identifies the limitations to this approach as well as highlights the potential benefits if integrated into trace analysis in operational laboratories.

4.2 IMPACT STATEMENT AND AIMS

The ability to produce a viable extract pool as well as generate a DNA profile following direct PCR will allow for the benefits of both workflows to be exploited for a single sample. The increased sensitivity available through direct PCR amplification allows the production of the ‘best case’ DNA profile, while the ability to perform an extraction from the remainder of the tapelift allows accreditation or laboratory-based requirements to be adhered to. The data presented within this

chapter provides empirical data to support the introduction of dual workflows to latent DNA samples through the incorporation of tapelifts.

4.2.1 Aims

Phase 1: "*To determine whether the generation of multiple concordant profiles is possible from a single tapelift sample following direct PCR amplification*"

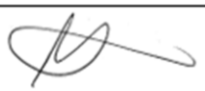
Phase 2: "*To investigate the potential of obtaining data from both extraction-based PCR and direct PCR from a single tapelift to facilitate a more seamless introduction of direct PCR into laboratories that require a sample to be re-testable*"

4.3 CAN TAPELIFTS FACILITATE DUAL WORKFLOW OR TECHNICAL REPLICATES FOR DIRECT PCR?

4.3 Manuscript: Exploring tapelifts as a method for dual workflow STR amplification

Manuscript published: *Forensic Science International: Genetics,* 2022

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**80**	**100**	**75**	**100**	**80**		03.08.21
DT	10	-	25	-	10		03.08.21
AL	10	-	-	-	10		05.08.21

4.3.1 Abstract

Although a version of direct PCR is implemented in forensic laboratories for reference material, its incorporation into workflow for the analysis of touch DNA, as a form of latent DNA, from casework exhibits is not. In addition to concerns about increased sensitivity causing more complex mixtures or the generation of more genetic data implicating an individual superfluous to the context of the alleged event, the complete use of the collected sample in the PCR as template has meant that there is no possibility for data reproducibility when needed. Here it is proposed that the use of tapelifts in touch DNA collection can facilitate replicate direct PCR analysis from a single sample allowing the sample to be re-tested. If all portions of the tapelift result in profiles with allelic and LR concordance, these sub-samples may be accepted as technical replicates, thus meeting any accreditation guideline requirements. Furthermore, we assess the use of a single tapelift for both direct PCR and extraction-based PCR workflows to illustrate the potential for benefits of both systems to be facilitated.

DNA was deposited by three donors onto six substrates with five sample replicates of each condition. Separation of each tapelift into three portions for three direct PCRs ensued using VeriFiler™ Plus. Separation of single tapelifts into three direct PCRs showed no statistical difference in donor allele calls or RFU, or subsequent LRs associated with their profiles. Comparison of profiles within the single tapelift showed more similarity, with high mixture-to-mixture match likelihoods, than when

these sub-samples were compared with profiles generated from other samples. This allows each sub-sample taken from the tapelift to be considered as technical replicates.

For dual workflow facilitation assessment, one donor deposited DNA through touch onto six substrates with five research replicates of each. Separation of single tapelifts into two portions, one for direct PCR and the retention and use of the remaining portion for extraction and subsequent PCR, showed no significant difference in allelic yield and subsequent donor comparison LRs. Comparison of deconvoluted profiles produced from a single tapelift showed high mixture-to-mixture match likelihoods, supporting DNA donor concordance. This indicates that removing a portion of a tapelift for direct PCR amplification, while processing the remainder through standard processes, allows increased sensitivity through direct PCR while offering the preparation of an eluate suitable for repeated analyses.

4.3.2 Introduction

The integration of direct PCR into casework has been in much debate [3, 204, 319, 381]. During its infancy, the technique's limitations were not well discussed and its introduction to forensic casework was, rightly, slow. It is now well understood that the technique is incredibly advantageous for a niche set of conditions [193, 247, 260, 313], whereas standard practice is still preferable for the bulk of forensic workload. Included in these niche conditions is having a small surface area with sub-optimal template source (for sample-direct direct PCR) [264, 267, 289], or reference samples (for dilution-direct PCR) [247, 382]. Three key limitations to direct PCR influencing its niche's boundaries are the inability to re-test the sample or retain an eluate, the lack of quantification data able to be obtained, and the inability to remove PCR inhibitors. This report discusses and explores the limitation surrounding the issue of a lack of reproducibility in direct PCR due the to the technician's inability to retain an eluate or re-test the sample using the same or alternate panels, utilising the sample-direct PCR method.

Legislation and/or internal accreditation guidelines often state the need, wherever possible, for human specific quantification data to be associated with a DNA extract, and/or the defence or technician to have the ability to re-test the sample [273, 274, 383-386]. This workflow restriction, due to the nature of direct PCR, causes additional interpretational challenges above the incapacity to re-test the sample. These include the inability to identify whether there will be insufficient template for amplification, too much template, the presence of an inhibitor within the sample, or DNA from too many contributors for interpretation. As the sample is exhausted during the single amplification event, optimisation of the PCR and CE injection methods cannot be performed, this is in contrast to samples that undergo standard processing.

Despite these disadvantages, it is possible to obtain highly informative DNA profiles from exhibits from which standard methods are known to consistently fail, such as the analysis of touch DNA [106, 133, 315]. These occurrences are not limited to shedder [163] or sample type [143, 289] as the technique has been comprehensively studied under an extensive variety of conditions [204, 264, 265, 313]. The ability for direct PCR technology to be incorporated into a larger pool of latent DNA exhibit types, while maintaining the ability to optimise the PCR or retain a portion of sample for additional analysis, would strengthen the argument for its niche application into casework and facilitate its incorporation under current legislative or accreditation guideline requirements.

The definition of replicate in its verb form is to 'make an exact copy', or to 'reproduce' [387]. In the application of reproducible results from replicate sampling of a single extract, in the context of forensic 're-testability' [273], the replicate is a technical replicate [383]; that is, a repeated measurement of the same sample is performed [388]. Injecting an identical PCR product multiple times will produce subtle differences in their electrophoretic mobilities or DNA masses detected in subsequent EPGs [389]. This can be in terms of RFU or called alleles with low level contributors. In addition to this, variability in PCRs performed from the same extract is also well-documented [390]. Because of this, the verb form of 'replicate' is not strictly possible [383, 391, 392], however the understanding of a technical replicate allows for this. For much research, 'replicate' sampling is the testing of multiple samples of the same type processed using the same method, but not strictly the same sample; referred to in this text as a 'research replicate' and more widely as a biological replicate [388]. This is done to observe how that sample type behaves multiple times and be able to exclude any outliers to make comparisons to other datasets stronger [392]. Therefore, within a typical forensic workflow (also applicable to research workflow) replication of a test can occur at different stages of the profiling process:

- Replicating a CE run from a common aliquot of PCR products, which would typically be performed to investigate CE run issues such as non-resolution of peaks or CE artefacts such as spikes
- Replicating a PCR from a common DNA extract, which is commonly performed in forensic analysis in order to obtain further information on the genotypes of the DNA donors
- Replicating an extraction for a common item. There are two ways in which people may think about these types of replications. One would be to carry out a second extraction on a substrate that has already undergone a DNA extraction in an attempt to obtain more DNA. We refer to this as a second extraction rather than a replicate extraction as the conditions of the substrate being extracted have been changed from the state it was in prior to the first extraction. The second way to think about a replicate extraction would be that a second

sample is taken in deliberately close proximity to the first in an attempt to recreate the conditions of the first sample. This is perhaps better referred to as a replicate sampling. Replicate sampling may be performed if some quality issue has occurred that renders the first sample unusable. Note that this idea of a replicate sampling should be thought of differently to the idea of taking additional samples to undergo extraction, with the distinction coming from the intent in taking the sample i.e. a replicate sample having the desired purpose of replicating the results of the first sample, while the additional sample having the purpose to generate more information about the exhibit without any desire to necessarily replicate the same result as another extracted sample.

For the purpose of this study the word 'replicate' will represent the technical replicate definition where multiple subsets of the same sample are taken rather than different samples of the same type. In the context of replicate reproducibility for DNA profiles, to be considered true replicates the same contributors must be present in terms of number and representation in all profiles; this does not mean identical alleles providing concordance is observed. There are also research replicates within the study, which will be referred to as 'samples' for this study. We claim that the while the need for technical replicates is not met by additional sampling (which generates research replicates) it can be met from replicate sampling (which generates technical replicates). We will demonstrate this point by showing that technical replicates from replicate sampling produce profiles that are much more similar to each other (under the sampling scheme we use) than profiles from additional sampling (research replicates).

When tapelifts are taken for direct PCR analysis, only a portion is placed into the PCR while the rest is usually discarded [294, 315]. It has been previously proposed that the use of tapelifts could enable multiple cuttings of the same lift to be taken and treated as replicate samples for analysis [315]. Collection of DNA template from exhibit types is traditionally based on the porosity of the substrate surface; porous substrates are tapelifted while non-porous substrates are swabbed [133, 300]. It has recently been shown that tapelifts are highly inefficient at collecting the cell-free component of touch DNA, unlike swabs [326]. Due to this, the analysis of tapelift and swab performance was tested on the substrates used within this study to allow any results obtained in this work to be compared to and reflected onto results in other published work with different collection media. It is proposed that taking replicate samples of a single tapelift will result in concordant DNA profiles and will address the current issue of reproducibility and re-testability with direct PCR, allowing its introduction into forensic casework workflow without breaking accreditation or legislative requirements [273, 274, 383-386].

4.3.3 Methods

This work is separated into three parts to address different components of the introduction of direct PCR into casework exhibit workflow through tapelifting. Part A compares the efficiency of swabbing and tapelifting touch DNA samples from substrates used within this study. Part B compares triplicate cuttings from the same sample, all processed by direct PCR, to determine the technologies' reproducibility. Part C utilises both extraction-based and direct PCR workflows from a single tapelift sample and determines the concordance of resultant profiles

Ethics

Approval was obtained prior to sample collection from the SBREC (reference 8109) of Flinders University. Informed consent forms were signed and collected from volunteers.

Exhibits and volunteers

All items were cleaned with a 2% sodium hypochlorite bleach solution, followed by water then 100% (v/v) ethanol. Negative control samples were performed in triplicate for all items. Items were as follows: ziplock bag; rubber mallet handle; electrical tape mounted on a glass slide; circuit board; twine; and cotton fabric.

Three volunteers, one from each shedder status, as described in Kanokwongnuwut *et al.* 2018 [22], were asked to wash their hands with water only, wait 30 mins returning to their regular activities, and deposit DNA onto an item for 15 secs [163]. The volunteers were asked to wait at least one hour before washing their hands in preparation for the next deposition cycle. Deposition depended on the sample type: the mallet, ziplock bag, and circuit board were handled as per normal use, while the electrical tape and fabric deposition was a single fingermark with medium pressure, and the twine was twisted between the thumb and index finger. Each volunteer touched ten replicates of each item, with one donor touching fifteen replicates.

Collection of DNA from exhibits

Swab Samples

Samples that underwent swabbing for direct PCR amplification were double-swabbed using nylon ultra-fine dental applicators (City Dental, Adelaide, Australia) pre-moistened with 2 µL 0.1% Triton™ X-100 (Sigma, VIC, Australia). The targeted surface area was covered a total of three times, or until the swab went dry. Swab head pairs were removed into a 0.2 mL thin-walled PCR tube using a sterile scalpel blade for direct PCR amplification. This was performed for five replicates of each substrate from each volunteer (N=90).

Tapelift Samples

DNA-free Lovell tape (Lovell Surgical Supplies, VIC, AUS) was used for all tapelifting. Quintuplet samples of each substrate from the volunteers were processed by either A) tapelift for separation into triplicate direct PCR amplifications (all volunteers), or B) for separation into both direct PCR amplification and an extraction (donor 3 only). Pictographic representation of 2.3.2 is shown in Figure 4.1.

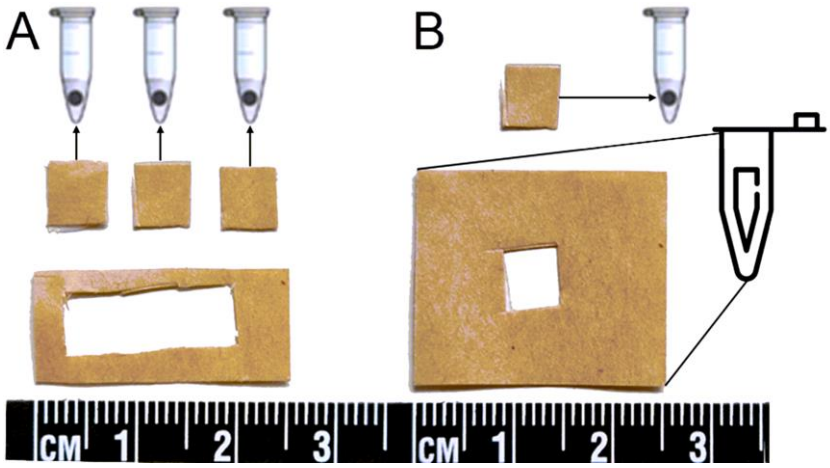


Figure 4.1. Representation of Lovell tapelift processing method from A and B from 2.3.2; the brown paper backing remains on the tape for visualisation purposes within the figure only. Image A shows the separation of three parts of the tapelift into separate PCR tubes. Image B depicts the removal of a part of a tapelift for direct PCR and the remainder being added to a 1.5 mL tube for standard DNA extraction.

<u>Separation of Lovell tapelift into three direct PCR amplifications (A).</u>

A Lovell tapelift tape (dimensions 20 mm x 25 mm) was cut in half prior to tapelifting items designated for this workflow. The tapelift half was used to collect deposited DNA from the surface of an item with tapelifting continuing until either the entire surface of interest was sampled three times, or the tape was no longer sticky.

From this tapelift, three 5 mm^2 samples were cut from the middle section of the tapelift; each sample was placed into a separate 0.2 mL thin-walled PCR tube for direct PCR amplification (Figure 4.1 A). For comparison against the direct PCR swab dataset the middle direct tapelift sample results were used.

<u>Separation of Lovell tapelift into direct PCR and extraction samples (B).</u>

The entire Lovell tapelift was used for tapelifting of items designated for this workflow; quintuplet samples per substrate touched by one donor, donor 3. Tapelifting was performed as above.

One 5 mm^2 sample was cut from the middle section of the tapelift and placed into a 0.2 mL thin-walled PCR tube. The remainder of the tapelift was placed in a 2 mL Lo-Bind Eppendorf for extraction (Figure 4.1 B).

Extraction and PCR

Extractions were performed using DNA-IQ (Promega Corporation, VIC, AUS), with a final elution volume of 60 µL. DNA quantification was performed using the Investigator Quantiplex Pro RGO (QIAGEN, VIC, Australia). All extracts were amplified, regardless of the quantified concentration. PCR post-extraction was performed with VeriFiler™ Plus (ThermoFisher Scientific, VIC, Australia), following manufacturer's recommendations, with a total DNA input of 0.5 ng, or the full 17.5 µL of spare volume where the quantified concentration was too low.

Samples that underwent direct PCR were amplified with VeriFiler™ Plus following manufacturer's recommendations, with the exception of 2 µL Prep-n-Go™ (Life Technologies, VIC, Australia) and Low-TE being used in place of water.

Separation and data analysis

All samples were separated on a 3500 Genetic Analyser (Thermo Fisher Scientific, VIC, AUS) and the output analysed using GeneMapper ID-X software. For Part A, peaks were recorded if ≥ 50 RFU and considered for heterozygosity if ≥ 150 RFU; profiles were considered 'informative' when they consisted of ≥ 12 autosomal donor alleles. For Part B and C all profiles were exported and run through STRmix™ for profile deconvolution. These were compared against donor and co-habitant's reference profiles for LR calculations. The STRmix™ deconvolution results from each portion taken from the same sample were also compared against one another using the mixture to mixture protocol with DBLR™ [393]; single-sourced and mixed DNA profiles were not separated for any of the analyses. All linear regressions, logistic regressions, LR tests of logistic regressions, Kolmogorov-Smirnov (KS) tests and Fligner-Killeen analyses were performed using R software [333].

4.3.4 Results and Discussion

All negative controls returned no alleles in subsequent profiles.

Part A: Analysis of swabs and tapelifts on a range of porous and non-porous surfaces for performance assessment.

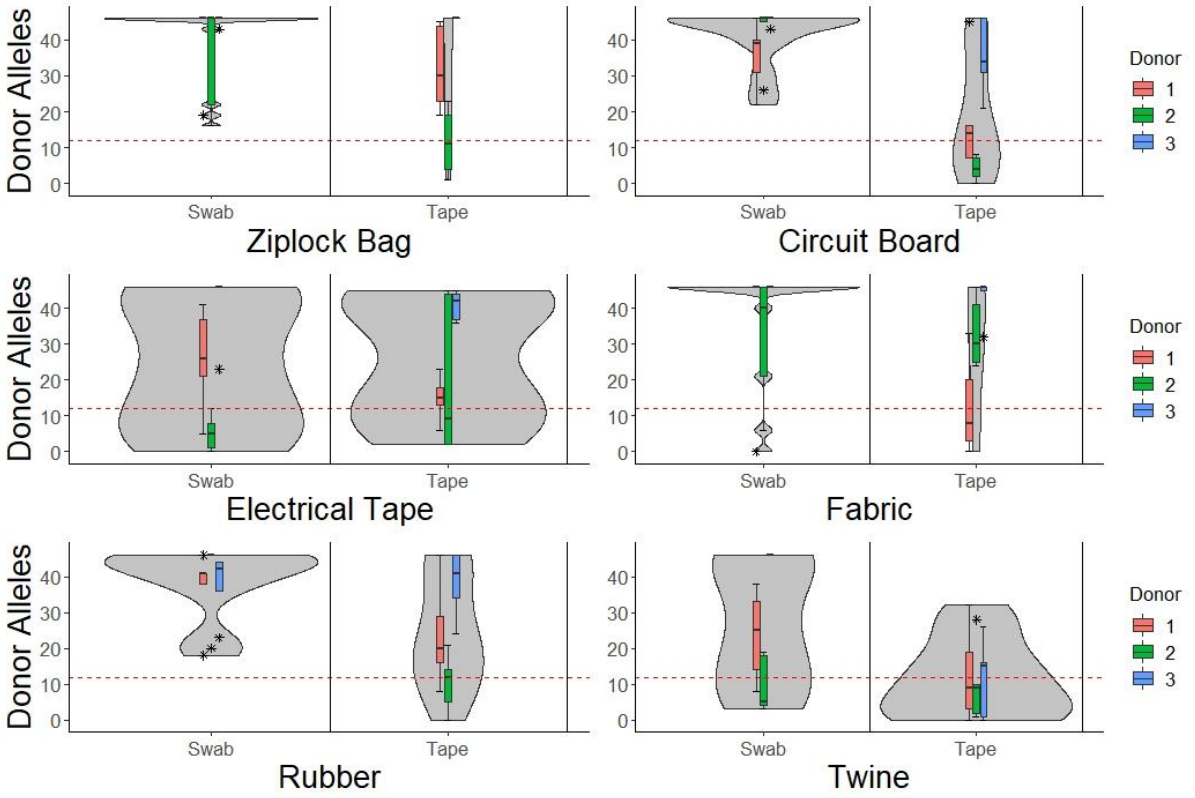


Figure 4.2. Violin plots showing the number of donor alleles observed in each profile following direct PCR amplification from either a swab or tapelifting sampling approach (N=180). Each substrate is separated. Boxplots of individual donor results are overlayed and a threshold of 12 alleles is shown as a red dashed line. Donor 1 (orange) is an intermediate shedder, donor two (green) is a low shedder, and donor 3 (blue) is a high shedder.

The distribution of profiles exhibiting each allele call number (donor) for each condition is shown in Figure 4.2. This dataset consists of three donors with five research replicates of each collection media per substrate type for all donors (N=180). The largest variance in allelic return was observed from donor 2, the low shedder. Donor 3, the high shedder, consistently produced DNA profiles with high allele calls across all substrate types and collection media, with the exception of the tapelift from twine. Substrate types also showed trends when considering individual donors. For example, the circuit board tapelift results from donor 1 and 2 failed to produce informative alleles consistently, which is in contrast to when they were swabbed and performed well.

Sampling from twine resulted in significantly less alleles than other substrates (p= 0.0144) with ziplock bags resulting in the highest allelic yield (average 35 alleles). When considering sampling media, swabbing produced more donor alleles on average than tapelifting from each surface type with the exception of electrical tape where performance was equal; significance was observed in the twine (p= 0.0122), rubber (p= 0.00933), and circuit board (p= 0.000772). Tapelifting of the circuit board suffered added complexity due to ineffective access to the DNA template because of its surface's landscape; swabs were able to access this template more easily than the tapelift. It was expected that the tapelifts would out-perform the swabs for the fabric and twine substrates due to their porosity [198, 394], however as the sampling surface area was extremely small and collection could be highly targeted, the swab still performed well. This is not expected to translate to all real-life exhibits where matters of surface area and sparser DNA template requires consideration, however some laboratories have moved away from tapelifting any exhibits in preference to swabbing as internal validations have shown that double swabbing outperformed tapelifting with their procedures (personal communication). The capacity within this work to target known cellular deposition areas is also a limitation of the data being directly translated to forensic exhibit sampling as sampling of touch DNA is performed 'blind' based on best assumptions in a casework setting.

With the exception of twine samples, sampling from all other surface types resulted in informative profiles, on average, for all donors. This supports the use of tapelifts through direct PCR as a viable way of producing informative STR profiles from a variety of surface types irrespective of porosity.

Part B: Determination as to the consistency and reliability of utilising tapelifts through direct PCR to allow a level of reproducibility within the technique.

Before comparisons between replicate samples taken from the same tapelift are performed, it is important to consider all data collectively. When considering this dataset, donor and surface type is significant for the number of donor alleles obtained in the DNA profiles produced ($p= 2.2x10^{-16}$ and $p= 4.04x10^{-8}$ respectively). This is also true for the donor RFU ($p= 6.263x10^{-8}$ and $p= 6.886x10^{-6}$ respectively) and subsequent LRs calculated against a donor reference profile ($p= 2.416x10^{-12}$ and $p= 8.35x10^{-4}$ respectively).

The LR of each replicate to donor match was calculated to determine whether the replicate samples taken from a single tapelift could be considered technical replicates of the sample. This was performed for all donors and substrates and can be seen in Figure 4.3. Each boxplot represents the triplicate samples from a single tapelift. There were five tapelifts performed for each substrate per

donor (N=270). This figure visually shows that the LRs obtained were close within the same tapelift and more divergent between tapelifts (as demonstrated in Figure 4.3 D).

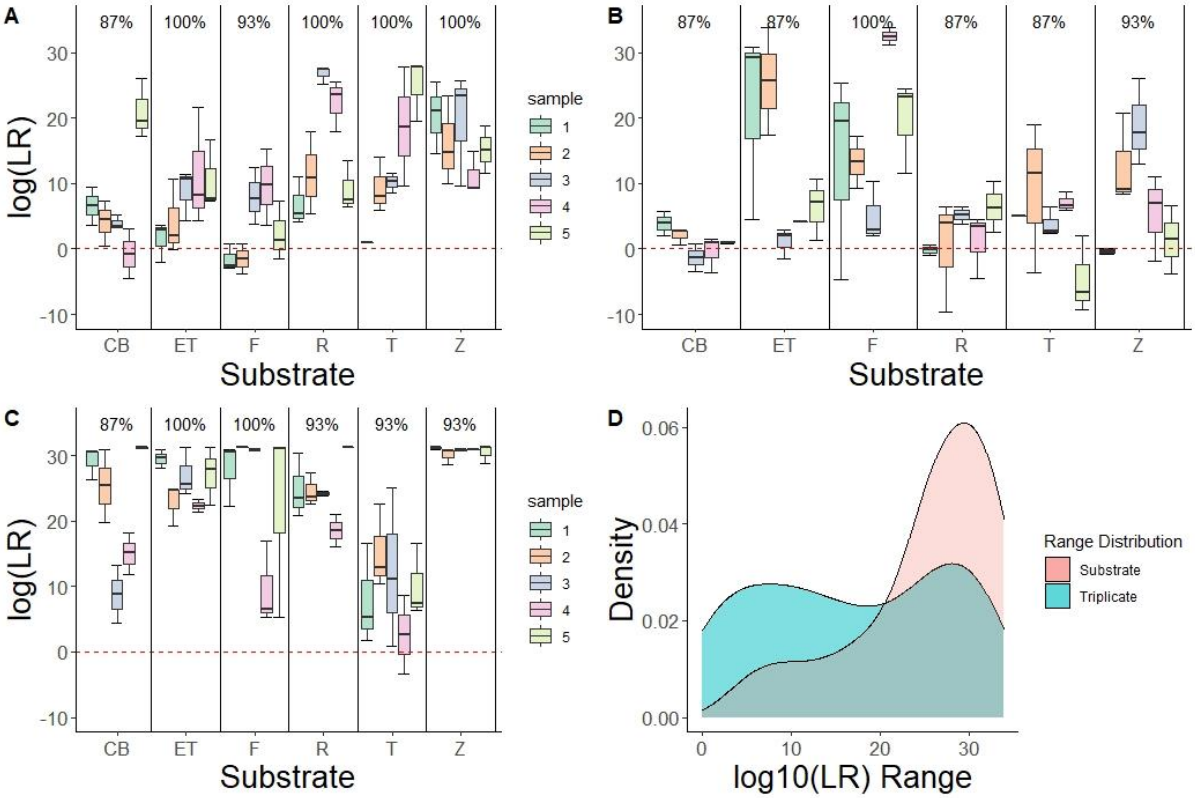


Figure 4.3. Log10(LR) of the profile-donor 'match'. Each boxplot represents the triplicate samples taken from each tapelift (1-5) for each substrate for donor 1 (A), donor 2 (B), and donor 3 (C). Proportion of the dataset shown (i.e. for those where the LR was not 0) is annotated above each column. N=270 (N=90 per panel). Panel (D) shows the distribution of the LR ranges (highest – lowest logLR) generated between the triplicate profiles from a single tapelift (blue), and all profiles generated for each substrate and donor dataset (pink).

To determine how similar each of the triplicate samples were, in terms of profile strength (LR), the range of the LRs generated from each component of the tapelift were calculated for each sample. In addition to this, the range of LRs generated over each profile from all samples within a substrate per donor were also calculated (i.e. range of donor 1 circuit board, range of donor 1 electrical tape, etc). The distribution of the LR ranges is shown in Figure 4.3, where the lower ranges (left) represent profiles that are closer together in composition (Figure 4.3 D); the distributions of donor allele and RFU ranges are provided in Supp. Figure 4.1. A KS test performed on these distributions showed that there was a significant difference between the LR ranges when considering intra-sample and inter-sample profiles (p= 0.01646) with the intra-sample profiles exhibiting a smaller LR range. The same was performed and observed for log(RFU) (p= 0.001538). As the KS test is utilised for continuous variables it was not performed on the number of alleles, furthermore LR is the most widely reportable

statistic for casework settings, so it was determined to be the most pertinent value to analyse with this method and with the Flinger-Killeen discussed below.

To test the difference in the pairwise variance in the LRs obtained for the three replicates from a single tapelift compared to between tapelifts, a Fligner-Killeen test was performed across the dataset. For the pairwise comparison of technical replicates, LRs from all components within a tapelift were compared against one another and the difference was recorded (three values per tapelift: 270 values in total). For the research replicate variance values, all portions of each tapelift with the same donor and substrate were compared against all other portions within those parameters, with the exception of portions within the same tapelift sample (90 values per donor/substrate: 1620 values in total). There was non-homogeneity observed in the variance of the logLR differences between the proposed technical replicates and the research replicates ($p= 2.513 \times 10^{-8}$). As it is understood that donor and surface type can impact results, this difference in variance test was performed on subsets of each substrate type and donor with results displayed in Table 4.1.

Table 4.1: P-values of pairwise LR comparisons using the Fligner-Killeen test for each donor and sample type subset amplified. This shows whether difference in variance within a tapelift sample (technical replicates) and between tapelift samples (research replicates) is significant.

DONOR/SAMPLE	C	ET	F	R	Z	T
1	0.00482	0.550	0.422	0.0021	0.813	0.242
2	0.488	0.171	0.0144	0.765	0.191	0.062
3	0.00317	0.0270	0.197	0.0118	0.472	0.940

We have shown that the comparison of technical and research replicates to their known DNA donors produce LRs that are more tightly clustered between the technical replicates than for research replicates. Another point we wished to investigate is whether the composition of profiles themselves are more tightly clustered in technical than research replicates. We do this by representing the closeness of samples in the plots seen in Figure 4.4. The plots in Figure 4.4 consist of a number of triangles. Each point of a triangle represents a sample that is compared to two other samples, either within the same technical replicate (Figure 4.4 left) or between research replicates, but from the same donor and substrate Figure 4.4 right). The x-axis position of the points of the triangles are the log of the total RFU in the profiles. The y-axis positions are determined by the level of support for the samples being compared having commons DNA donors. This is represented by calculating a mixture-to-mixture LR with propositions:

- The mixtures have a common donor
- The mixtures have no common donors

The $\log_{10}$(LR) obtained from the mixture-to-mixture comparison of any two samples is at the centre-point of the line connecting those samples. The y-axis positions of the triangle points are then determined from the samples' total RFU values and the mixture-to-mixture LRs. As the composition of profiles in a set of three becomes close, then the triangle size will contract (with three identical samples contracting down to a single point). The area of the triangles has been graded from green (small area) to red (large area) to make interpretation easier. In order to avoid plot overcrowding a random set of 25 triangles are shown in Figure 4.4 for each plot, for an overlay of all comparisons made within the dataset see Supp. Figure 4.2 and Supp. Figure 4.3.

In Figure 4.4 we see that the technical replicates produced profiles that are closer in composition, judged on DNA amounts measured by RFU and allelic composition, than research replicates. This is seen by small green triangles in the technical replicate plot (Figure 4.4 left) and larger triangles shifted more to the red colours in the research replicate plots (Figure 4.4 right).

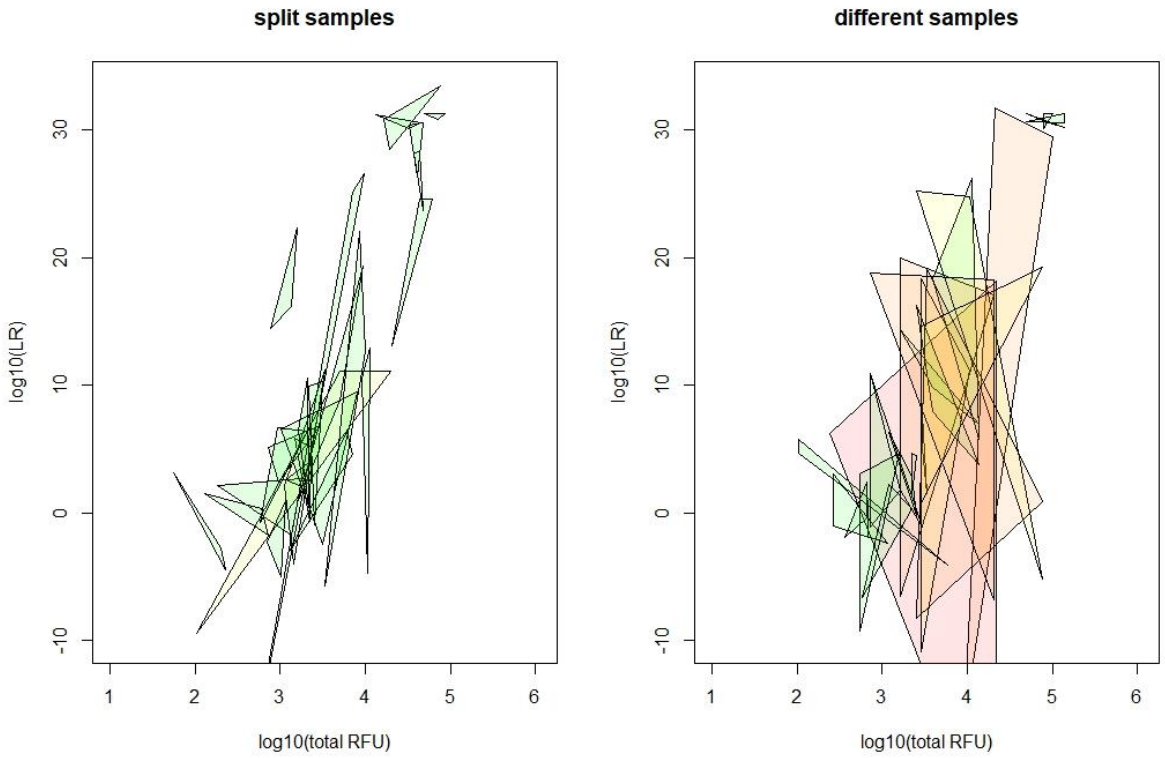


Figure 4.4. Profile to profile match LR of triplicate replicates taken from a single tapelift (left) and three profiles taken from the same donor and substrate but not the same tapelift (right). Colour scaling indicates the area within the triangle made between the three samples 'matched'. Green indicates high profile-profile-profile composition similaraity with red indicating a lower similarity. A random set of 25 triangles were drawn.

These data indicate that separating a tapelift as described in Figure 4.1 allows for reproducible DNA profiles to be obtained from a single sample. This satisfies the requirement for defence re-testability or retention of sample template for alternate downstream processing. In addition to this, the expectation is that concordant profiles would be obtained by processing all replicates with direct PCR for low template exhibits. As there were no controlled mixture depositions, through multiple handlers, to assess how this method would perform with mixed DNA contributions in a casework setting there is limitation in its translation to all sample types; however approximately 30% of the dataset resulted in mixed DNA profiles, due to the nature of touch DNA transfer mechanisms, and concordance was observed within these profiles as shown with the mixture-to-mixture analyses.

Part C: Assessment as to the viability of utilising both direct and extraction-based PCR workflows from a single tapelift.

Data presented in Part C are based on Donor 3 only. When considering the number of alleles amplified in samples taken from the same substrate and donor, differences between the direct portions taken in Part B and Part C were observed. This is not unexpected due to the variable nature of touch DNA deposits and subsequent PCR success [4, 108, 131]. When considering Donor 3, there was significance in the difference of the number of alleles observed between the datasets with respect to electrical tape ($p=2.12x10^{-05}$), twine ($p=8.1x10^{-04}$), and ziplock bags ($p= 0.0115$).

There was no statistical difference between the direct PCR and PCR post-extraction workflows with respect to the number of donor alleles amplified ($p= 0.939$) (Figure 4.5) or the LR of resultant profile to donor reference ($p= 0.328$). This was unexpected as previous literature suggests that these substrate types do not perform as well as observed when touch DNA is taken through an extraction, however depending on DNA deposition it is possible [326]. It does, however, not affect the argument being considered: whether both workflows can be performed on the same sample and result in concordance with any genetic data produced.

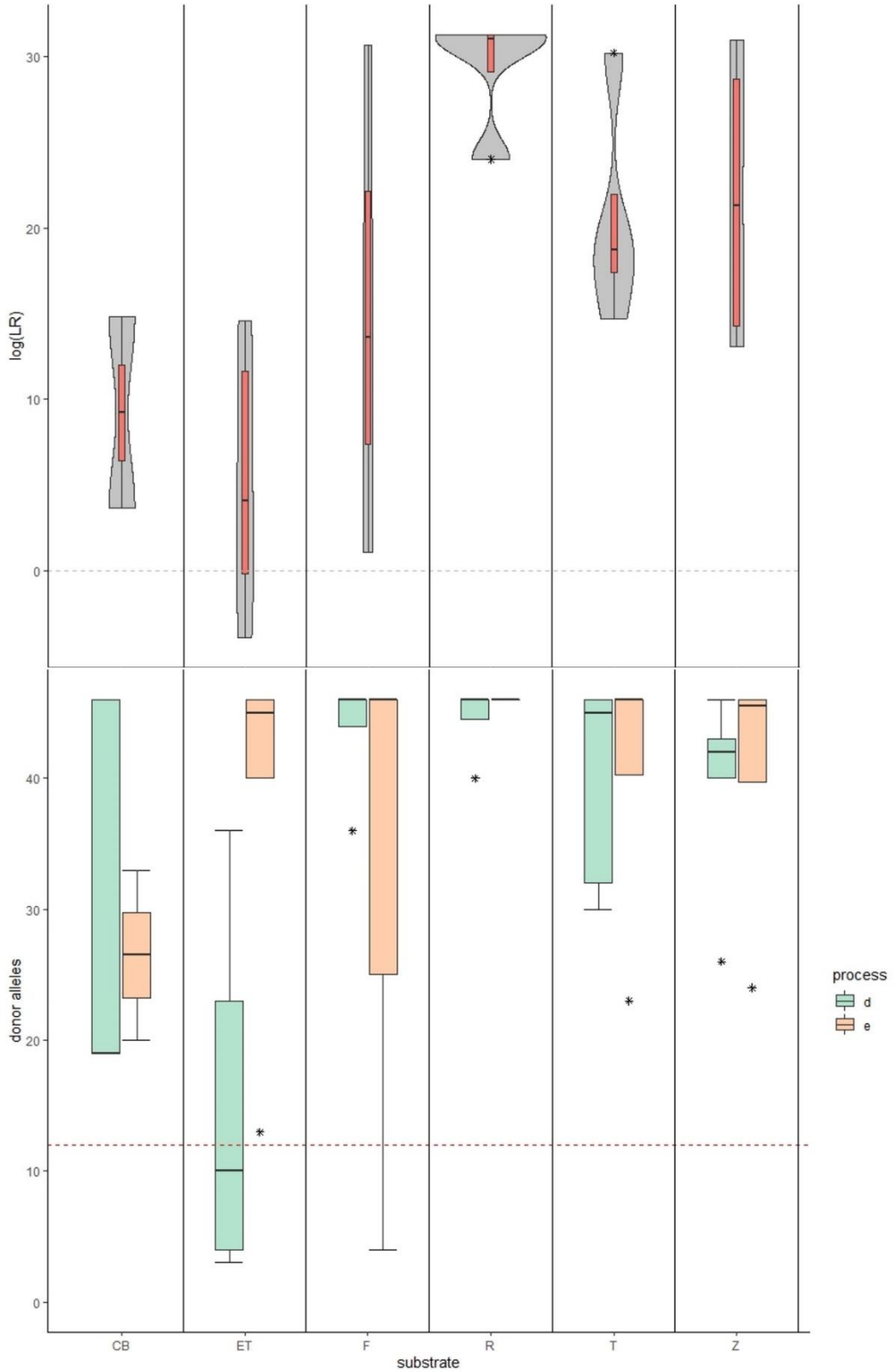


Figure 4.5. Profile to profile 'match' LR (log10) between the direct and extraction portions from a single tapelift for each substrate type (top) and the donor alleles amplified for each substrate type separated by workflow (bottom).

For the discussion as to the viability of using both a direct and extraction workflow from a single tapelift sample, the consideration for portions to be technical replicates is not relevant. Instead, only the issue of donor concordance is of relevance. The mixture-to-mixture LRs between the paired tapelift portions processed through the direct and extraction workflows were calculated to determine whether there was strong support for the contributors within each profile being the same, compared to being different (Figure 4.5). The analysis supported the presence of common DNA donors for all sample comparisons except for one, an electrical tape sample, indicating profile concordance in the individual tapelift samples across the direct and extract workflows. There were two reasons for the non-concordance in the one electrical tape sample pair. Due to the direct portion containing only 4 alleles from a single donor and the post-extraction portion exhibiting 13 donor and 2 non-donor alleles, one of these alleles was compared to the second, minor, contributor within the post-extraction sample. This occurred at locus D1 where there was no representation of Donor 3 causing this alternate comparison to be made. In addition to this at THO1 the profile produced following direct PCR exhibited one allele of the expected heterozygous pairing while the profile generated following PCR post-extraction exhibited the other.

Literature suggests that it would be possible to utilise a nucleic acid staining dye to visualise the tapelift and screen it for cell numbers to facilitate educated direct-portion sub-sampling or to inform between 2.3.2.1 and 2.3.2.2 methods [343]. By combining results from cell counts, in light of Kanokwongnuwut *et al.* 2021 [326] and this body of work, laboratories could optimise the downstream processing of tapelifts following collection. The incorporation of nucleic acid staining, however, is time-consuming and limited with respect to template source quality or the presence of inhibitors [326, 343].

These data support the combination use of direct PCR and extraction-based PCR from a single tapelift for exhibits. This allows for the generally accepted benefits of both workflows to be exploited: direct PCR results in an increase in sensitivity [193, 204] resulting in the potential for greater amplification data from low template DNA and a reduction in cost and time[204, 319], while the benefits of a standard workflow such as the removal of inhibitors to the amplification process, possibility to generate quantification data, and eluate acquisition to allow further retesting [204, 319] are also facilitated.

4.3.5 Conclusion

Although commonly used for porous surfaces, tapelifts are a viable sampling option for the non-porous substrate types included in this study. The determination as to whether direct PCR could be considered reproducible, for the translational use into casework exhibit analysis, was considered in a number of ways.

The number of alleles, RFU, and log10(LR) calculated from the comparison against the donor reference profile, of the profiles generated from triplicate replicates within a tapelift showed no statistical difference from one another. When separating the dataset by donor and substrate type the range in the proposed technical replicate samples and research replicates was calculated for donor alleles, RFU, and LR calculated following comparison against a donor reference profile. This showed a significant difference, following a Kolmogorov-Smirnov test, between the range distributions of the technical and research replicates for both RFU and LR. This indicates that the profiles generated within a tapelift were more similar to one another than when compared to samples from different tapelifts and supports their inclusion as technical replicates. To assess the difference in the variance between the donor match LR more closely the variance of each pairwise comparison both intra- and inter- tapelift sample was conducted for each donor and substrate combination. The Fligner-Killeen test that showed the difference in LR variance was significant between the proposed technical replicates and the research replicates as the distributions of LR ranges showed that the proposed technical replicates had lower variation in their LRs than research replicates.

We assessed the similarity of profiles produced from samples taken from the same tapelift and compared these to samples from different tapelifts. This aligns with the current forensic thinking of resampling versus additional sampling. We found that the composition of the samples within a tapelift were much closer in DNA amount and genotypes than samples from different tapelifts of the same substrate and donor. This was done by utilising mixture-to-mixture comparison that produces an LR that considers whether the mixtures have common DNA donors versus different DNA donors. The expectation would naturally be that the split samples from the same tapelift will produce profiles that are more similar than those produced from samples of different deposition events. This is indeed what was found in our study. The reason for the greater difference between separate deposition events is likely to be differences in DNA that accumulates on the hands of volunteers in the 30-minute period between hand washing and deposition, and also micro differences in the deposition events (i.e. time or pressure or area of contact). One possible future experiment could be to trial separate deposition events that occur immediately after each other, or to trial deposition events that occur at the same time but with different contact areas (e.g. thumb and forefinger). Each of these have some limitations and the differences in experimental deposition setup will be expected to produce

differences in the DNA profiles also. Ultimately if all differences between separate depositions could be removed, the result would be that the separate samples that produced profiles of a high similarity would align with the split tapelift samples produced in this work. Our work supports triplicate replicates taken from the single tapelift being considered as technical replicates for the purpose of DNA amplification and analysis, thus fitting legislative or accreditation guideline requirements for DNA template retention for subsequent testing.

There was concordance in the contributor data between the direct and extraction workflow portions taken from a single tapelift. If direct PCR is not successful on the first portion taken, an extraction can be performed on the remaining tapelift for DNA purification and eluate production. Although it is not being suggested that this dual PCR workflow be performed on all touch DNA samples, when template is unknown, evidentiary samples are limited within a case, and performing direct PCR is believed to be of niche beneficial application, the methods described can be employed confidently. These data support the use of tapelifts as a facilitator for dual workflow analysis of DNA template from casework exhibits.

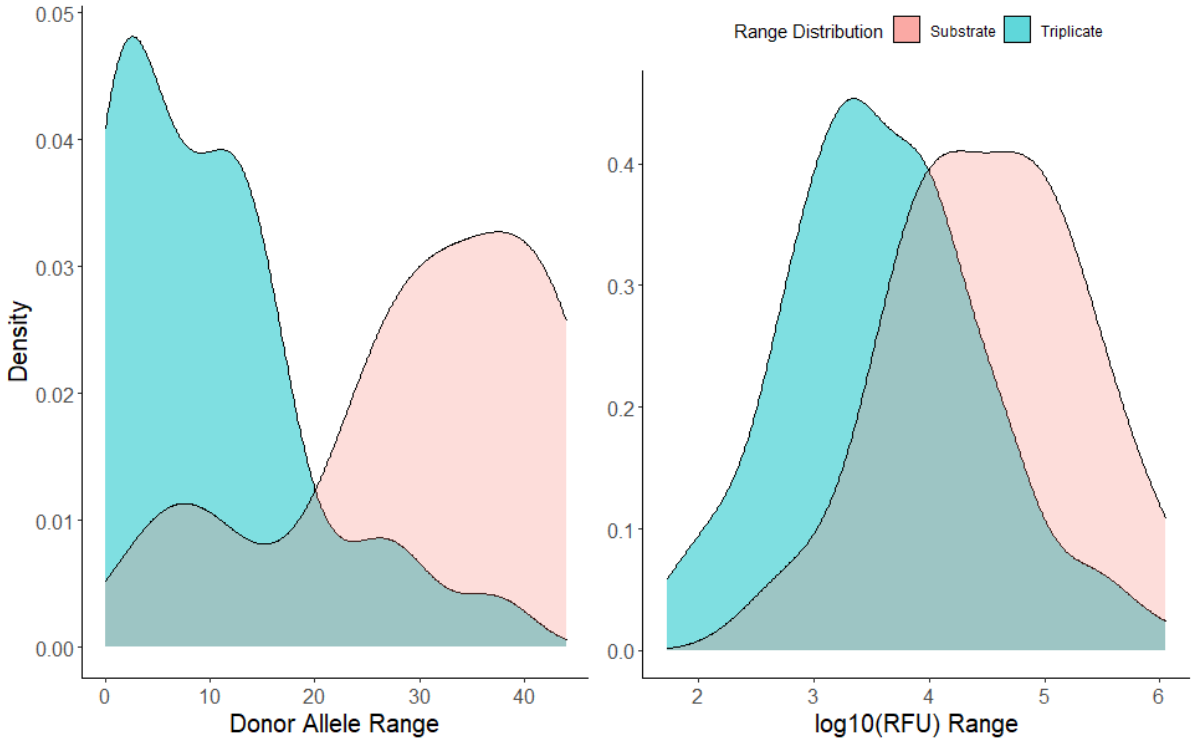
4.3.6 Conflict of Interest

The authors declare no conflict of interest.

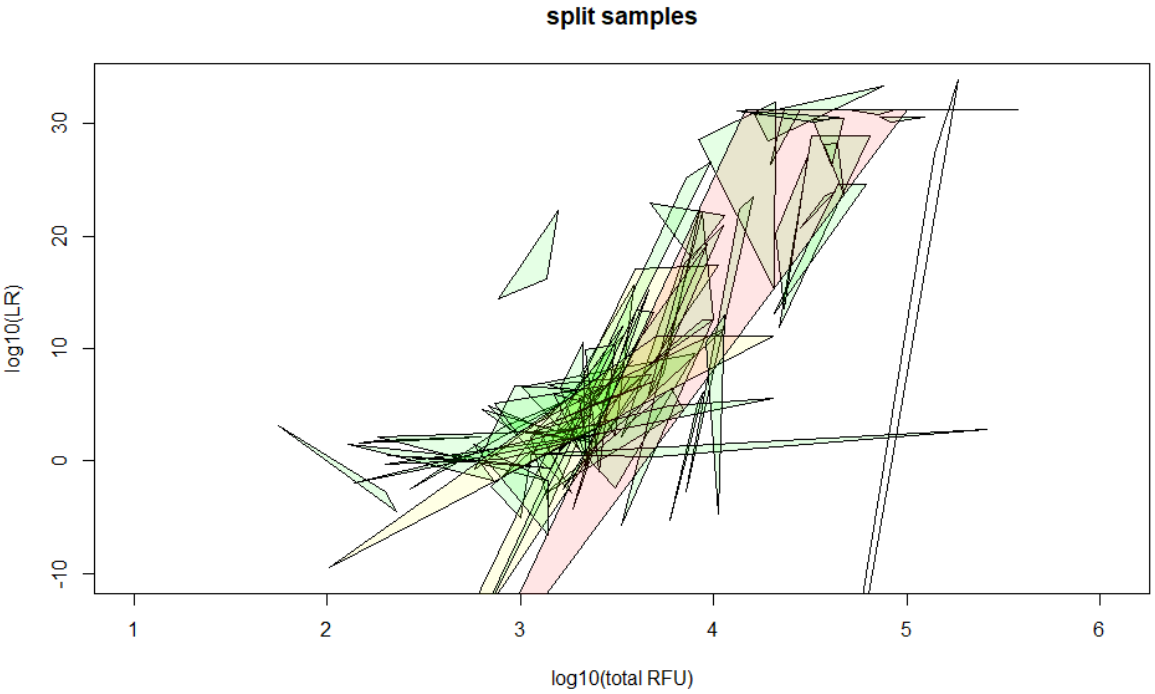
4.3.7 Acknowledgements

This research is supported by the Attorney General's Department through the Ross Vining Memorial Research Fund. We would also like to thank all volunteers that participated in this study.

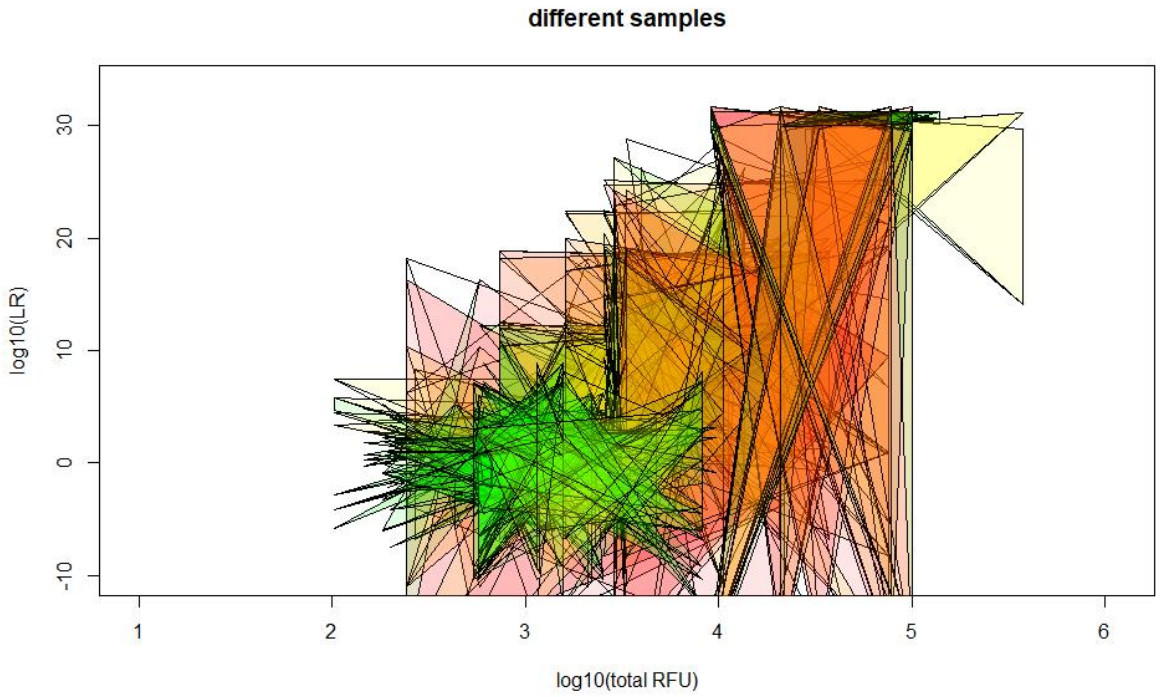
4.3.8 Supplementary Material



Supp. Figure 4.1. The distribution of the donor allele and log(RFU) ranges generated between the triplicate profiles from a single tapelift (red), and all profiles generated for each substrate and donor dataset (blue).



Supp. Figure 4.2. Profile to profile match LR of triplicate replicates taken from a single tapelift. Colour scaling indicates the area within the triangle made between the three samples 'matched'. Green indicates high profile-profile-profile composition simialraity with red indicating a lower simialrity.



Supp. Figure 4.3. Profile to profile match LR of three profiles taken from the same donor and substrate but not the same tapelift. Colour scaling indicates the area within the triangle made between the three samples 'matched'. Green indicates high profile-profile-profile composition simialraity with red indicating a lower simialrity.

4.4 CONCLUDING COMMENTS

The data presented in Chapter 4 is the first time multiple STR PCRs have been performed from a single sample with direct PCR methodology. It has been shown that not only is replicate sampling possible, but these replicates can also be considered technical replicates of one another showing profile concordance with respect to donor inclusion and LR strength. This supports the inclusion of direct PCR methods for the analysis of forensic casework samples when its niche application is beneficial to the likelihood of obtaining DNA profiles suitable to be up-loaded to the DNA database of choice. The aim "*To determine whether the generation of multiple concordant profiles is possible from a single tapelift sample following direct PCR amplification*" has been addressed within this work. In addition to this, the novel examination of whether a single tapelift could support dual workflows provided valuable results. These data suggest that it is possible to obtain concordant DNA profiles, with respect to donor inclusion, when a clipping from a single tapelift is processed by direct PCR methods with the remainder following extraction. This is beneficial to forensic laboratories when it is expected that no DNA profile will be obtained post-extraction, but there is a requirement for a sample pool to be retained. This dual workflow should be performed when the success of STR amplification is unsure through standard extraction-based workflows or if limited case-related items are available; it is not suggested that this be performed in all cases. The aim "*To investigate the potential of obtaining data from both extraction-based PCR and direct PCR from a single tapelift to facilitate a more seamless introduction of direct PCR into laboratories that require a sample to be re-testable*" has been addresses within this work.

CHAPTER V:

APPLICATION OF DIRECT PCR TO SUB-OPTIMAL SAMPLE TYPES

Manuscripts enclosed:

Martin, B., Blackie, R., Linacre, A. (2019). "*DNA profiles from matchsticks*." Australian Journal of Forensic Sciences

Martin, B., Plummer, A. Linacre, A. Henry, J., (2020). "*Direct PCR of fired shotgun casings: a South Australian evaluation*."
Australian Journal of Forensic Sciences

5.1 PREFACE

Although understanding the benefits and limitations of direct PCR for latent DNA samples from several sample types and donor shedder categories provides pivotal data for laboratories and the scientific community, as previous chapters have explored, there is more to consider when analysing casework samples. Depositions thus far have not been places under treatments or stresses that have the potential to reduce amplification efficiency or template viability. Pushing direct PCR to the limit of evidentiary possibility will allow the data, guidelines, and considerations produced to encompass a larger pool of sample types. Investigation into challenging sample types examined expands the boundaries of what is currently known to be possible in forensic investigations. This chapter is split into two phases, each of which examines aspects relating to the application and performance of the direct PCR technique to touch DNA deposits from a range of challenging exhibit types.

The first phase of the chapter considers the application of direct PCR to arson investigation whereby a struck matchstick has been the source of ignition. Comparison of STR profiles generated by extraction and direct PCR from handled matchsticks, as well as direct PCR from struck matchsticks, supports the introduction of direct PCR into matchstick analysis. Results are reported in a peer reviewed publication in the *Australian Journal of Forensic Sciences.*

The second phase of the chapter investigates the possibility to obtain an STR profile using direct PCR from spent ammunition. Current research is focussed on improving the *recovery* of DNA from ammunition through various means, including a swab and rinse technique [135]. This, although better than current methods often employed, still exhibits low success rates. Other research soaks the ammunition to improve sensitivity [395], however this is corrosive to striations on the casings [396]. The direct PCR method described here is a non-destructive technique that can be utilised in casework where limited samples are available and microscopic analysis is also vital to perform. Preliminary and supplementary studies are presented around one aspect of the research reported in a peer reviewed publication in *Australian Journal of Forensic Sciences.*

5.2 IMPACT STATEMENT AND AIMS

The paper “DNA profiles from matchsticks” has shown the niche application advantage of direct PCR to latent DNA samples even after they have been compromised. This work represents the first instance of touch DNA being put under challenging circumstances, through the lighting of a matchstick, and direct PCR being performed without altering cycling conditions. It was determined that direct PCR facilitated the amplification of donor alleles from matchstick samples at a greater rate than standard extraction-based PCR. Moreover, as LCN techniques were not followed with either the

extraction-based PCR or direct PCR amplification, stochastic effects were synonymous to latent DNA profiles generated post-extraction. This study provides supporting evidence for the introduction of direct PCR into arson investigation whereby a match was the ignition source. The tapelifting and direct PCR amplification method described within this work has been used in subsequent studies [192, 317].

The paper "Direct PCR of fired shotgun casings: a South Australian evaluation" coupled with the additional data presented in this chapter provides some preliminary insights into how and why direct PCR should and should not be utilised as a workflow for the analysis of spent ammunition. Round type and size are key variables that impact the workflow that would be most advisable. This work represents a starting platform for additional research to enable both DNA profiles and microscopic analysis of striations for firearm examination.

5.2.1 AIMS

Phase 1: "*To determine whether direct PCR is a viable alternative to LCN and extraction-based PCR workflows for the analysis of struck matchsticks*"

Phase 2: "*To examine the application of direct PCR to the examination of spent ammunition in comparison to other methods*"

5.3 PHASE 1 TESTING DIRECT PCR SENSITIVITY AGAINST STRUCK MATCHSTICKS

5.3 Manuscript: DNA profiles from matchsticks

Manuscript published: *Australian Journal of Forensic Sciences*, 2019

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**45**	**100**	**90**	**100**	**70**		03.08.21
RB	45	-	10	-	10		05.08.21
AL	10	-	-	-	20		05.08.21

5.3.1 Abstract

Matchsticks may be found in forensic investigations, and here we report on a method to generate informative DNA profiles from a matchstick. Matchsticks were struck or held emulating striking, cellular material was removed using a tapelift method and DNA profiles were generated from a small portion of the tapelift using either direct PCR amplification or post-extraction PCR. Performing an extraction from the tapelift prior to PCR resulted in 62% of profiles being informative, compared with 86% held and 97% struck matchsticks that underwent direct PCR. Incorporation of a tapelift method into matchstick analysis allows effective collection of cellular material for the generation of genetic information from the striker of the matchstick.

5.3.2 Introduction

As DNA extraction processes lose up to 76% of a starting template [2], touch DNA often gives poor STR profiles [131]. Direct PCR circumvents this extraction process to allow all DNA within a sample to be available as a template for PCR [143, 258]. Direct PCR has been applied to a range of substrates; such as hair [262], fingernail [260], or a swab taken from an exhibit [143, 193, 264]. Tapelifts have also been used as a template source for direct PCR [267, 294, 307]. The type of tape has been shown to significantly alter the quantity of DNA available for future PCR [198, 397]. Matchsticks play a role in ignition attempts, such as in bushfires or car fires [398]. Potentially, due to the lack of reliable methodology for processing this sample type, the retrieval of DNA from matchsticks has played a role in court on a few occasions [399]. LCN typing has been applied; although additional PCR cycles increase

stochastic effects, thus affecting the reliability data [220]. Direct PCR has been shown to increase the sensitivity of the amplification process without altering cycling conditions [259]. We report on a study incorporating tapelifting methods into the analysis of matchsticks in both post-extraction PCR and direct PCR amplification.

5.3.3 Methods

Seven volunteers (IND 01- IND07) were asked to wash their hands, without soap, and wait 15 mins before handling a matchstick in one of two ways. The sample was either: (i) an unstruck matchstick handled for up to 15 s, or (ii) a matchstick removed from its box, struck, and left to burn until extinguished. Volunteers produced six unstruck matchsticks, to allow for three replicates of either extraction-based PCR and direct PCR methods, and five struck matchsticks for direct PCR amplification.

Sampling was performed using a strip approximately 25 mm × 30 mm of 25 mm width Sellotape brand adhesive tape. An area of approximately 3 mm^2 was cut from the tapelift and placed into either a 0.2 mL thin-walled PCR tube, to be processed by direct PCR, or into a 1.5 mL Eppendorf tube, to undergo a DNA extraction. Extractions were performed using the DNA IQ System (Promega, NSW, AUS) using the '1–3 2 mm punches of FTA paper' method.

All amplifications were performed using the ProFlex thermal-cycler (ThermoFisher Scientific, VIC, AUS) with the Identifiler Plus kit (ThermoFisher Scientific), in 25 µL, following the manufacturer's protocol, with the exception of 2 µL of Prep-n-Go™ (ThermoFisher Scientific) and Low TE Buffer (ThermoFisher Scientific) in place of water. Into the PCR of the extracted samples 10 µL of extract was added to obtain a maximum DNA template for the reaction. PCR product (1 µL) was added to 8.7 µL Hi-Di formamide and 0.3 µL 600 LIZ (Thermo Fisher Scientific) and separated on a 3500 Genetic Analyser (ThermoFisher Scientific). Data were analysed using GeneMapper ID-X (version 1.4).

5.3.4 Results and Discussion

DNA extraction vs direct PCR methodologies

An average of 53% (17/32) of alleles were generated from samples where an extraction process was performed. This compared with an average of 85% (27/32) alleles recorded in DNA profiles of unstruck matchstick samples processed by direct PCR. DNA profiles were considered informative (i.e. can be uploaded for comparison to a database) if 12 or more STR alleles were recorded, discounting amelogenin [264]. An average 62% of profiles generated from samples processed by extraction prior to amplification were informative, compared with 85% of profiles produced though direct PCR. There was clear variation in the number of alleles present in each profile

between the volunteers. Shedder status of the volunteers was previously tested [163] with IND 01, IND 02 and IND 04 designated high shedders, IND 07 an intermediate shedder, and IND 03, IND 05 and IND 06 low shedders. Of the samples that underwent extraction prior to PCR, a full profile was obtained from all three replicates for IND 01 (high shedder), with two other volunteers producing profiles that were informative across replicates. This compared with samples from three volunteers producing full STR profiles for all replicates and five of seven volunteers producing informative profiles across replicates.

The trends observed within the data support the shedder status designations as the percentage of the profile observed for the high shedders was greater than that of the low shedders, when unstruck matchstick samples were processed by either process (Figure 5.1). There was little variation between volunteers in the average number of alleles present in the profiles of samples processed by direct PCR, with the exception of IND 03 and IND 05 who are low shedders. The variation in the number of alleles within profiles between volunteers was much larger in samples that underwent extraction prior to amplification. This indicates that the shedder status of an individual has less effect on the resulting data produced with direct PCR methods, compared with data produced after samples have undergone extraction prior to amplification; the loss of DNA from the extraction affects the amplification and subsequent analysis of DNA samples donated by low shedders to a greater extent than high shedders, who have a larger quantity of DNA template available before the extraction.

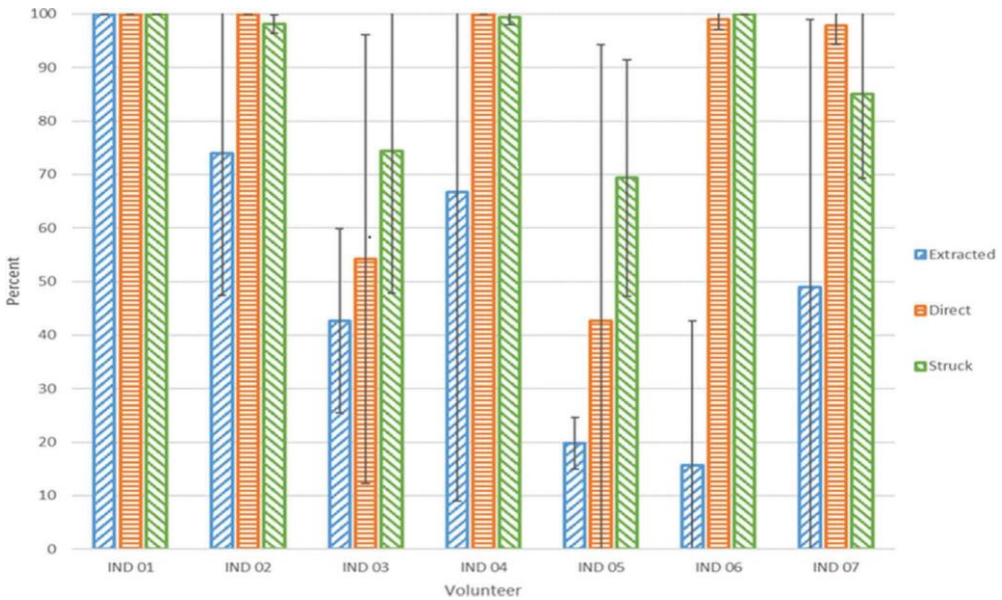


Figure 5.1. Average percentage of the DNA profile present (out of a total of 32 alleles) across each sample type and each volunteer. Error bars represent the standard deviation present between the three replicates of each sample type per volunteer.

Struck matchsticks

There was little difference observed in the resulting profiles from the unstruck and stuck matchsticks that underwent direct PCR. The clear reduction in genetic information present in profiles of unstruck matchstick samples that underwent extraction is still prominent when compared with struck matchsticks that followed direct PCR protocol. The same relationship was observed, with respect to the number of informative profiles within the dataset, between the unstruck and struck matchsticks following direct PCR protocol (Figure 5.2). An average of 86% of the STR profile (28/32) was recorded for struck matchsticks, using direct PCR, with 97% of the profiles being informative.

Mixed DNA profiles were present in 57% of the samples processed by direct PCR and 94% of the samples that underwent extraction prior to amplification. In each case, only a few non-donor alleles were present and these were from a clear minor contributor. Alleles could be attributed to individuals who shared common areas with volunteers. This is not unexpected in the analysis of trace DNA.

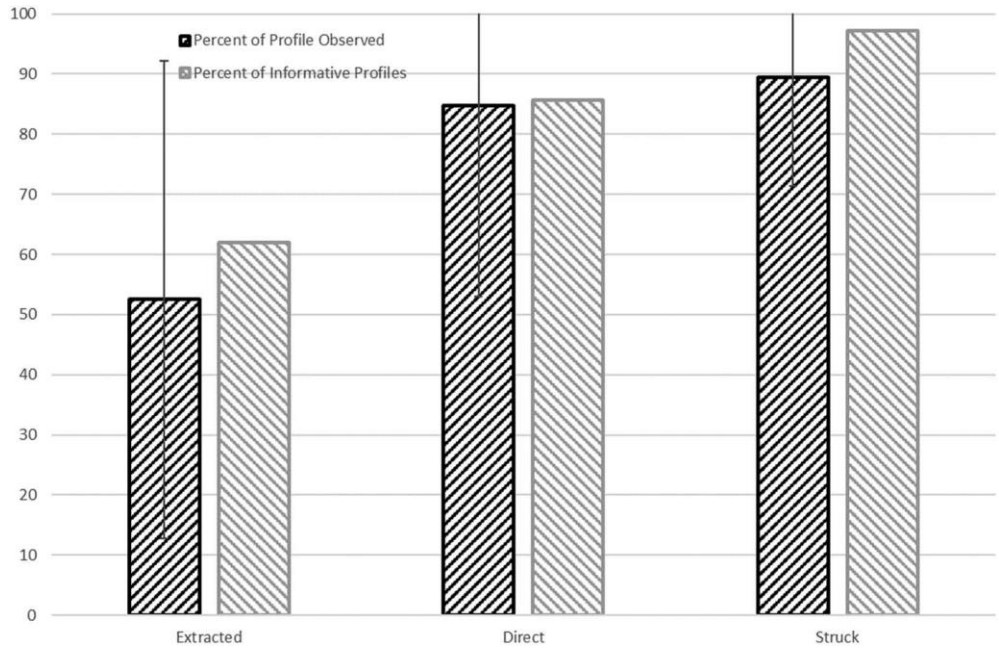


Figure 5.2. Average percentage of donor's profile present and average number of informative profiles across all volunteers between sampling types.

5.4 Conclusion

These data provide strong evidence that informative DNA profiles are obtainable from struck matchsticks using a tapelift technique in combination with direct PCR. Fewer informative profiles were obtained after using an extraction-based PCR protocol; this is concordant with previous research [294, 307, 397]. Tapelifts are a viable alternative to extracting the whole matchstick when applying either extraction-based PCR or direct PCR. As only a small portion of the tapelift is used in either the extraction protocol or direct PCR protocol described, replicate sampling is possible, thus removing a criticism of direct PCR.

5.5 Conflict of Interest

No potential conflict of interest was reported by the authors.

5.6 Funding and Acknowledgments

Funding for this research was provided by the Attorney General's Department through Forensic Science SA and the Ross Vining Memorial Research Fund. Funding sources had no involvement in the study design, collection and interpretation of data, writing of the report, or the decision to submit the paper for publication. We would like to acknowledge and thank all volunteers who participated in this study for the donation of their time and DNA.

5.4 PHASE 2 STR ANALYSIS OF AMMUNITION WITHOUT SOAKING OR EXTRACTION

5.4a Preliminary study

5.4a.1 Introduction

Ammunition traditionally returns poor DNA profiles following swabbing and extraction processes. Alternate methods that have been explored incorporate the soaking of the cartridge case in a lysis buffer and using this liquid for the extraction process [400, 401]. Although this has been shown to yield increased allelic returns in subsequent profiles, compared to swab-based extraction, it corrodes micro-topographies present within the bullets surface [402]. Because of this, following a seizure, triaging of the bullet casings requires a decision to either perform microscopic analysis or perform DNA analysis. Ideally both DNA profiling and microscopic analysis can be performed from the same sample.

To investigate the potential of obtaining up-loadable DNA profiles from fired ammunition, preliminary studies were conducted to apply the direct PCR workflow to a range of ideal sample types. This was extended with an internal validation study performed with FSSA for the purpose of optimising profile development for an exhibit seized during a case. Further studies have shown that alteration to PCR conditions, in the form of mastermix preparation, can aid in the stabilisation of direct PCR against inhibitors within the collected sample.

5.4a.2 Methods

Exhibits and volunteers

One volunteer prepared and fired all samples. Four ammunition types were examined to determine whether direct PCR could be a viable workflow for the analysis of cartridge cases. These were as follows: 12G shotgun casing with a polymer case and brass head; 7.62 mm painted steel casing, .45 nickel casing, and 9 mm brass casing. Quintuplet samples of each ammunition type under each condition were prepared. Ammunition was either unfired or fired with three handling conditions. The handling conditions involved 1) no handling whereby the ammunition was removed from the box and immediately packaged or fired using gloves, 2) minimal handling whereby the ammunition was removed from the box and loaded into the weapon quickly, and 3) ten mins of handling whereby the volunteer rolled the ammunition in their hand for 10 mins prior to loading the weapon. Once packaged, the samples were stored on a desk for 6 weeks prior to DNA collection.

Collection, amplification, separation, and data analysis

Each sample was double swabbed using a nylon ultrafine micro applicator moistened with 0.1% Triton™ X-100, with the exception of the 9 mm brass casings where only one swab was used. This swab head was removed using a sterile scalpel blade into a 0.2 mL PCR tube for direct amplification. Amplifications were conducted using Identifiler® Plus following manufacturers protocol, with the exception of 2 µL Prep-n-Go™ and Low TE in place of water. PCR product (1 µL) was injected, with 8.5 µL Hi-Di Formamide and 0.5 µL 600 LIZ®, and separated on a 3500 Genetic Analyser.

Data analysis of CE output was performed using GeneMapper® IDX v1.4. Peak thresholds were 50 RFU and 150 RFU for true allele and heterozygous consideration respectively.

5.4a.3 Results and Discussion

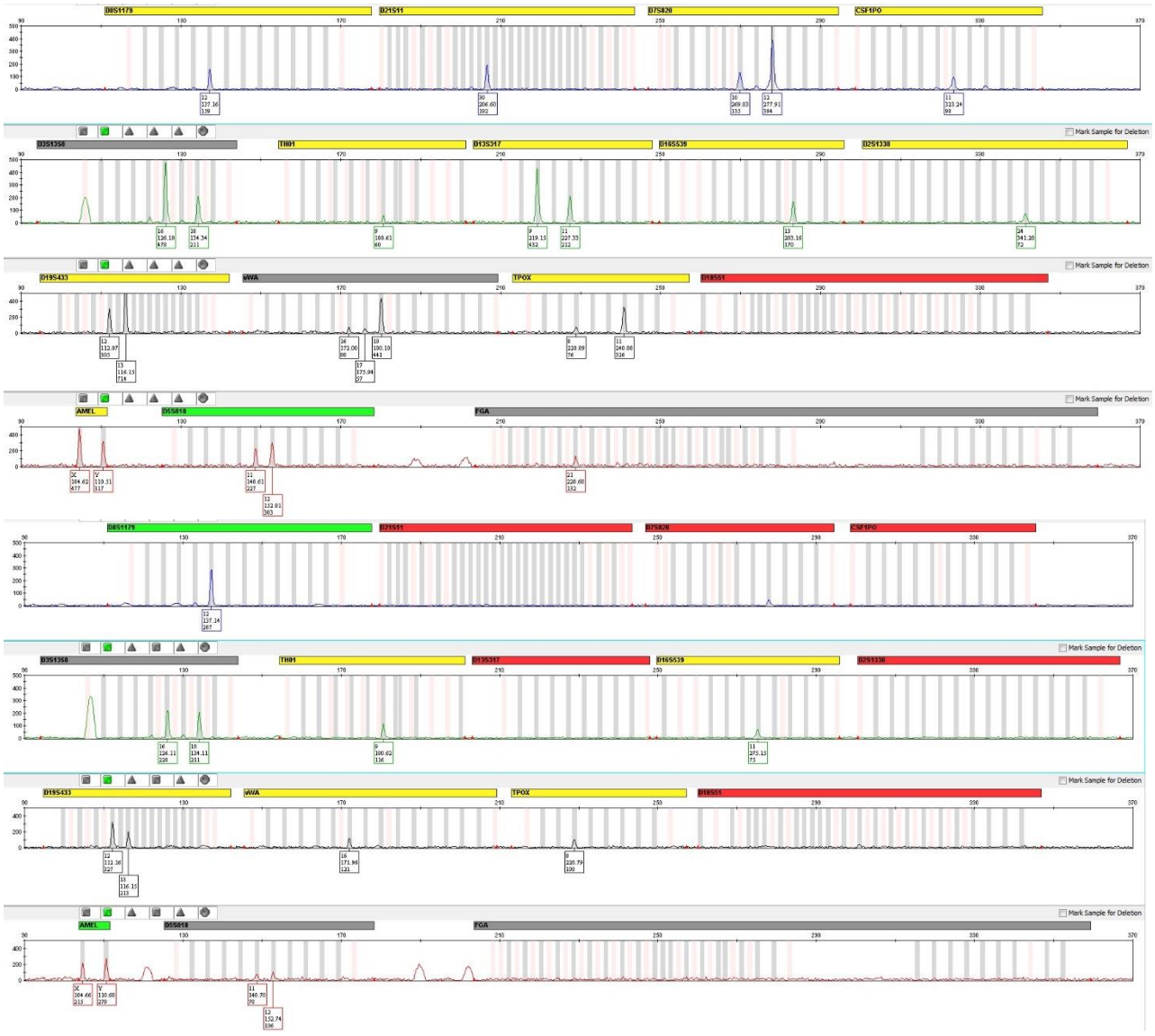


Figure 5.3. Example of STR profile generated from a 12G casing both unfired (top) and fired (bottom).

Following the direct PCR amplification of swabbed cartridge casings, much variation in the quality of resultant profiles is observed (Table 5.1). All negative controls, except one 12G untouched and unfired (A), were negative; the single 12G A profile that returned alleles was a complete STR profile of the DNA donor handling all casings. The largest proportion of sampled casings to return profiles considered up-loadable were from the 12G long hold, both fired and unfired, and the 7.62 mm steel long hold, unfired. Firing the cartridges resulted in a loss of amplified genetic material (Table 5.1;Figure 5.3). Brass bullets returned no profiles, with the exception of one that returned 11 autosomal alleles that matched the DNA donor. This was not unexpected as there is literature that suggests brass can cause DNA degradation through oxidation [403].

Table 5.1: Average alleles attributed to the donor observed in a DNA profile following direct PCR amplification with Identifiler® Plus sampled 6 weeks post-firing. A: untouched and unfired, B: untouched and fired (using gloves), C: Minimal handling (loading of weapon only) and unfired, D: Minimal holding (loading of weapon only) and fired, E: 10 mins handing and unfired, F: 10 mins handling and fired.

	Condition	Attributed Alleles	% profile observed	Informative /5
12 G	A	6.4	20	1
	B	0	0	0
	C	7.4	23.125	1
	D	2.4	7.5	0
	E	19.2	60	4
	F	18.4	57.5	4
7.62 mm Steel	A	0	0	0
	B	0	0	0
	C	2.8	8.75	0
	D	6.4	20	1
	E	21.3	67.5	4
	F	8.4	26.25	1
0.45 Nickel	A	0	0	0
	B	0	0	0
	C	9.8	30.625	2
	D	7.8	24.375	2
	E	17.8	55.625	3
	F	2.8	8.75	0
9 mm brass	A	0	0	0
	B	0	0	0
	C	0	0	0
	D	0	0	0
	E	4	12.5	1
	F	0	0	0

Direct amplification of all bullet types tested resulted in non-allelic peaks appearing in consistent locations within the STR profile. These were OL in most profiles (Figure 5.4), however on occasion a peak could fall within a bin in D3 (13), FGA (17), THO1 (5). These have been observed in similar locations for nickel, steel, and brass bullet types, and each ammunition type have their own unique artefact peaks also. There is migration of artefacts in the EPGs observed between ammunition (Figure 5.5); this is, however, a small dataset and an increase to this would be advantageous to address the consistency of artefact location. The artefacts run in the same dye lanes no matter the kit (i.e. the large green artefact appearing within D3 in Identifiler Plus™ appears in D8 (similar bp size) in GlobalFiler™.

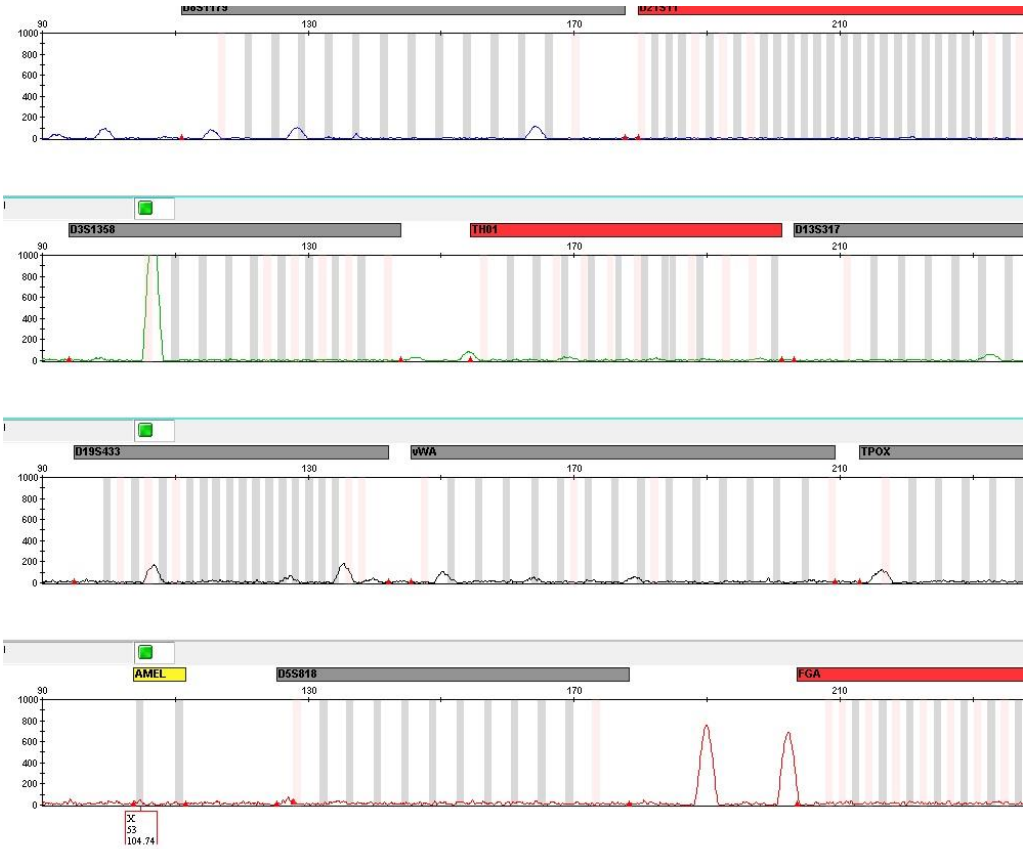


Figure 5.4. Example of OL peaks generated from the direct PCR amplification of cartridge cases. This sample was a .45 nickel casing post-firing.

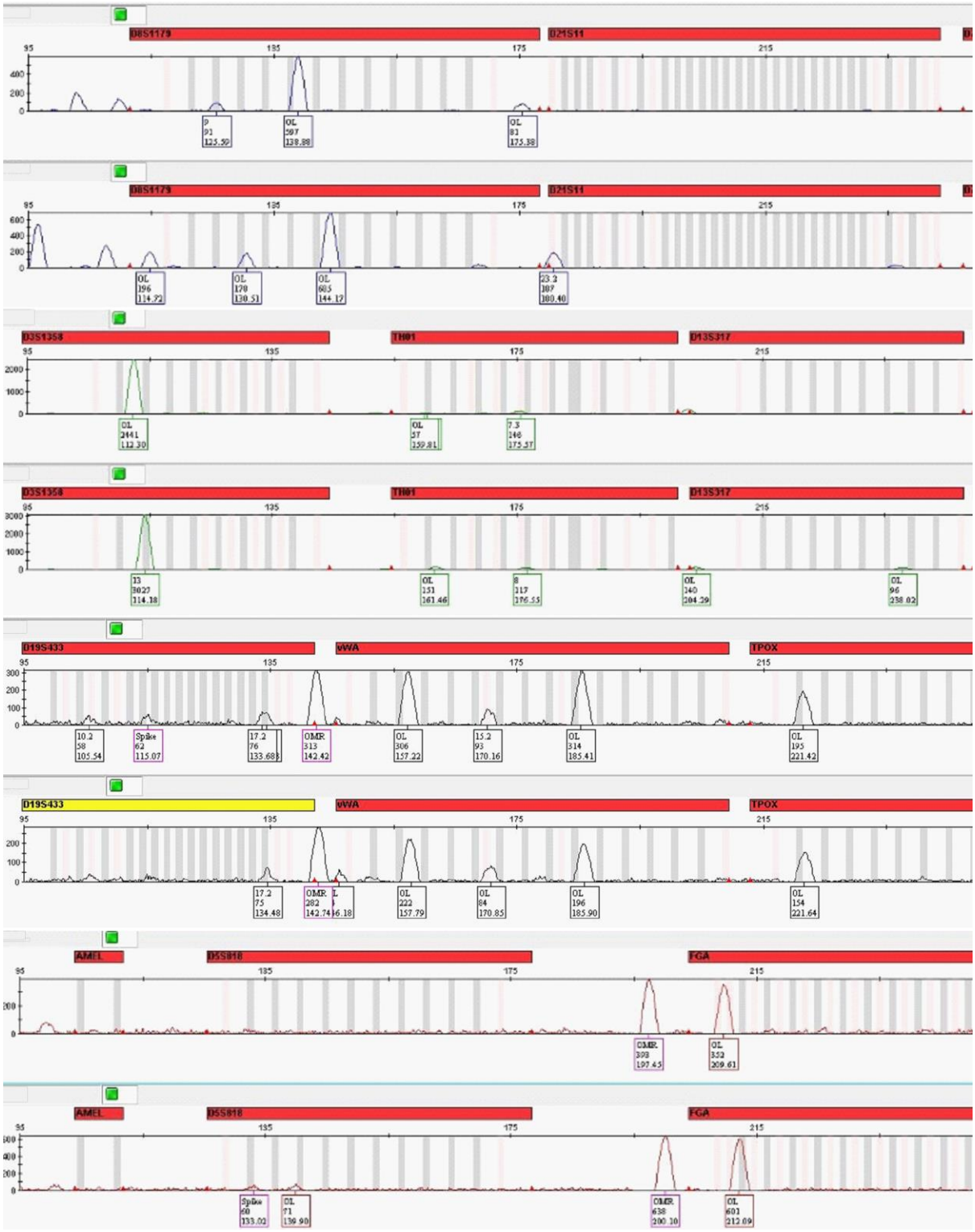


Figure 5.5. Exemplars of artefacts observed in Identifiler Plus™ profiles due to the direct PCR amplification of fired ammunition samples.

5.4a.4 Conclusion

Although profiles were able to be obtained 6 weeks post deposition and firing, direct PCR amplification was not able to reliably produce an up-loadable profile. Generation of any genetic material from fired cartridge cases is useful, especially considering the casings experience no damage due to the sampling process, however further investigation into sampling methods and PCR constituents may provide improved profiles from these sample types. The observation of artefacts within the post-firing sample profiles generated following direct PCR is important to note. As these appear to be consistent, future research into categorising these, in terms of location and morphology, more acutely is important.

5.4b Manuscript: Direct PCR of fired shotgun casings: a South Australian evaluation

Manuscript published: *Australian Journal of Forensic Sciences*, 2020

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**60**	**90**	**80**	**20**	**30**		03.08.21
AP	30	10	-	-	30		02.08.21
AL	-	-	-	-	10		05.08.21
JH	10	-	20	80	30		03.08.21

5.4b.1 Abstract

Recovery of touch DNA from fired ammunition casings may provide vital forensic evidence for investigation and/or prosecution of firearm offenses. Previous studies employing a direct PCR approach, where the traditional DNA extraction process is bypassed, have demonstrated an improved profiling success rate from some types of ammunition. To assess the potential value of these techniques to recover touch DNA from fired shotgun ammunition, direct PCR was evaluated for its ability to recover DNA profiles from fired 12 gauge Buckshot OOSG shotgun cartridges as compared to routine swabbing with DNA extraction. In this study, swabs subjected to direct PCR gave a significantly lower recovery of alleles than those undergoing DNA extraction. The presence of PCR inhibitors that were produced or deposited during the firing process may be the cause of these poorer results. The success of a direct PCR approach may therefore be directly related to the type of ammunition or firearm used and should therefore be specifically tested for individual case scenarios prior to employing it in forensic casework.

5.4b.2 Introduction

Offenses involving firearms are generally serious in nature and the ability to link touch DNA on fired ammunition casings to an individual can be vital in a police investigation and/or criminal prosecution. Recovery of touch DNA from fired ammunition is known to be problematic due to low amounts of deposited cellular material as well as possible heat, friction and chemical effects during the firing process [138, 400, 404, 405]. Improved sensitivity and robustness of DNA profiling kits in

conjunction with development of methods which maximise the availability of amplifiable DNA has enabled the profiling success of touch DNA from ammunition to be improved [405-407]. Also, there is growing interest in the use of direct PCR for ammunition [137, 289] as the success of this approach for STR profiling of touch DNA on other substrates has been widely reported [143, 193, 253, 260, 262, 289, 295]. The apparent success of direct PCR can be attributed to it ensuring that all recovered DNA is available to the PCR and circumventing the extraction process where significant losses of DNA can occur [408, 409]. However, one limitation of this technique is that inhibitors from the sample substrate, normally removed by the DNA extraction process, may enter the PCR and affect downstream profiling success.

The investigation of direct PCR for the recovery of STR profiles from fired ammunition is limited to a recent study by Thanakiatkrai and Rerkamnuaychoke [137]. Herein, direct PCR using Identifiler® Plus was shown to double the average number of alleles recovered from 19 x 9 mm Luger, 7.62 x 39mm AK47, and 5.56 x 45 mm NATO bullets in comparison to a conventional DNA extraction protocol. Unlike these ammunition types, 12 gauge shotgun cartridges have a significantly larger surface area and are typically composed of a plastic casing with a brass head. To date, there are no published studies regarding the success of direct PCR for fired 12 gauge shotgun casings and therefore an evaluation would be required before this technique was employed for casework involving this ammunition type.

Spent ammunition products are often located at crime scenes where firearms have been used, and 12 gauge ammunition is commonly used in firearm crime in South Australia. Difficulty in recovering touch DNA from these 12 gauge ammunition products presents a significant gap in the ability to add value to the investigation. Currently, direct PCR of spent cartridges is not validated for use at FSSA and the SOP for recovering DNA from these exhibits is to swab the entire outer surface with a Puritan® micropopule swab and subject this swab to DNA extraction. We therefore undertook this evaluation to compare the direct PCR approach to FSSA's standard swabbing and DNA extraction procedure. This was to confirm whether or not a direct PCR approach offered an improved chance of recovering DNA profiles from such exhibits.

5.4b.3 Methods

Preparation of the ammunition

Winchester brand Buckshot OOSG 12 gauge cartridges (Figure 5.6) from sealed boxes were loaded with DNA from an anonymous donor by holding them in unwashed hands for 1 min (40 cartridges) or 10 mins (40 cartridges). A further 20 unhandled cartridges were included to determine the baseline DNA level straight from the box. For each of the 1 min and 10 min hold times, 20

cartridges were left unfired and the remaining 20 were fired using a Boito single-barrel shotgun. Fired casings were collected under DNA-free conditions. DNA from the anonymous donor was provided with informed consent and was approved by the Research and Development Committee of FSSA.

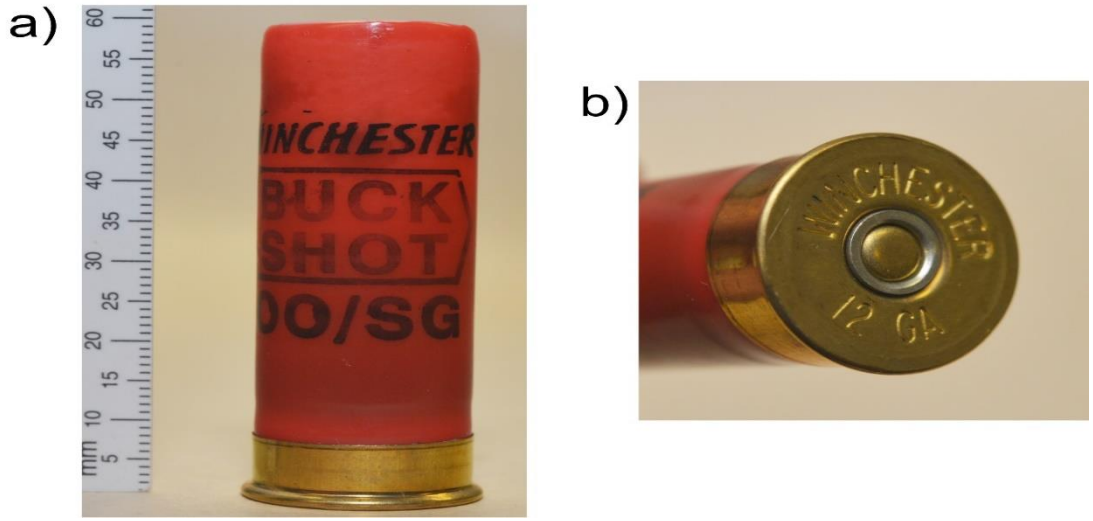


Figure 5.6. Winchester OOSG buckshot 12 Gauge ammunition (unfired) depicting a) overall construction and b) detail of the brass cartridge head. Notably, the cartridge is constructed with a sharp edge at the intersection of the plastic and brass components and recessed stamped characters of the manufacture markings on the brass.

Sampling of DNA

Of the 20 cartridges in each cohort (1 min hold/unfired; 1 min hold/fired; 10 min hold/unfired; 10 min hold/fired; unheld/unfired control), 10 were swabbed a micropopule (MPOP) trace DNA collection swab (Puritan®, Guilford, ME, USA) pre-wetted with 100% isopropanol. The swab head was removed and subjected to DNA extraction. The other 10 casings/cartridges were double-swabbed with ethylene oxide treated nylon ultra-fine micro-applicators (City Dental, Adelaide, Australia) after applying 5 µL of 0.1% Triton X-100 (Sigma, Australia) directly to the ammunition surface. The two swab heads were removed using a sterile scalpel blade, placed into a 0.2 mL thin-walled PCR tube, and subjected to direct PCR.

Generation of DNA profiles

DNA was extracted from MPOP swabs using the DNA-IQ system (Promega, Madison, WI, USA) on a Hamilton AutoLys STAR liquid handling platform as per the manufacturer's instructions. The final DNA extract volume was 60 µL. The quantity of recovered DNA was determined using the Quantifiler™ Trio DNA Quantification Kit (ThermoFisher Scientific, Waltham, MA, USA). Both extracted DNA and swabs for direct PCR were profiled using the GlobalFiler™ PCR amplification kit (ThermoFisher Scientific) using a ProFlex thermocycler (ThermoFisher, Scientific) in 25 µL reactions with 29 cycles of PCR as per the manufacturer's instructions. For DNA extracts, either 400 pg of DNA or 15 µL of extract

was added to the PCR. PCR products were electrophoresed on an Applied Biosystems® 3500xL Genetic Analyser (ThermoFisher Scientific) using 1 µL of PCR product to 10 µL of HiDi™ formamide/GeneScan™ 600 LIZ. Separated fragments were analysed using GeneMapper® IDX v1.4 software (ThermoFisher Scientific) with a peak amplitude threshold of 50 RFU.

Data analysis

The number of donor alleles recovered for each sample was determined where 43 was the total number of alleles expected in the donor's profile. The average peak intensity of the donor alleles across each profile was also determined by dividing the summed RFU of all peaks by the number of donor peaks observed. The number of non-donor alleles and their average peak intensity was also recorded.

5.4b.4 Results and Discussion

The number of donor and non-donor alleles recovered from the fired and unfired 12 gauge cartridges held for either 1 min or 10 mins and analysed with either the direct PCR or the MPOP swabbing protocol are presented in Figure 5.7. For the unfired cartridges, full donor profiles were obtained from 11/40 (28%) samples and only one sample yielded no profile. The average number of donor alleles recovered was approximately 37/43 (86%) for the 10 min hold time and approximately 25/43 (58%) for the 1 min hold time. There was no significant difference in the number of donor alleles recovered using direct PCR as compared to the MPOP swabbing method (p= 1.0 (10 min hold) and p= 0.62 (1 min hold); Mann Whitney U-test, two tailed). There was also no significant difference in the peak heights of the donor alleles for profiles generated with the different methods (p= 0.24 (10 min hold) and p= 0.41 (1 min hold); Mann-Whitney U test, two tailed) (data not shown).

For the fired cartridges, full donor profiles were only detected in 2/40 (5%) samples and 7/40 (18%) yielded no profile. Six of the seven failed profiles were in the direct PCR cohort. The number of donor alleles recovered from the fired cartridges was significantly reduced for the direct PCR approach compared to the MPOP swabbing method. For the 10 min hold, the average number of donor alleles recovered was 16/43 (38%) for direct PCR as compared to 27/43 (63%) for the MPOP approach (p= 0.04; Mann Whitney U-test, one tailed). For the 1 min hold time, the average number of donor alleles recovered was 6/43 (14%) for direct PCR as compared to 20/43 (47%) for the MPOP approach (p= 0.006; Mann Whitney U-test, one tailed). Despite the reduction in allele recovery using direct PCR, the average peak intensity of donor alleles was not significantly different to that of the MPOP protocol (p= 0.12 (10 min hold) and p= 0.08 (1 min hold); Mann-Whitney U test, two-tailed) (data not shown).

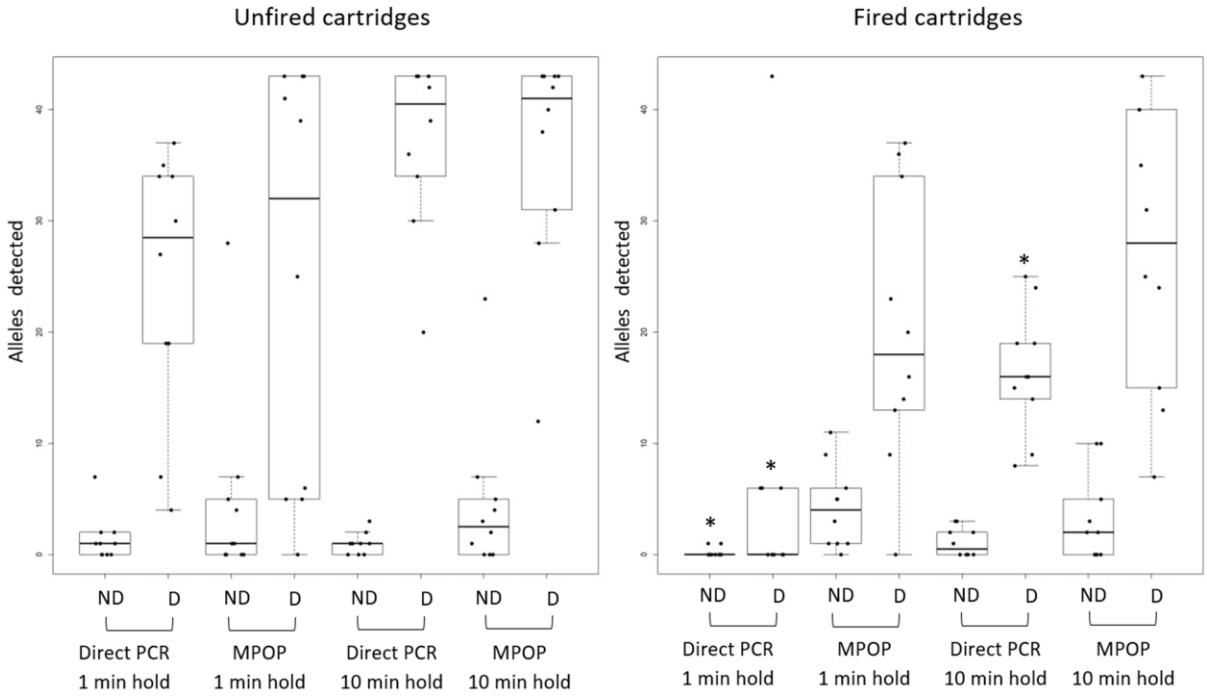


Figure 5.7. Box plots representing the number of donor (D) and non-donor (ND) alleles recovered from fired and unfired 12 gauge Buckshot OOSG cartridges. The dots indicate individual data points. Cartridges were either swabbed with ultra-fine micro-applicators and subjected to direct PCR or swabbed with a Puritan® MPOP and subjected to DNA extraction with Promega DNA-IQ. There are 10 replicates in each sample group. Asterisks indicate results that are significantly for direct PCR as compared to the MPOP method at p<0.05 (Mann-Whitney U-test, one-tailed).

As the MPOP and direct PCR approaches gave similar outcomes for the unfired ammunition, the reduction in the recovery of donor alleles for the fired ammunition using the direct PCR approach suggests that the firing process has directly affected its success. The reason for this is not known but it is possible that the firing process produced or deposited a chemical residue to the surface of the casings which resulted in downstream inhibition of the polymerase during direct PCR amplification. The presence of PCR inhibitors on the cartridge surface would be expected to have less effect on the MPOP samples (as was observed) due to their partial or complete removal during the DNA extraction process. As the average percentage of donor alleles recovered using the MPOP method was slightly reduced for the fired ammunition as compared to the unfired for both hold times, it is possible that some co-extraction of PCR inhibitors has also occurred with this approach. Alternatively, the reduction in donor DNA relative to the unfired ammunition could be caused by physical loss of DNA from the surface of the cartridge during the firing process.

Non-donor alleles were detected in 60% of the profiles generated in this study and ranged from 1 allele through to a total of 28 alleles per profile (average = 2.5 alleles per profile) (refer Figure 5.7). The number of non-donor alleles recovered was not significantly different between the MPOP

and direct PCR approaches with the exception of the fired 1 min hold cohort. Here, the number of non-donor alleles detected was significantly lower for the direct PCR method (p= 0.001, Mann Whitney U-test, one tailed). However, detection of DNA in general was particularly poor within this direct PCR cohort and this result is therefore not unexpected. The presence of non-donor alleles on 30% of ammunition samples tested straight from the box (average = 0.5; range 0 – 3 alleles per profile) (data not shown) indicates that some of the DNA detected in resultant profiles may originate from the ammunition manufacture process. However, as the frequency and extent of non-donor DNA was higher for the handled ammunition, this suggests that most DNA originated via secondary transfer from the donor's hands, firearm, or environment. The level of non-donor DNA observed in this study is reflective of that observed previously [4,8]. Therefore, caution must be exercised when interpreting profiles obtained from ammunition (both fired and unfired) as a DNA match may not be synonymous with criminal activity.

5.4b.5 Conclusion

The successful recovery and profiling of touch DNA from ammunition is critical to many forensic investigations. This study specifically evaluated the efficacy of direct PCR to generate DNA profiles from fired 12 gauge Buckshot OOSG cartridges commonly encountered during casework. When compared to the MPOP swabbing and DNA extraction protocol currently employed by FSSA, the direct PCR approach resulted in a significantly poorer profiling outcome. As this was not observed for unfired ammunition, it seems that a PCR inhibitor(s) is produced or deposited onto the cartridges during the firing process. As both the plastic and brass surfaces of the fired cartridge were sampled for direct PCR, further investigation is required to determine whether the PCR inhibitor is specific to either one of these surfaces.

This outcome of this study differs to that observed by Thanakiatkrai and Rerkamnuaychoke [137] where direct PCR gave improved profiling success with three types of fired, smaller gauge metallic ammunition. The reason for the difference in outcome is not known but may relate to the ammunition type, the firearm, the methodology used to recover DNA, or that different PCR amplification kits were used.

This highlights the importance of conducting an evaluation which is specific to the individual case scenario or exhibit prior to the application of a new technique in real-life casework. This ensures that it offers benefits and outcomes additional to those of currently employed techniques.

5.4b.6 Acknowledgements

The authors wish to thank staff in the Forensic DNA Analysis group at FSSA for laboratory analyses, Dr Duncan Taylor for assistance with preparation of the graphical figure, and Dr Andrew Donnelly for helpful casework discussions.

5.4c Secondary study

5.4c.1 Introduction

As the cause of amplification reduction for direct PCR samples over extraction has been observed with fired larger surface area ammunition is currently unknown [321], it is important to investigate whether it can be improved with simple alterations to protocols. If it can be improved due to differences in the PCR constituents the previously supposed proposal, that the reduction of success is due to inhibitors found and collected from the surface following firing, is supported. If it is improved through differences in cycling conditions or collection methods then it is likely that the template quantity is not sufficient; as the extraction process is well understood to cause the loss of DNA template [2, 3, 201] and this workflow produced higher DNA data than its direct PCR counterpart [321] this is unlikely.

Although commercially available STR have improved buffer systems over the years to better cope with inhibitor presence [255], research groups have produced their own specially designed PCR buffer systems to determine whether amplification could be improved [137, 257] and, as discussed in Chapter 1.6, surfactants or additives have also been investigated [134]. As the desire is to streamline the incorporation of methods into forensic practise that have the ability to increase DNA profiling data results the use of buffers within commercial kits is ideal, and any alteration to the PCR should come through additives in place of water. To investigate this, Prep-n-Go™ was added to direct PCR amplifications using GlobalFiler™ for fired 12 G ammunition and a comparison to previously obtained direct PCR results and post-extraction amplification results (Chapter 5.4b) is proposed.

5.4c.2 Methods

Exhibits and Volunteers

An anonymous donor handled 40 12 G cartridges (Winchester brand buckshot OOSG), with unwashed hands, for ten mins. Of these cartridges, 15 were unfired and 25 were fired using a Biito single-barrel shotgun.

Sampling and processing of DNA

MPOP trace DNA collection swabs (Puritan®, Guilford, ME, USA), pre-moistened with 100% isopropanol, were used to sample ten fired cartridge casings. These swab heads were removed and

placed into an LHP tube for extraction by DNA-IQ system (Promega, Madison, WI, USA), following manufacturer's instructions, on a Hamilton AutoLys STAR liquid handling platform. The final elution volume was 60 µL.

The remaining 30 were double-swabbed with a nylon ultrafine microapplicator after applying 5 µL of 0.1% Triton™ X-100 directly to the ammunition surface. The two swab heads were removed, using a sterile scalped blade, directly into a 0.2 µL PCR tube for direct PCR amplification. Direct PCR amplification was performed on 10 fired, and 10 unfired casings following manufacturers guidelines, with the other 5 being amplified with 2 µL Prep-n-Go™ within the PCR mix. Both extracted DNA and swabs reserved for direct PCR were profiled using GlobalFiler™ PCR amplification kit. For DNA extracts, either 400 pg of DNA or 15 µL of extract was added to the PCR for amplification. Following amplification, 1 µL of PCR product was added to 10 µL of HiDi™ formamide/GeneScan™ 600 LIZ for separation on the 3500XL Genetic Analyser. Separation data was analysed with GeneMapper® IDX v1.4 software with a peak height threshold of 50 RFU.

Data analysis

The number of donor alleles recovered for each sample was determined; a maximum of 43 was expected in the donor's profile. A GLM was performed between the following: unfired direct PCR with no Prep-n-Go™ and unfired direct PCR with Prep-n-Go™; fired direct PCR with no Prep-n-Go™ and fired direct PCR with Prep-n-Go™; fired PCR post extraction with no Prep-n-Go™ and fired direct PCR with Prep-n-Go™.

5.4c.3 Results and Discussion

Table 5.2: Comparison between samples processed with and without Prep-n-Go™ within the PCR constituents. Replicates of 10 were performed for samples without Prep-n-Go™ and 5 replicates were performed with Prep-n-Go™.

	10 min unfired		10 min fired		10 min fired	
Workflow	Direct				Extraction	Direct
Prep-n-Go™	WITHOUT	WITH	WITHOUT	WITH	WITHOUT	WITH
Average Donor Alleles	37.3	32.8	16.5	25.6	27.3	25.6
Standard Deviation	7.60	8.87	5.56	8.68	12.59	8.68
p-value (GLM)	0.326		0.0146		0.789	

There was no significant difference in the number of donor alleles amplified between unfired direct PCR samples either containing or not containing Prep-n-Go™ (Table 5.2). When the casings were sampled post-firing, however, Prep-n-Go™ addition within the PCR showed a significant increase in the number of donor alleles amplified. As data generated and presented within the enclosed

manuscript (Martin, B., *et al.*, Direct PCR of fired shotgun casings: a South Australian evaluation. *Australian Journal of Forensic Sciences*, 2020: p. 1-7) indicates that, with GlobalFiler® amplification following manufacturers protocol, standard extraction-based PCR workflow outperformed direct PCR when no Prep-n-Go™ is added. To see whether the inclusion of Prep-n-Go™ increased the recovery of alleles to a comparable standard to that of the extraction samples datasets were compared. There was no significance noted in the number of donor alleles amplified between the extraction-based workflow and the direct PCR with Prep-n-Go™ workflow.

Conclusion

There is the potential that the size of the swab heads is influential in the quality of the resultant profile; MPOP heads are much larger and can facilitate increased cellular recovery over the ultrafine swab heads. Considering this, comparison with other ammunition types should be performed using the methods described above. As no significance was observed between the workflows, with the addition of Prep-n-Go™ to the direct PCR, either workflow can be utilised for optimum non-destructive cartridge case sampling and processing. This also highlights the value or increasing a PCRs robustness against inhibitors and the need for additional research in the additives space.

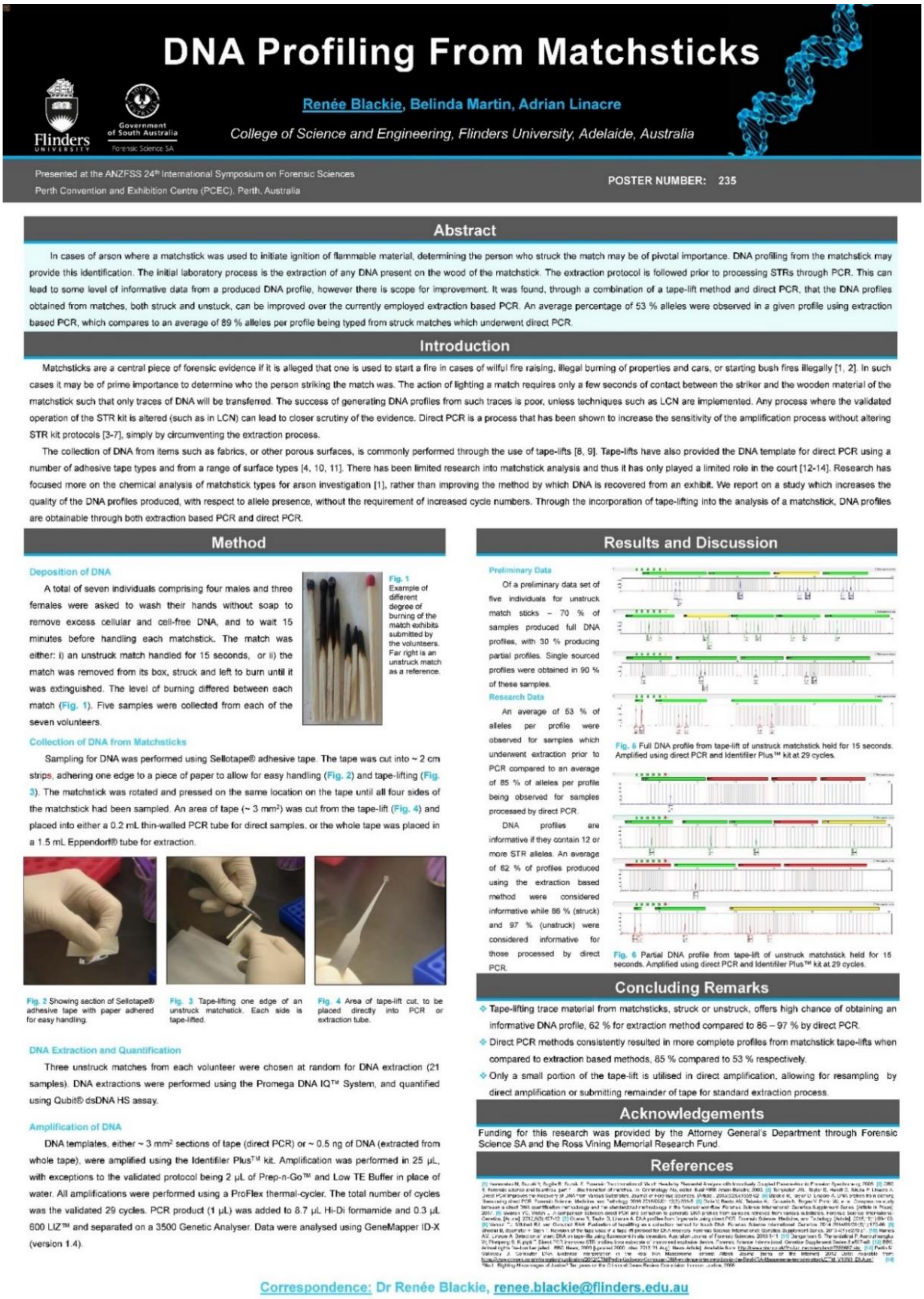
5.5 CONCLUDING COMMENTS

The data presented in 5.3, Phase 1, supports the introduction of direct PCR amplification of tapelift samples from struck matchsticks, such as those found in an arson incident. Direct PCR resulted in an increased number of alleles per profile compared with PCR post-extraction. Additionally, struck matchsticks returned profiles considered up-loadable to a DNA database for 97% of samples. The aim "*to determine whether direct PCR is a viable alternative to LCN and extraction-based PCR workflows for the analysis of struck matchsticks*" has been addressed within this work.

The data presented within 5.4, Phase 2, provide an insight into the difficulty of amplification of latent DNA workflow choice when considering spent ammunition. Initial data suggests that direct PCR is preferable to extraction-based PCR methods for smaller cartridge sizes. Issues surrounding inhibition from primer exposure or gunshot residue (GSR) did not seem of concern when considering the benefit of increased template addition with the direct PCR methodology. For larger cartridge cases, such as the 12G, standard workflow is still ideal due to the inability to collect the available template for direct PCR purposes. There may be merit in exploring alternate collection methods, such as tapelifts, that allow a more direct approach, however this was not within the remit of the study. The introduction of Prep-n-Go™ to the direct PCR process increased the allelic yield significantly, therefore if direct PCR is utilised, even for larger cartridge sizes, it is valuable to include this reagent in the PCR set up. The need for further investigation and categorisation of the artefacts observed in direct PCR EPGs of fired cartridges is identified and future examination is encouraged. The aim "*to examine the application of direct PCR to the examination of spent ammunition in comparison to other methods*" was addressed within this work.

5.6 APPENDICES

5.6.1 Poster presentation at "The Australian and New Zealand Forensic Science Society" conference, Perth, Australia, 2018. 'DNA Profiling from Matchsticks'



CHAPTER VI:

ANALYSIS OF DNA FOLLOWING EXTREME CONDITIONS: POST-DETONATION PROCESSING

Manuscripts enclosed:

Martin, B., Kanokwongnuwut, P., Taylor, D., Kirkbride, P., Armitt, D., Linacre, A. (2020) "*Successful STR amplification of post-blast IED samples by fluorescent visualisation and direct PCR.*" Forensic Science International: Genetics

Martin, B., Kaesler, T., Kirkbride, P., Linacre, A. (2022) "*The influences of dusty environments on the STR typing success of post-detonation touch DNA samples*." Forensic Science International: Genetics

6.1 PREFACE

Following the success and limitations observed and discussed in Chapter V, it was desirable to determine where the boundaries of touch DNA amplification lie using direct PCR under specified conditions. From 5.4, the surface area analysed was small in subsequent studies, unless otherwise stated, and Prep-n-Go™ was added to all direct PCRs. As IED samples experience incredible pressure and heat exposure [410, 411] following detonation, determination of whether direct PCR methods, developed through the work presented in previous chapters, could improve the STR EPG results or advise triage team was of both academic and industry interest. This chapter explores a workflow involving nucleic acid staining and direct PCR for the analysis of post-detonation IED samples. A number of sample types, typical of those encountered in an IED, were exposed to detonation and direct PCR STR profiling ensued. Results are reported in a peer reviewed publication in *Forensic Science International: Genetics.*

In addition to this a secondary study addresses questions raised as to how translatable these results are to different environments in which detonations may occur. Through careful design, experimentation can produce data that facilitates consideration of complex interactions such as between methodology, external uncontrollable variables, and variables of interest. Here, questions surrounding how well direct PCR can facilitate amplification of touch DNA to produce informative profiles given extreme pressures, influence if inhibitors, and other variables are explored. Results are reported in a manuscript currently under review in *Forensic Science International: Genetics.*

6.2 IMPACT STATEMENT AND AIMS

The paper “Successful STR amplification of post-blast IED samples buy fluorescent visualisation and direct PCR” has revolutionised our understanding of post-detonation STR amplification potential. Its novel use of DD for the monitoring of cellular retention, movement, and loss due to an experimental procedure supports its inclusion in studies for its ability to add another level of analytical power to results. The results within this work are foundational to future research with industry and support direct PCR use for counterterrorism operations.

Furthermore, the data produced following this initial study continued to exceed expectations based on previous literature. The methods utilised and considerations outlined can greatly improve DNA-based intelligence data provided to counterterrorism operations. These data form the basis of supporting research expansion to other genetic analyses and compromised sample types.

Data generated with respect to post-detonation IED components has become invaluable for counterterrorism agencies, both locally (DSTG) and abroad (CSTTO). It has provided guidelines as to the types of samples that should be prioritised in evidence collection following an event, the triaging potential obtained through nucleic acid stain visualisation, and the application of direct PCR to optimise response team workflows.

6.2.1 Aims

"*To investigate the potential for direct PCR to provide informative STR data from post-detonation IED samples*"

"*To investigate the difference an outdoor, dirty, environment has on direct PCRs capability to provide informative STR data from post-detonation IED samples"*

6.3 BLAST CHAMBER: POST-DETONATION SAMPLING USING DIRECT PCR

6.3 Manuscript: Successful STR amplification of post-blast IED samples by fluorescent visualisation and direct PCR

Manuscript published: *Forensic Science International: Genetics*, 2020

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**40**	**45**	**30**	**90**	**40**		03.08.21
PK	20	45	30	10	30		04.08.21
DT	-	-	25	-	7.5		03.08.21
KPK	15	10	15	-	7.5		03.08.21
DA	10	-	-	-	5	Armitt, David Digitally signed by Armitt, David Date: 2021.08.05 09:21:57 +09'30'	05.08.21
AL	15	-	-	-	10		05.08.21

6.3.1 Abstract

IEDs present a number of challenges in terms of the generation of forensically relevant information. Inhibition to PCR from sub-optimal sample types as well as from specific substrates has historically meant that extraction prior to PCR has been required. Improvements to STR kit buffers lead to the successful introduction of direct PCR to the analysis of IED-relevant samples, however none of these samples have been exposed to detonations. This study presents data to support the use of direct PCR in the analysis of IED components post-detonation. VeriFiler™ Plus generated informative profiles, containing ≥ 12 autosomal alleles, from samples touched for a maximum of 15 s that were then exposed to a detonation from plastic explosive placed as close as 100 mm. Of the 37 recovered touched items or fragments, 28 contained autosomal alleles from the donor with 18 (49%) presenting informative profiles that matched the DNA donor. This compared with results following STR PCR

post-extraction with one of 11 amplified post-detonation touch DNA samples being informative. The use of DD staining and visualisation before and after detonation allowed for analysis as to cell loss or damage as a result of the detonation itself and aided in the triaging of samples to be selected for DNA profiling. This is the first record of cellular visualisation and comparison before and after detonation with accompanying STR results on a range of sample types typical of IED constituents. Following comparison of DD visualised cells and STR amplification success, chemical analysis of plastic and electrical tape samples supported substrate-specific inhibition. These data represent the first instance of informative DNA profiles being produced from post-detonation samples using direct PCR, as close as 100 mm from the charge.

6.3.2 Introduction

IEDs are a devastating tool readily utilised by terrorists and other criminals worldwide. In the construction of IEDs, which is often performed with cheap and widely available items, DNA from the maker of the IED may be deposited on the components during construction, placement, and deployment. Provided that the DNA can be analysed, genetic data may provide pivotal information in the prosecution of offences and intelligence for the fight against acts that involve the use of an IED. However, the ability to generate a DNA profile from touched items after they have been subjected to the massive forces and temperatures of an explosion is potentially fraught with difficulties.

A number of studies that have obtained genetic information from IED components post-detonation have used samples spiked with known amounts of pristine cellular material in the form of epithelial cells or buffy coat [139, 143]. Varying success was obtained with both extraction and direct PCR amplification workflows using STR analyses. The benefit of spiking with buccal cells is that it is possible to give an approximate number of cells deposited on each item. However, realistically, buccal cells are unlikely to be deposited on IED components. This means that while buccal cells may be used for a proof-of-concept, or as controls, in order to mimic real-life performance, real-life deposition is required. In reality, it is corneocytes that will be transferred onto IED component surfaces if they are touched by human hands [112]. To determine with accuracy whether DNA profiles can be obtained from items post-detonation, components of IEDs touched by volunteers should be used.

The analysis of retrieved touch DNA from IEDs has previously utilised both direct STR PCR [141, 142, 267] and STR amplification post-extraction [139, 314]. Increasing the number of PCR cycles has been performed on PVC and circuit board samples [267], half-volume amplification has been performed on material recovered from PVC, circuit boards, and electrical tape [141] and latent DNA

detection by fluorescent dyes prior to amplification has been performed on PVC and electrical tape [141, 142]. Altering the amplification methods, such as cycle number or kit volume, may make incorporation into forensic laboratory workflow difficult due to strict validation guidelines and high workflow automation, therefore it is not ideal to alter the manufacturers' guidelines. While these studies highlight the potential to analyse trace DNA on such substrates, they are restricted in any conclusions that can be made regarding the reality of post explosion sample analysis.

There have been very few studies that have been able to obtain any STR alleles in profiles post-detonation; these have both utilised DNA extractions within their workflow [139, 412]. Phetpeng *et al.* used consensus profiles from 195 pieces of IED evidence to obtain a single profile from all samples. They compared a number of sampling and processing methods, using Identifiler® Plus as the STR kit, and found that the most efficient method produced an average of 6.02 alleles per profile from their touch DNA post-detonation samples, with 9 returning full profiles [139]. Another study, performed by Tasker *et al.* used an epithelial cell suspension, containing approximately 800 cells per sample [412]. They obtained less than 13 alleles (30%) per profile, using GlobalFiler®, for the majority of post-detonation samples [412]. They found the degradation of samples was mild to moderate from their Quantifiler® Trio data. As well as STR amplification SNP analyses, by MPS, and insertion/null markers (INNUL) amplifications were performed. Tasker *et al.* found that INNUL amplification produced the most complete genetic profiles when compared to their MPS and STR amplifications [412]. Data from INNUL may provide some intelligence information, however there is no STR database equivalent for these markers.

The standard analysis in forensic DNA testing starts with an ex- traction process; this typically uses an automated liquid handling system. The end point is quantifiable DNA in a purified form. Performing an extraction reduces the inhibitors present within a sample, however, significant DNA template loss also occurs [2, 3, 201, 259]. An alternative is to use direct PCR as this eliminates the extraction process and helps retain all DNA template for amplification [136, 259, 260, 262, 263, 265, 292, 293, 295, 347]. It has been shown that direct PCR often improves genetic material recovery on touch or trace DNA samples [143, 193, 264, 267, 289, 315]. Although increased template is available for PCR, inhibitors are also present in the amplified sample. A trade-off between inhibitor presence, in direct PCR workflow, and potential DNA template loss, through extraction, must be considered. This is especially true for samples that may contain large quantities of inhibitors, such as post-detonation IED exhibits. Direct PCR has been used on samples that may contain large amounts of possible inhibitors,

such as blood [257, 284, 285], however direct amplification of post-detonation touched IED samples has not been performed to observe inhibition within these relevant sample types.

A relatively new concept for touch DNA analysis is the application of DD to a sample for the visualisation of cellular material [162, 163, 353, 413]. A recent report used DD and SYBR® Green on touched IED mock exhibits that had not undergone detonation. High and low partial DNA profiles were obtained after this DD staining using direct PCR amplification [142] and an average of 12.6 alleles (out of a possible 32) were obtained after visualisation with SYBR® Green [141]. In that article there was a suggestion that DD may inhibit PCR, therefore the use of SYBR® green was advised [141]; this has not been experienced in our laboratory, therefore DD was used in this study. Although previous studies have visualised cellular material on samples that have not undergone detonation, they support the potential for latent DNA detection using fluorescence prior to sample collection on post-detonation samples.

We report that cellular material deposited by touch could be visualised after it had been exposed to a controlled explosion and STR data could be obtained effectively from it. The visualisation of deposits has the potential to greatly aid the triaging of samples for inclusion or exclusion for downstream processing. In this work, touch DNA was stained before detonation and the location of cells recorded. After detonation it was possible to observe how much of the initial cellular deposit remained. STR results from both direct PCR and post-extraction PCR workflows are compared.

6.3 Methods

Many studies have shown that the surface type of exhibits analysed plays a role in the amount of genetic data obtained [139, 142, 289, 314]. As well as the exhibit type, the collection and processing method used plays a significant role in the quality of resultant STR profiles obtained from touch DNA samples [139, 142, 249, 264, 267, 289]. Because of this, an amalgamation of the most efficient sampling and processing methods has been per- formed on a range of sample types typical of those found within IEDs.

Ethics

Prior approval was obtained from the SBREC at Flinders University (reference 8109).

Exhibits and volunteers

Five substrates were chosen to emulate potential real-life exhibits used in the construction of an IED comprising: twin-core insulated wire; a peeled battery casing mounted on aluminium sheeting;

aluminium sheeting; electrical tape (non-adhesive surface); and thick polypropylene plastic sheet. Henceforth the sheath of the twin-core wire will be referred to as “wire” and the polypropylene plastic sheet will be referred to as “plastic". A triangular substrate unit was constructed, consisting of these five substrates (Figure 6.1). All items were cleaned with 3% sodium hypochlorite bleach, wiped, sprayed with nuclease- free water, wiped, and allowed to air dry in an isolated clean room. Each sample was then irradiated with UV for 15 min to ensure no amplifiable DNA was present on the items prior to the deposition of DNA by the volunteers.

Figure 6.1. Five sample units, comprised of twin-core insulated wire, a peeled battery mounted on aluminium sheeting, aluminium sheeting, electrical tape, and polypropylene plastic. Each sample within the sample unit contains reference points for DD visualisation and sample ID information.

Two volunteers (designated PRI 01 and PRI 02) each touched every substrate unit as described below (Section 8.3.2.3) in six replicates giving a total of 80 samples; two units from each volunteer were not exposed to a detonation in order to provide a set of touch controls. Other positive controls of each substrate unit were created in quadruplet using saliva from one volunteer; two were exposed

to detonation and the others were not. Negative controls of each substrate were performed in quadruplicate; two were exposed to detonation and the others were not. The six touched replicates that were exposed to detonation were separated into two groups for a total of three replicates per volunteer per group, these groups being: direct PCR STR, and standard extraction to STR PCR methodologies. One replicate of each negative control substrate and each positive control substrate, from each volunteer, were separated into the previous categories. One saliva positive control unit, one touched control unit per volunteer, and one negative control unit were separated into the previous categories.

The shedder status of both volunteers had been previously determined following the method of Kanokwongnuwut *et al.* [163]. Both volunteers were found to be intermediate shedders, representational of the largest portion of the previously sampled population [155, 163].

Deposition of DNA

The two participants were asked to touch each sample for a maximum of 15 s after an undefined time post-handwashing during which time regular activities, such as office work, were performed. This is to facilitate DNA deposition typical of that encountered in casework. The volunteers were asked to wait 15 min between touching each substrate to allow cellular build up on their hands. These times were chosen as previous studies have shown that after 15 min sufficient DNA is present on an individual's hands to allow its successful profiling [149, 150, 264].

Detection of latent DNA

DD (Promega, Madison, WI, USA) 10,000x stock solution was diluted with 0.01% Triton™ X-100 (Sigma, Victoria, Australia) to a working solution of 20x [161]. Onto each substrate, 5 µL of the 20x DD solution was placed and spread over the surface. Previous studies have shown that DD does not inhibit PCR amplification when it is applied in this quantity. The DD solution was allowed to air-dry before it was visualised with a Dino-Lite EDGE AM4115T-GFBW digital fluorescence microscope (AnMo Electronics Corporation, New Taipei City, Taiwan) with a 480 nm LED light source and a 510 nm emission filter. Images of cellular material were taken at 50x and 220x magnifications at each of the three locations.

Cellular material from three 220× images from each location were scored (from level 0–4) visually using the exemplars given in Figure 6.2. These cell scores were averaged across the nine 220x images per sample to establish the average distribution of cells across the item prior to detonation. The same process was carried out post-detonation, using the same locations where cells were

detected pre-detonation to ensure comparable images pre- and post-detonation. Where fragmentation of samples occurred, and positions could not be matched fully to pre-detonation images, the sample contained less than the previous nine images and the average cell score was adjusted accordingly. All small fragments (~ 25 mm^2 to 200 mm^2) were visualised and sorted into a group containing fragments that contained cellular material or a group containing fragments where no cellular material was detected. Fragments containing cellular material were compared to pre-detonation images and designated to their sample code appropriately. Fragments that could not be identified by comparison to pre-detonation images were labelled as 'unknown'.

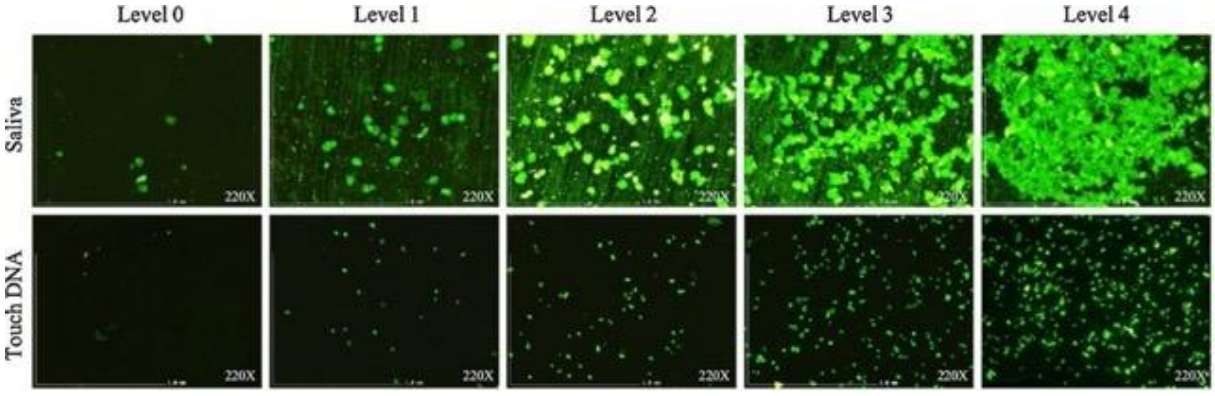


Figure 6.2. Visual cell scoring guide with saliva DNA samples and touch DNA samples of each designation.

Exposure of samples to detonation

Samples were exposed to detonations of explosive charges conducted in a blast chamber. The substrate units were used in six explosive events with 180 g of PE4 plastic explosive as an approximately spherical charge, suspended approximately 1.5 m above the floor and initiated with an electric bridge wire detonator. Samples were suspended at different distances from the charge, between 100 mm and 1,250 mm, measured from the surface of the charge to the centre of the closest face of the sample unit; due to the dimensions of the blast chamber, separation of sample from explosive could not exceed 1,250 mm. After detonation occurred, samples that remained intact or fragments that could be identified as specific samples were placed in their designated transport box. Other fragments were individually packaged for transport and future designation by DD visualisation and comparison.

Collection of DNA from samples

Each sample was double-swabbed using a nylon ultra-fine micro-applicator (City Dental, SA, Australia), with the exception of small fragments where one swab was used. Each swab head was moistened with 2 µL of 0.1% Triton™ X-100 (Sigma). The swab heads were removed, using a sterile scalpel blade, into either a 0.2 mL thin-walled PCR tube (Eppendorf, NSW, Australia) for direct amplification or an automated liquid handling platform (LHP) tube for extraction.

DNA extraction and quantification

DNA extractions were performed using the DNA IQ™ System (Promega, Madison, Wisconsin) on an automated LHP with a final ex- traction volume of 60 µL. The DNA in all samples was quantified after the DNA extraction process using the Quantifiler Trio™ DNA quantification kit (ThermoFisher Scientific, Waltham, Massachusetts).

STR DNA amplification

Direct PCR was performed on each sample using the VeriFiler™ Plus kit (ThermoFisher Scientific). Amplifications were performed in 25 µL following the manufacturer's protocol, with exception of 2 µL of Prep-n-Go™ (ThermoFisher Scientific) and Low TE Buffer (ThermoFisher Scientific) replacing water. All amplifications were performed on a ProFlex™ thermal-cycler (ThermoFisher Scientific). PCR product (1 µL) was added to 9.6 µL Hi Di formamide and 0.4 µL 600 LIZ® (ThermoFisher Scientific) and separated on a 3500 Genetic Analyser (ThermoFisher Scientific).

All extracted samples that returned a quantification value with the addition of ten that returned no quantification data, selected on the basis of largest number of cells recorded pre-extraction, were processed for STR typing. Amplification of extracted DNA samples were per- formed as above with 15.5 µL of the extract added to the PCR. Due to the 2 µL Prep-n-Go™, the maximum volume of extract able to be added to the PCR was 15.5 µL for a final PCR volume of 25 µL.

Data analysis

Data were analysed using GeneMapper® ID-X (version 1.4). The number of donor and non-donor alleles were counted and the average RFU per donor and non-donor peak was calculated across each dye lane. The quality of the profiles, with respect to peak morphology, peak balance and artefact incidence were observed. Peaks were recorded if they were of 50 RFU or above. Peaks were considered for homozygosity if they were of 150 RFU or above. Profiles containing ≥12 autosomal alleles were considered informative (up-loadable to a DNA database). A regression analysis was

performed analysing the effect of surface type on the number of peaks, taking into account the distance from the charge by considering the 'cells after' scores.

Chemical analysis of substrates

IR spectrometry was performed on the electrical tape, twin-core insulated wire, and plastic substrate using a Nexus 870 spectrometer and a Nicolet ATR accessory equipped with a single-pass diamond crystal (ThermoFisher Scientific). After analysis, each item was soaked in dichloromethane in order to extract plasticisers and the treated plastics were re-analysed using IR spectrometry. The dichloromethane extracts were analysed using an Agilent 7890 gas chromatograph equipped with a 5975 triple-axis mass spectral detector. An HP5-MS column (Agilent Technologies, VIC, Australia) with dimensions 30 m x 250 µm × 0.25 µm was used for gas chromatographic (GC) separation and mass spectrometry (MS) was carried out in electron ionisation mode at 70 eV (scan range 40 m/z–450 m/z). GC analysis was con- ducted in split mode (1:10) with an injection port temperature of 300°C. Flow was held constant at 1 mL/min with He carrier gas. The temperature program was 100−300 °C at a heating rate of 30 °C per min and then the oven was held at 300 °C for 5 min. Accurate mass MS analysis of the dichloromethane extract was performed on an AxION 2 Time of Flight Mass Spectrometer Perkin Elmer, VIC, Australia equipped with a Direct Sample Accessory Perkin Elmer operating at 350°C with a nitrogen gas pressure of 90 psi a resolution of approximately 8000 at m/z 622.0290. The mass spectrometer scanned across a range of 100−1000 m/z in positive ion mode, with a spectral acquisition rate of 1 spectrum/second. Calibrant masses of 121.05087, 322.0500, 622.0300 and 922.0100 m/z were obtained using an APCI tuning mix (Agilent Technologies). Analysis was carried out by placing a drop of the diluted dichloromethane extract (5 µL) on Perkin Elmer stainless steel sample mesh.

6.4 Results and Discussion

Diamond Dye use for triaging

Using DD to stain and visualise cellular material, items can very quickly be screened to determine whether cellular material is present and, if it is, whether subsequent efforts to recover it using swabs has been successful. In the investigation of a deployed IED, significant fragmentation of the device may take place and thus it may be necessary to examine a large number of fragments. As only a few of which may have an exploitable deposit of cellular material present, visualisation of cellular material can help reduce the labour involved in processing items and reduce the number of

items subjected to downstream DNA processing that do not have useable deposits of cellular material present, thus saving analysis costs.

In the research described here, substrates were stained with DD prior to detonation rather than post-detonation (as would be carried out in normal investigations) in the expectation that the impact of a detonation upon cellular deposits could be evaluated. Data generated from this method showed that DD-cell conjugates are completely stable under extreme physical pressure and high temperatures, as experienced during a detonation.

Cell scoring data comparison pre- and post-detonation

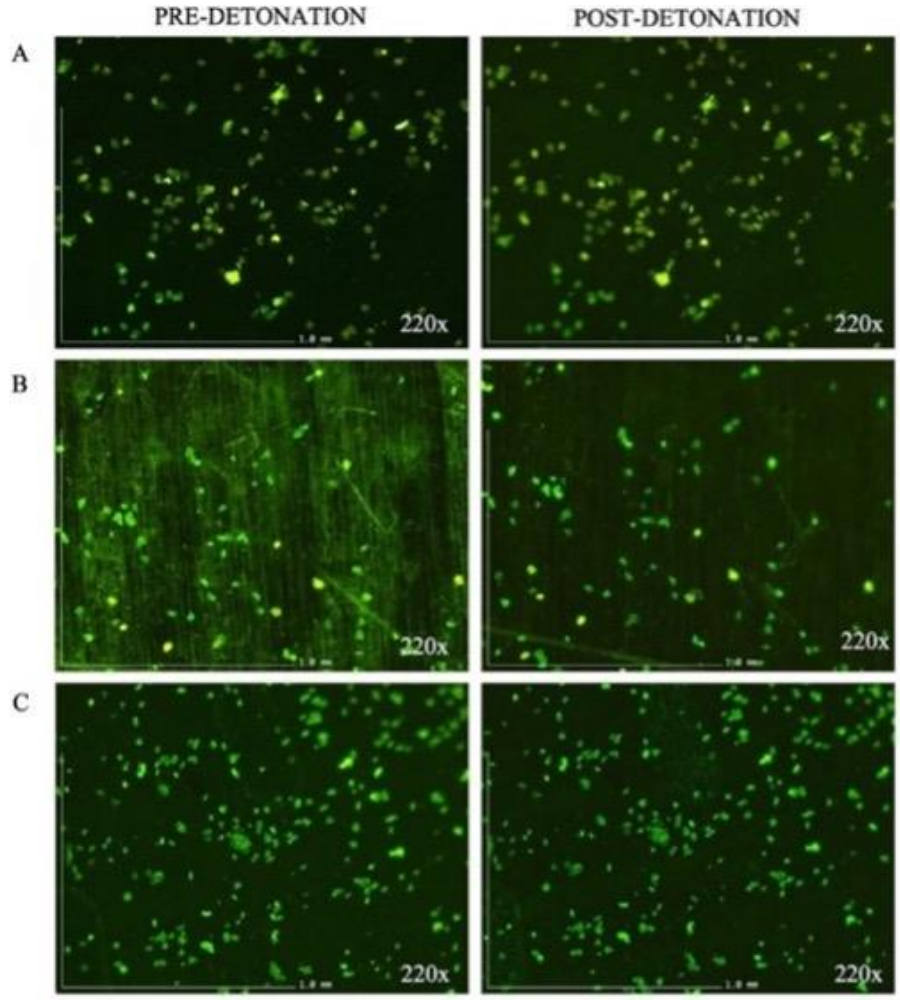


Figure 6.3. Example of pre-detonation (left) and post-detonation (right) images matched at the same location on a battery (A), aluminium (B), and electrical tape (C). Images taken at 220x magnification.

The process of depositing touch DNA onto IED components, staining with DD to record the location and number of cells on each substrate, exposure of these substrates to an explosive detonation, re-visualisation of substrates to record cell retention, and subjection each substrate to either direct PCR or PCR post-extraction for STR profile analyses allows realistic conclusions to be drawn on the effects the detonation process has on substrates containing touch DNA. As exactly the same location could be identified pre- and post-detonation on each collected substrate, providing total cell loss had not occurred, the number of cells lost due to the detonation process could be determined. An example of the comparison of pre- and post- detonation substrate images is shown in Figure 6.3.

There was no significant difference in the average number of alleles per profile observed between the two DNA donors ($p > 0.5$). This was expected as both were intermediate shedders, therefore it would be expected that they would deposit a similar amount of cellular material onto each item. When test substrates were further than 250 mm from the explosion, cell loss was insignificant. At 250 mm from the charge cell loss was noted, with cell loss being considered significant at 100 mm ($p= 0.00014$) (Figure 6.4). Note that with distance the cell scoring increased in an approximately exponential fashion (e.g. a score of 1 was an increase by a factor of approximately Euler's number from a score of 0) and the proportion of cellular material remaining for each sample was calculated as the exponent of the post-detonation cell score minus the pre-detonation cell score. Morphological changes to cellular material were also noted (Figure 6.6). At ≤ 100 mm from the charge the substrate samples themselves experienced severe physical damage or were fragmented to the extent that they could not be recovered. It can be concluded with confidence that cell movement and loss does not occur readily from samples ≥ 250 mm from the charge of an IED.

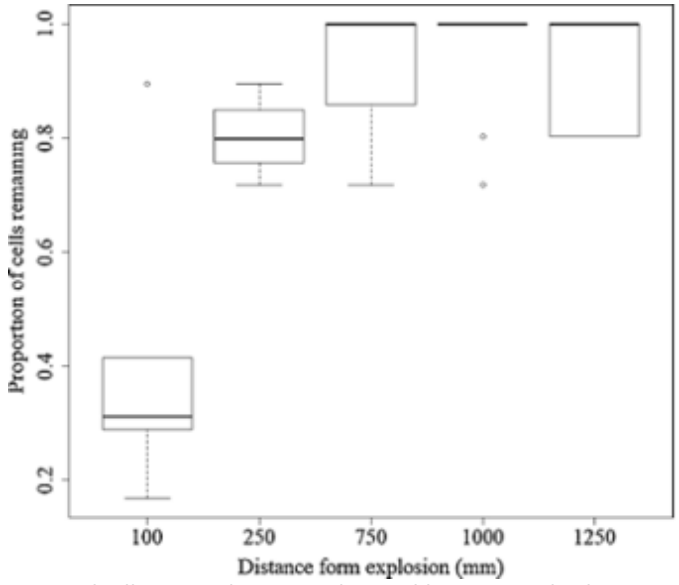


Figure 6.4. Proportion of the original cells retained on a sample post-blast against the distance each sample was positioned from the charge. Units placed right next to the charge (within 100 mm) retained relatively few cells. This is in contrast to the units placed ≥ 250 mm from the charge where over 70% of cells remained in their original location.

STR profiling data – direct PCR against PCR post-extraction

There was an expectation that DNA deposition would differ between substrates as a myriad of variables play a role in DNA deposition. To account for this variation in the analyses of resultant DNA profiles, with respect to the number of peaks observed and their RFU values, the cellular material deposited by the donors was visualised and scored. The method used is representational of the touch deposition expected in the construction of an IED. Substrate units were triangular in shape, therefore when suspended and detonated not all faces were exposed to identical conditions. As the trends we observed with respect to the role distance plays on cell loss, subsequently discussed, were seen over larger distances and each sample surface was within a 25 mm radius of the measured surface it was concluded any difference experienced due to direct/indirect exposure to the charge would be minute within each substrate unit when compared between other substrate units at different distances.

Direct PCR

All samples that were not exposed to a detonation, baseline samples, produced informative profiles (≥12 autosomal alleles); average cell scores per baseline sample were equivalent to pre-detonation samples ranging 0.89-2.44. There was a reduction in the number of donor STR alleles observed post-detonation on all substrate types compared with samples that had not been exposed to a detonation. As very little cell loss was observed, an average score loss of 0.49 was observed, this

reduction in amplified STR alleles is most likely due to inhibitory or DNA degradation effects caused by the detonation process. These effects are not sufficiently severe to result in an inability to produce STR profiles as 49% of the samples analysed resulted in informative STR profiles (76% produced amplified alleles), however the inhibition/ degradation post-detonation was significant (p= 0.0034) between the wire and plastic samples when compared with the other substrate types (Figure 6.5). As samples that were touched and stained but not exposed to a detonation returned STR profiles for wire and plastic positive control samples, inhibition from the substrate due to an effect of the detonation is likely.

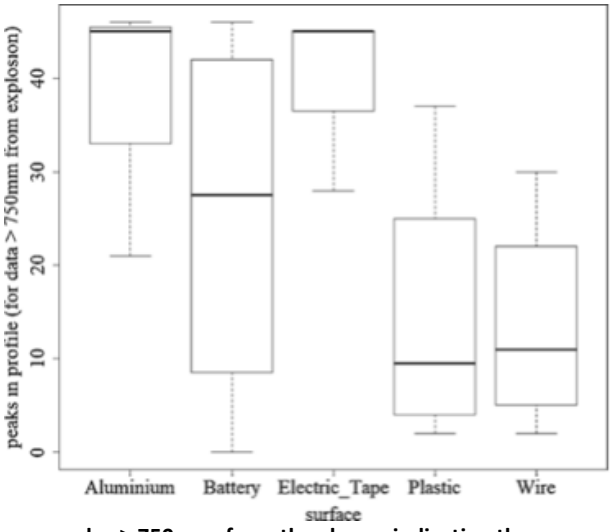


Figure 6.5. Boxplot of data from samples ≥ 750 mm from the charge indicating the average number of alleles per profile obtained from each surface type.

Partial profiles, containing between 1–29 autosomal alleles, from samples as close as 100 mm to the charge were able to be obtained with a full DNA profile obtained from an electrical tape fragment 250 mm from the charge Supp. Figure 6.1 and Supp. Figure 6.2). The STR data indicate that with a direct PCR workflow it is possible to produce STR profiles from post- detonation samples that had been touched for as little as 15 s pre-detonation.

There was a general relationship between the amount of cellular material visualised and the resultant STR profile when no substrate-specific inhibition occurred and considering distance from the charge (Supp. Figure 6.3). Because of this, staining with DD and visually analysing the samples can give an indication of the potential STR profile able to be obtained post-amplification. The relationship observed between the cell score and resultant STR profile was not completely described by a linear

relationship (R^2 = 0.65). This may be due to a number of factors. The quantity of DNA within each visualised cell is likely different due to the DNA within touch deposits, which could contain keratinocytes and/ or corneocytes in different stages of degradation. The staining does not indicate the quality of DNA, merely its presence. A low cell score with higher quality DNA compared with a high cell score with heavily de- graded DNA could therefore give similar STR profiles, in terms of alleles amplified. Additionally, the presence of any inhibitors due to the detonation may not have been uniform across all samples, especially considering those closer to the charge against those further from the charge.

Of the 37 touch DNA post-detonation samples and fragments analysed, 18 returned profiles considered informative, 10 returned profiles with 1–11 autosomal alleles, and the remaining 9 generated no alleles. Due to the quality of the buffer and the efficiency of the enzyme within VeriFiler® Plus, samples with very low cell scores were still amplified successfully with many alleles present, therefore most samples with a cell score above 1, with the exception of wire and plastic samples, resulted in informative DNA profiles (83%).

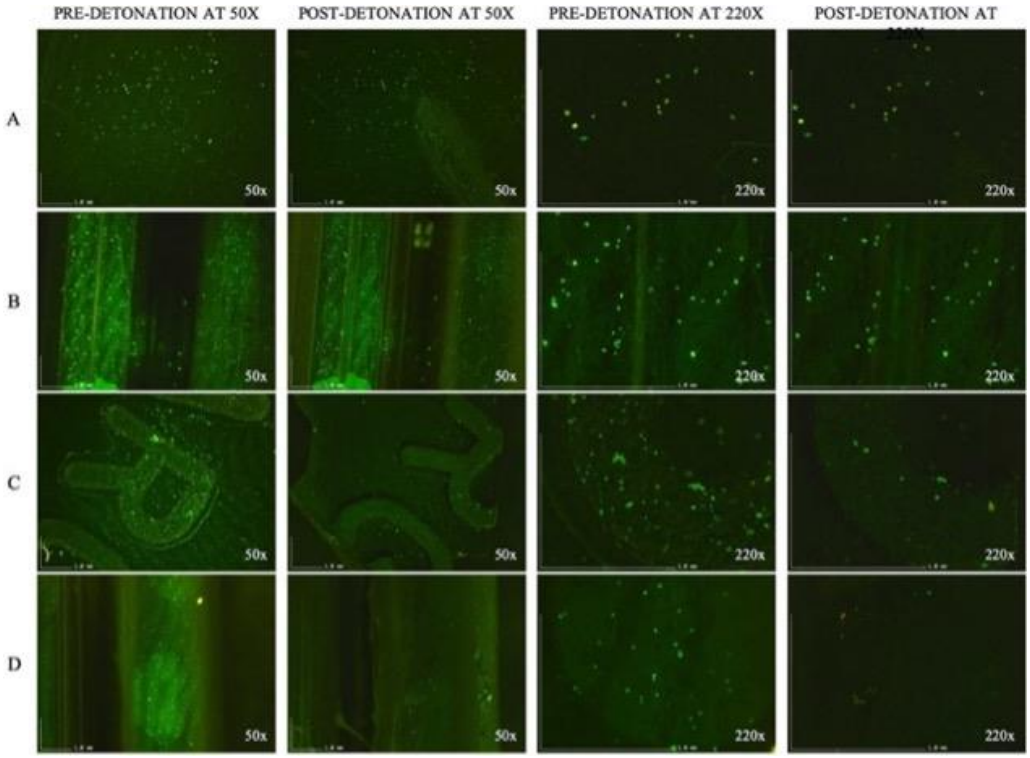


Figure 6.6. Comparison of pre- and post-detonation from plastic and twin wire samples from 750 mm and 100 mm from the charge; plastic 750 mm (A), wire 750 mm (B), plastic 100 mm (C) and wire 100 mm (D). The images show at 50× and 220× magnifications.

PCR post-extraction

The successful generation of DNA profiles by direct PCR is in contrast to the substrates analysed by post-extraction PCR. Of the 27 detonated touch DNA samples only three returned quantification values. Of the 11 detonated touch DNA samples that underwent PCR post-ex- traction only one returned an informative profile, one presented 7 autosomal alleles and all others failed. The only sample to return an in- formative profile post-extraction was from a wire sample and presented more alleles than any wire sample amplified with direct PCR. This indicates that when processing wire, and likely other plastic samples, an extraction pre-PCR could offer a better chance for obtaining DNA data, however as only one post-detonation post-extraction wire sample was processed this is not conclusive. All other sample types should be analysed by a direct PCR workflow as results show strong support for the conclusion that the application of this method to post-detonation samples gives the highest chance for genetic data yield.

Substrate specific effects

After an IED detonation event, many evidence recovery operatives in hostile environments are restricted in regard to the time that they can spend in the blast scene collecting samples (personal communications, DSTG). Therefore, information as to the types of samples most likely to return informative STR profiles can provide valuable guidance. Previous research suggests that the type of substrate analysed plays a role in the resultant profiles [1,5,7,23] and our STR data generated post-detonation supports this.

There was no significant difference between the cell loss observed and the surface type ($p > 0.18$). However, this does not correlate with expected profiling success as STR profiles observed for the wires and plastic samples contained far fewer alleles per profile than the other sample types tested. A boxplot comparing the number of peaks detected and the surface type was created. To negate the confounding effect that distance from the charge has, the boxplot considers data with the minimum distance of 750 mm (Figure 6.5).

Both the plastic and wire surface types returned results indicating the reduction in the number of alleles present per profile from these sample types was significant ($p= 0.03302$ and $p=0.00411$ respectively). This indicates surface-specific inhibition or surface-specific DNA de- gradation. As the plastic and wire samples that did not undergo detonation produced STR profiles comparable to the other substrates, the inhibition or degradation observed within the STR profiles of post-detonation samples is due to these substrates being in proximity to a detonation.

Chemical analysis of the wire, plastic and electrical tape was carried out. Using IR spectrometry, both the electric tape and twin core insulated wire were identified as plasticised PVC while the plastic substrate was identified as plasticised polypropylene. GC–MS and accurate mass MS indicated that the plasticiser in the electric tape and plastic substrate was dioctyl phthalate while accurate mass MS indicated that the twin-core insulated wire contained trioctyl trimellitate. It is not clear why the detonation caused the wire and plastic but not the tape to become inhibitory to PCR. The phenomenon is not linked to the type of plasticiser, as the plastic and the tape have the same plasticiser, and it is not linked to the type of polymer in the substrate, as the tape and the wire are both made from PVC. It may be that the detonation event is causing migration of the plasticiser out of the wire but not the electrical tape, thus presenting the opportunity for plasticiser to contaminate the cellular material collected from the wire and to inhibit PCR. It may also be that the PVC in the wire suffers degradation and releases a product, such as hydrogen chloride gas, that interferes with subsequent PCR, although the reason why PVC tape and PVC wire behave differently is not clear. As plasticiser is widely used in many common plastic objects that may be used in IEDs, further investigation of this phenomenon is continuing in our laboratory.

Limitations

The explosive behaviour of the charge during experiments was observed using high speed video photography. Even though steps were taken to ensure that the charges had the same spherical shape and mass, some variation in the development of the detonation's fireball was noted between experiments. Additionally, as the detonation was con- ducted in a blast chamber, real-world inhibitors to the DD or PCR steps of the workflow were not present. Additional research in alternate environments would therefore allow for more rigorous and appropriate testing of the proposed workflow.

Due to the difference in fragment sizes obtained post-detonation, the surface area swabbed inevitably differed with each sample. The variation in fragment size of samples the same distance from the charge due to detonation has been previously observed [8]. The surface area visualised was also not the only areas swabbed on a substrate; larger substrates were swabbed in areas not visualised to maximise template collection. Although this is representational of casework workflow it presents challenges with data analyses in terms of comparison between samples. Samples with localised high cellular material concentration and those with lower cellular material concentration across the entire exhibit surface will score differently with the applied method but may contain the same number of

cells across the sampled area as a sample with the inverse cellular distribution; however, areas representative of the surface coverage with respect to cellular deposition were targeted for the cell scores where possible. It is more likely that variable spread, and variable swabbing areas would confound the data.

6.5 Conclusion

These data represent the first instance of successful DNA profile generation from post-detonation samples using direct PCR. Samples as close as 250 mm from the charge generated a full DNA profile, with substantially less profiling success achieved with cellular material located 100 mm from the charge. Therefore, there is a real chance for successful genetic data generation from forensic exhibits collected post- detonation.

This study shows the contribution that DD staining and visualisation can aid triaging in post-detonation examination casework. Application of the dye and subsequent visualisation is a rapid process and is per- formed in ambient light. The ability to produce genetic data in the form of an STR profile from post-detonated samples has been successfully shown and the implications and potential benefits are obvious.

The success of obtaining genetic information from these samples post-detonation gives confidence that a wider study of IED-relevant exhibits could be successful and is therefore warranted. As substrate- specific inhibition was observed, the analysis of a larger range of exhibit types post-detonation will allow for increased data as to the types of exhibits that are the best to prioritise for collection if time pressures are imposed. In addition, if such an expanded study of substrates indicates that particular substrates are prone to causing inhibition of PCR the information will aid decision-making as to which samples collected should be processed using direct PCR and which should be subjected to extraction and clean-up.

A specific study into the effect that distance from the charge has on the cellular material, in terms of loss and morphological changes and resultant viability of STR profiling, will also present data that will in- form triaging teams. Research as to the number of touch DNA cells required for successful STR profiling is also important for informing sample triage teams if DD staining is incorporated into workflow.

6.6 Conflict of Interest

None declared.

6.7 Funding and Acknowledgements

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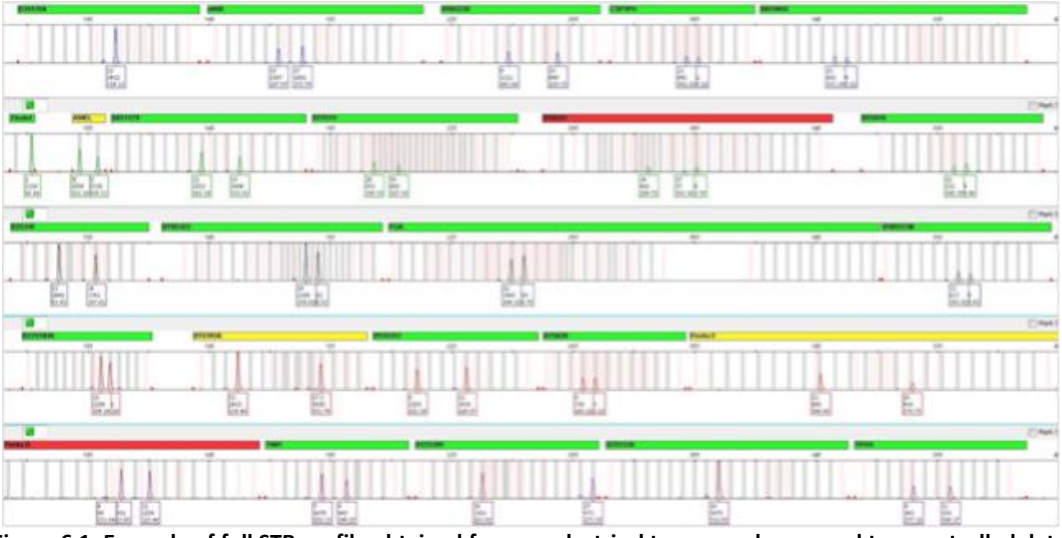
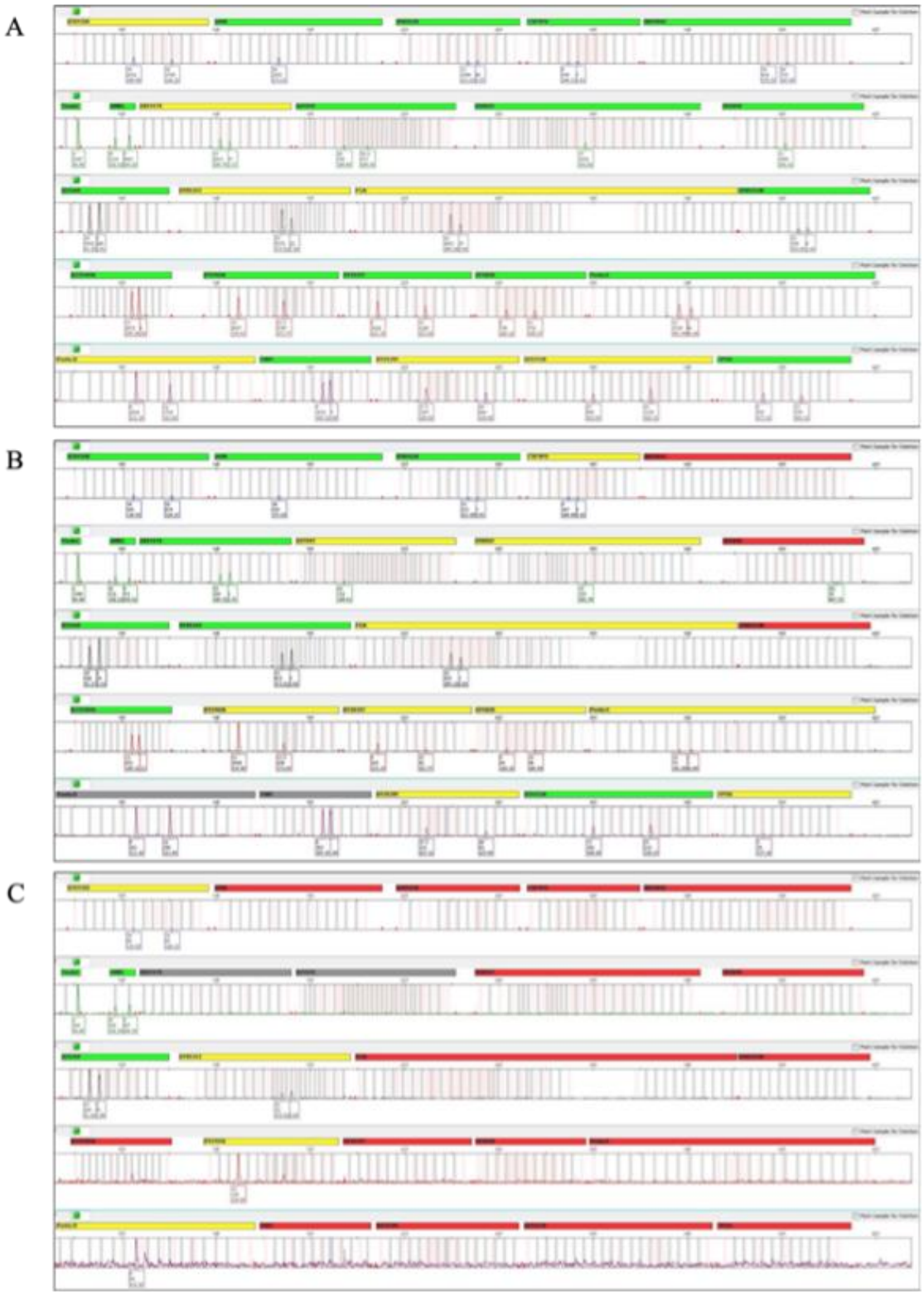
We would like to acknowledge Jessica Champion and Jenifer Young for their contribution to this work. Additionally, thanks go to our DNA donor volunteers and the team at DSTG.

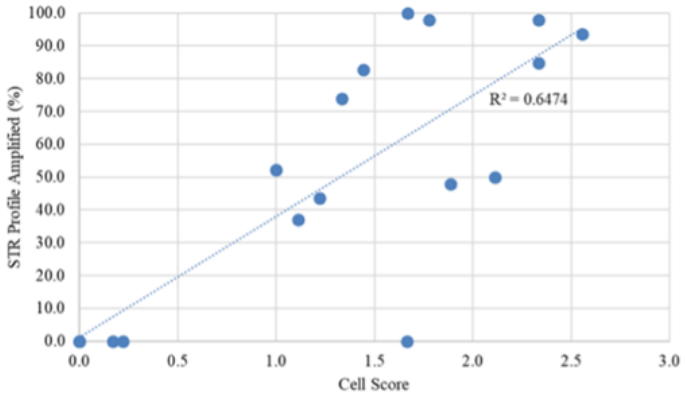
6.8 Supplementary Material



Supp. Figure 6.1. Example of full STR profile obtained from an electrical tape sample exposed to a controlled detonation of plastic explosive. The sample was 250 mm from the charge.



Supp. Figure 6.2. Example of three STR profiles obtained from post-detonation samples; (A) an aluminium sample 1000 mm from the charge with a cell score of 1.78 , (B) a mounted battery sample 750 mm from the charge with a cell score of 1.44, and (C) an insulated wire sample 750 mm from the charge with a cell score of 1.78.



Supp. Figure 6.3. Average cell score per sample against the resultant STR amplification success of post-detonation samples. Items were a piece of aluminium, a mounted battery casting, and electrical tape. Samples were 100 mm-1000 mm from a charge of plastic explosive.

6.4 OUTDOORS: POST-DETONATION SAMPLING USING DIRECT PCR

6.4 Manuscript: The influences of dusty environments on the STR typing success of post-detonation touch DNA samples

Manuscript published: Forensic Science International: Genetics, 2022

Statement of Authorship

	Conception/ Experimental Design	Laboratory work/ data Acquisition	Data Analysis/ Interpretation	Manuscript Preparation	Manuscript Editing	Signature	Date
BM	**80**	**60**	**90**	**95**	**70**		03.08.21
TK	-	40	10	5	10		05.08.21
KPK	10	-	-	-	10		03.08.21
AL	10	-	-	-	10		05.08.21

6.4.1 Abstract

As the use of improvised explosive devices (IEDs) in a broad spectrum of offences continues, it is vital that research is performed to assess the capabilities of the forensic DNA profiling technology currently available to provide information as to potential perpetrators. This work investigates some of the most important gaps in our understanding surrounding the poor success rates in DNA profiling obtained through the sampling of touch DNA on post-detonation IED samples. It has been previously suggested that the use of Diamond™ Nucleic Acid Dye may fix cells to a surface, therefore reducing the effect of an experimental process to remove or damage those cells. This was found not to be the case for samples undergoing a detonation as there was no difference in the resultant post-detonation profiles between the stained samples, stained prior to detonation, and unstained samples. The comparison of data from previously performed research, within an enclosed explosives chamber, to real-world outdoor detonation events in a rural and dusty environment was investigated. It was found that there was a significant difference between the environments for the aluminium but not for the battery or electrical tape substrates indicating that environment has the potential to influence STR

success through the introduction of PCR inhibitors; humic acid within rural natural dust was introduced here. No difference was observed in cell loss due to the detonation between environments and the dirt within the PCR was higher in the 'outdoor' samples. The effect on cellular retention and damage due to the sample's distance from the charge has been thoroughly investigated through incremental 100 mm exposure. Distance from the charge was found to affect every metric analysed; these being the cell loss from samples, the number of alleles amplified in resultant direct PCR profiles, and the total RFU of the subsequent profiles. These data outline the importance of this work allowing results to be assessed and triage decisions be made accordingly.

The analysis of wood, PVC pipe, a mobile phone with rubber buttons, a SIM card, and a circuit board showed that none of these samples at 400 mm from the charge caused substrate specific PCR inhibition. On-site collection teams do not need to triage collection based on these sample types as there was no significant difference observed in their ability to return DNA profiling data. Surface area and inhibitor presence are key variables to consider when determining STR processing workflow for post-detonation samples as for samples with larger surface areas within the outdoor environment PCR post-extraction is preferential to direct PCR.

6.4.2 Introduction

IEDs are used throughout the world in different capacities to perform acts of terrorism and other crimes [414, 415]. Depending on the circumstances under which these devices are found, or if they are detonated, the quantity and quality of the information produced post-genetic analysis can differ greatly. The importance of this difference depends on the application of the evidence; the requirements surrounding genetic data requirements for defence technical intelligence or prosecution purposes are different. Although methods for STR typing are extremely sensitive [330] and success has been gained in the sphere of touch DNA in recent years [199, 204, 264, 289, 315], STR data generated from IED items is extremely limited in the literature with reported results often leading to uninformative STR profiles. There is a large focus in the literature on fingermark recovery from IED components post-detonation or post-neutralisation, with fingermark recovery being possible to varying degrees for both [140, 416-419].

Previous studies have explored direct PCR and PCR post-extraction using a range of DNA sources such as saliva [143], buffy coat [139], blood [415], and DNA deposited through touch [141, 313, 416]. The quality of the DNA source and workflow choice used in previous studies has an effect on the extent of successful STR profiles generated within each study. That research has largely

focussed on touch samples that have not undergone detonation [141, 420] or samples spiked with saliva or high quality and quantity cellular material samples that had been detonated [314, 416]; the research on touch DNA post-detonation is limited [139, 140, 313, 421]. Touch DNA is the most realistic source to be encountered on IEDs and their components as construction, placement, and deployment would usually only involve handling [112, 313]. As a consequence, amplification successes reported in the literature may not be directly applicable to operational workflows where exploded or unexploded IED items are involved.

Recently, Al-Snan (2021) describes the collection, extraction, and GlobalFiler™ amplification of seized IEDs, made inert by specialists prior to testing [420]. Tapelifts, swabs, or direct cuttings of one sample from five cases was performed with C4, a plastic explosive charge, as the charge of choice; they had not been detonated. Extracts were vacuum concentrated to increase the concentration of the DNA template available for PCR. Of the five samples analysed, full DNA profiles were observed with four of them returning 2-3 person mixtures. Tonkrongjun *et al.* (2019) analysed mock IEDs, each comprising eight substrates, with five following standard PCR post-extraction protocols and five by direct PCR [141]. These were handled by volunteers for 'assembly', time not specified, and not detonated. They found that the direct PCR approach was preferable to standard practice, with 32% of their profiles being considered up-loadable to CODIS, and that substrate played a role in profiling success.

Post-detonation touch DNA template research is sparse, as previously stated. Vanderheyden *et al.* investigated the potential to detect identifiable fingermarks and amplify the associated touch DNA template following IED neutralisation or detonation [140]. They utilised an in-house multiplex STR kit of only nine loci and report that eight of 43 samples produced allelic data with five returning full profiles following the detonation of 7g C4; the charge carrier was also filled with 0.5 L of sand to absorb some of the energy from the detonation. It was found that the amount of DNA present on each item was not significantly different from one another post-detonation; the items were a metal can, suitcase, electrical tape, mobile phone, battery, detonator, push button, and circuit board. Although STR profiles were generated, this charge size is unrealistic and limits translation of results to expected outcomes in casework.

Hoffmann *et al.* analysed eight backpacks post-detonation using AmpFLSTR™ MiniFiler™ to amplify nine loci [421]. These backpacks were worn and handled by the DNA donor for 11 days prior to the detonation event. The production of consensus profiles, of all individual samples profiled per

backpack (7-10 samples depending on the backpack), resulted in seven of the eight backpack consensus profiles being complete and matching the DNA donor. Averaged individual sample success ranged from 5 loci to 9 loci (full) depending on the region of the backpack analysed. The charge for this work was 1.5 oz (42.5 g) of shotshell powder; this charge size is again on the low end of what may be expected in a real case, reducing the value of the results in terms of translating the methods used to real-world scenarios.

The aforementioned papers describe STR kits with limited targeted loci and small energetic material masses. When considering requirements for database searching, CODIS ≥ 8 loci [422] and NCIDD ≥12 alleles [423] for example, the limited locus representation within these kits may reduce the value of the profiles obtained when compared to being able to utilise STR kits with larger number of loci. Recently, a study utilising a larger energetic mass of 180g of plastic explosive (PE4), and an STR kit targeting 23 autosomal loci, VeriFiler™ Plus, was performed using touch DNA template in an enclosed explosive chamber [313]. In this study, five substrates (electrical tape, battery coating, aluminium, twin-core wire, and polypropylene plastic) were touched for a maximum of 15 s and built into an inert IED. Detonation ensued and collection and analysis of 37 recovered fragments resulted in 18 being considered informative (≥12 autosomal alleles) [70]. These samples were processed using direct PCR; PCR post extraction resulted in 1 of 11 profiles being considered informative. Although this provided valuable data as to the potential to obtain informative DNA profiles from these sample types, there were many questions surrounding genetic data yield following a detonation event that remained unanswered. One uncertainty of particular importance is that it is not currently known whether different results would be obtained from an outdoor detonation event. It is important for this translation to a more realistic environment to be performed; consideration as to the outdoor environment must also be made as an urban or rural setting is likely to affect the ability to obtain DNA profiles from subsequent IED fragments differently due to inhibitor presence/absence from environmental differences [269].

The relationship between cellular damage/migration due to increasing distance from the detonation's point of origin has not been thoroughly investigated. This is of importance as parts of the device may be offset from the charge depending on the design and purpose of the device, or if the device has been disarmed with a neutralisation technique [140, 416]. As the intensity of the pressure wave decreases quickly with distance, it is expected that the touched substrate's vicinity to the charge will directly impact the expectation of finding viable cellular template for downstream amplification

[411, 424]. Due to this, if a trend can be found that indicates the distance at which PCR viable cellular material can be collected reliably, triaging teams could be better equipped to make educated decisions about collection and sample processing. This trend may be dependent on surface type, due to their different capacities to inherently retain DNA, or the environment type in which the event occurs.

Due to touch DNA being of a poor quality [338] it is important to prioritise the collection of IED component with substrate types that will yield the most informative STR profiles downstream Effective post-detonation collection is important as the heat, shockwave, and a change in chemistry on the surface of substrates could all affect the success of downstream STR profiling. Previous research on DNA recovery from IEDs has suggested that direct PCR could provide an alternative workflow to PCR post-extraction to increase the workflow's sensitivity to touch DNA [141, 142]. This technique, however, may need to be restricted to a range of specific sample types post-detonation due to substrate specific inhibition to the PCR. Previous literature has found that certain substrates post-detonation, which include electrical wire and some plastics, cause inhibitory effects to the direct PCR workflow due to the release and subsequent collection of surface materials [313]. To guide evidence recovery teams as to what surface types should be prioritised for collection following an event, a larger variety of substrates than previously tested need to be investigated.

Cellular staining and visualisation for sample screening and template targeting is relatively new to forensic applications and allows the visualisation of deposited cellular material [163, 343]. Although its introduction into routine operational casework settings is currently questioned [343], its application to niche situations such as with the analysis of IED items has been proposed [141, 313]. Previous research has utilised a nucleic acid stain, such as SYBR® Green or DD, in experimental design to a) ensure cellular material was present initially and to monitor the loss due to detonation [313] or b) aid in targeted collection of latent DNA [141, 372]. In a), the consideration of DD's potential role in cellular fixation to a surface needs to be identified for multiple reasons. If DD does cause cellular fixation, this has the potential to reduce the impact of the results obtained by Martin *et al.*, 2020, as all samples were stained prior to detonation [313]. If there is no evidence that supports cellular fixation due to the application of DD, then this technique can be reliably incorporated into future experimental plans to accurately monitor cellular loss or migration due to extreme conditions, events, or stresses.

This body of work aims to further investigate the questions, and research extension suggestions, raised by Martin *et al.* [313] to provide a more in-depth understanding around the potential for DNA profiling from post-detonation IED samples for weapons technical intelligence or criminal prosecution purposes. Questions around DD fixation, how distance and additional substrates affect DNA data yield, and whether outdoor settings differ from a blast chamber environment are addressed.

6.4.3 Methods

Overarching methods

Ethics approval

Approval was obtained prior to sample collection from the SBREC (reference 8109) of Flinders University.

Exhibits and volunteers

To ensure no amplifiable DNA was present on the items, prior to DNA deposition by a volunteer, all items were irradiated with UV for 15 mins after being cleaned with 3% sodium hypochlorite bleach, followed by nuclease-free water, and finally being allowed to air dry in an isolated clean room.

The shedder status of each volunteer had been previously determined to be intermediate, representational of the largest portion of the previously sampled population [155, 163], following the method of Kanokwongnuwut *et al.* [163].

Deposition of DNA

Each substrate was handled in the same way for each trial, except when otherwise explicitly stated. The volunteers were asked to touch each sample for a maximum of 15 s after an undefined time post-handwashing. During this time regular activities, such as office work, were performed, with the instruction to not wear gloves or wash hands within 15 mins of deposition. The volunteers were asked to wait a minimum of 30 mins between touching each substrate as it has been previously shown that sufficient DNA to facilitate successful profiling is present on an individual's hand 15 mins post-handwashing [149, 150, 264]. This regime was used to allow DNA deposition to mirror the deposition expected to be encountered in casework.

Detection of Latent DNA

DD (Promega, Madison, WI, USA) 10,000x stock solution was diluted with 0.01% Triton X-100 (Sigma, Victoria, Australia) to a working solution of 20x [308]. Two DD deposition techniques were employed for different trials. For Trial 1 and Trial 2, 5 µL of the 20x DD solution was placed and spread over the surface of each substrate using a pipette. Previous studies have shown that DD does not inhibit PCR amplification when it is applied in this quantity [163]. For Trial 3, the DD solution was applied to the substrates with the spray system noted as SD16 in Young *et al.*, 2020, and utilised in Cook *et al.*, 2021, as the surface areas were too large to apply DD by a pipette [343, 425]. After drying, the substrates were visualised as described in Kanokwongnuwut *et al.* [163]. Images of cellular material were taken at 50x magnification and 220x magnification over the surface area of each sample prior to detonation. Post-detonation, these images were taken at the same magnifications with care to match the locations of each photo exactly to the pre-detonation images where possible. Following the capture of nine images, with cellular presence representational of the entire substrate, were selected at 220x magnification for cell scoring pre-and post- detonation. An example of the matched pre-and post-detonation images can be seen in Supp. Figure 6.4 and Supp. Figure 6.5.

Cellular material from nine 220x images from each location were scored (from level 0 to 4) visually following a previously utilised framework [313]. These cell scores were averaged across the nine 220x images per sample to establish the average distribution of cells across the sample prior to detonation. This was also performed post-detonation for the comparison of cell presence with pre-detonation images. Cell scores were adjusted post-detonation where fragmentation of samples occurred, as in Martin *et al.* [313], or positions visualised pre-detonation could not be matched fully with the post-detonation samples images. Fragments that could not be identified by comparison to pre-detonation images, but were stained with DD, were labelled as 'unknown'. Small (20 mm to 25 mm length or width) unlabelled fragments that contained no DD stain were not processed downstream.

Exposure of Samples to Detonation

Samples were exposed to detonations of explosive charges conducted in a specified controlled space outdoors. The substrate units were exposed to one of three explosive events with 160 g of PE4 plastic explosive as an approximately spherical charge placed on the surface of the ground (Figure 6.7). After detonation occurred, samples that remained intact or fragments that could be identified as

specific samples were placed in their designated transport box. Other fragments were individually packaged for transport and future designation by DD visualisation and comparison.

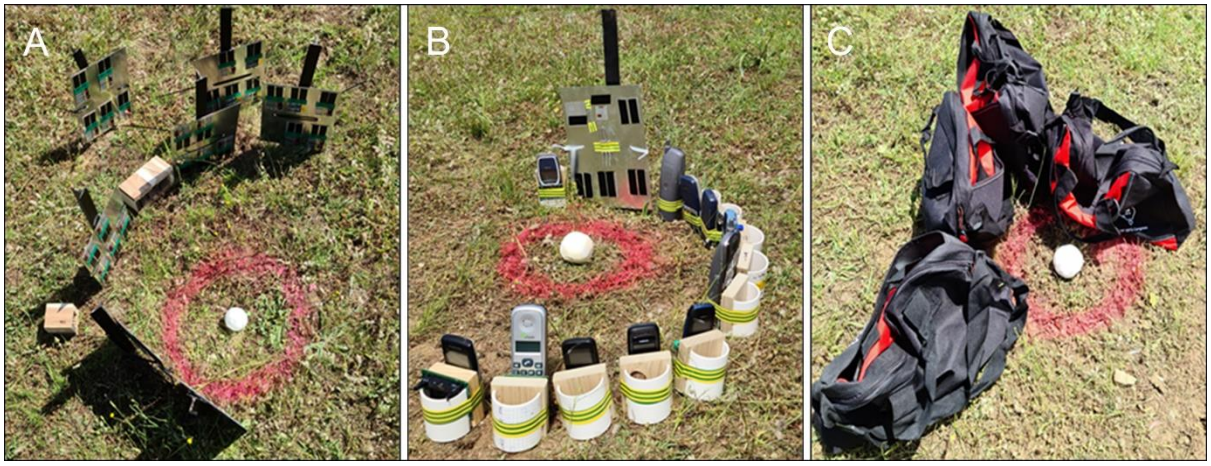


Figure 6.7. Detonation layout for each trial. Panel A is Trial 1 and 2 being the battery, aluminium and electrical tape samples slid into wooden holding units at 400 mm from the charge, and battery and electrical tape samples mounted on both sides of each witness plate placed 100-700 mm from the charge (in 100 mm increments). Panel B and panel C are Trial 3; panel B is the substrate extension units from Set 1 placed 400 mm from the charge and panel C is the backpack units from Set 2 placed immediately next to the charge.

Collection of DNA from Samples

Natural dry-soil dust was present on the surface of the substrates as a result of the outdoor rural environment in which the samples were detonated. The type of soil present within this environment was typical of a dry, hard, compacted clay and was disrupted due to the explosion. Due to this, prior to DNA collection, each sample was lightly blown in a laminar flow hood with the air compressor from the SD16 DD application system to remove excess dust. A preliminary study showed that the blowing of dusty samples allowed for direct PCR amplification where it failed in non-blown counterparts (data not shown). Collection of DNA from the samples was conducted as described in Martin *et al.* [313] for all direct PCR samples. For samples undergoing extraction, 20 µL of 0.1% Triton™ X-100 was added to the head of a nylon 4N6 flocked swab (Copan, Interpath, VIC, AUS) and the sample was swabbed until the surface area had been covered three times, or the swab-head was dry. The swab head was removed with a sterile scalpel blade into a 1.5 mL tube for subsequent extraction (Eppendorf, NSW, Australia).

DNA Extraction and Quantification

DNA extractions were performed manually using the DNA IQ™ System (Promega, Sydney, Australia), with an elution volume of 60 µL. Quantification of extracts were performed using the Quantifiler™ Trio DNA quantification kit (QIAGEN VIC, AUS).

STR DNA Amplification

Direct PCR amplifications were performed using VeriFiler™ Plus (ThermoFisher Scientific, VIC, AUS) in 25 µL following the manufacturer's protocol, with exception of 2 µL of Prep-n-Go™ (ThermoFisher Scientific) and Low TE Buffer (ThermoFisher Scientific) replacing water. Amplification, separation, and data analysis parameters were conducted as outlined Martin *et al.* [313]. For samples undergoing extraction, a suitable volume of sample extract was added to achieve 0.5 ng within the PCR, or the maximum of 17.5 µL was added. The lower bound threshold placed on extracts before profiling was not performed was 0.003 ng/µL. All samples were separated on a 3500 Genetic Analyser (ThermoFisher Scientific).

Data Analysis

Peaks were recorded if they were of 50 RFU or above. Peaks were considered for homozygosity if they were of 150 RFU or above. Profiles were considered informative if they contained 12 or more autosomal alleles. PCR dirtiness scores were given visually (from level 0 to 4) following a devised framework previously used [313], see Supp. Figure 6.6. Linear and logistic regressions, and likelihood tests, were performed using R software [333].

Trial 1: Indoor and outdoor comparison

Exhibits and volunteers

Three substrates were chosen to emulate potential real-life exhibits used in the construction of an IED comprising: a peeled battery casing mounted on aluminium sheeting; aluminium sheeting; and electrical tape. A substrate unit was constructed, consisting of these three substrates (Supp. Figure 6.7).

One volunteer, Donor 1, touched every substrate as described in section 2.1.3 in ten replicates giving a total of 30 samples. Positive controls of each substrate unit were created in duplicate using saliva. A negative control of each substrate was also performed. Five of the touch DNA replicates were stained with DD, as outlined in section 2.1.4, while five remained unstained. All samples were processed by direct PCR.

Exposure of Samples to Detonation

Samples were slid into a wooden holding unit (Figure 6.7 A), to hold the samples perpendicular to the blast; the positive and negative samples were placed in a separate holding unit. Having been mounted within these blocks, samples were placed at 400 mm from the charge, measured from the surface of the charge to the centre of the holding unit's face (Figure 6.7).

Trial 2: Distance

Exhibits and volunteers

From previously conducted studies two substrates were chosen to study the effect distance plays on cellular retention and subsequent STR profiling success [313]. These substrates were electrical tape and a peeled battery coating, both mounted on aluminium sheet (Supp. Figure 6.4). The collective of two electrical tape strips and one peeled battery coating forms what will now be termed a substrate unit.

One volunteer touched every substrate unit as described below (section 2.3.2) in 45 replicates giving a total of 180 samples; three units were not exposed to a blast in order to provide a set of non-detonation touch controls. Negative and positive controls, eight units each, were prepared; seven of each control were exposed to detonation while the last was not. The 42 touched substrate units, that were to undergo detonation exposure, were separated into seven groups each containing six units. These groups were as follows: 100 mm; 200 mm; 300 mm; 400 mm; 500 mm; 600 mm; and 700 mm from the charge. Three units from each group were mounted to the front of a witness plate, the other three on the reverse (Figure 6.7, panel A). One positive control, with saliva template, and one negative control unit was mounted to the front of the witness plate at each distance. All samples were processed by direct PCR.

Deposition of DNA

It has been previously reported that there is no significant difference in DNA deposition between the left and right thumbs [149, 163, 172]. Due to this, the volunteer was asked to touch the two identical substrate samples on the same side of the substrate unit simultaneously to deposit thumbprints with equivalent cellular material content.

Detection of latent DNA

Onto one thumbprint of each substrate type on each substrate unit (See Supp. Figure 6.7), 5 μL of the 20x DD solution was placed and spread over the surface using a pipette.

Exposure of Samples to Detonation

Samples were placed at different distances from the charge, having been mounted to witness plates, every 100 mm between 100 mm and 700 mm measured from the surface of the charge to the centre of the witness plate face (Figure 6.7 A).

Trial 3: Substrate extension

Exhibits and volunteers

Trial 3 was performed with two sets of samples. Donor 2 performed all DNA deposition events for both sets. Set 1 included five substrates comprising: a circuit board; a piece of wood; a SIM card; a mobile phone with rubber buttons (SIM card enclosed); and PVC pipe. Donor 2 handled each substrate with eleven replicates. A substrate unit was constructed, consisting of these five substrates after DNA deposition occurred (Supp. Figure 6.7). Five substrate units were classified for direct PCR processing, five for the extraction-based workflow, and one as a non-detonation touch control. One positive control substrate unit, with the addition of saliva, and one negative control substrate unit were also constructed and exposed to detonation.

Set 2 included four substrates comprising: a backpack, a drink bottle, a faux leather wallet, and a flip-style mobile phone. Quadruplet samples of each substrate were prepared and handled. For the detonation event all items were placed within the backpack. All samples were processed by direct PCR.

Deposition of DNA

Set 1: Alterations to the deposition of cellular material did not alter the deposition time. Instructions were given to the volunteer to touch the numbers on the mobile phone and press the glass surface to their face during the 15 s deposition time. In addition to this, the SIM card was requested to be placed into the back of the mobile phone following cellular deposition.

Set 2: The backpack was worn for two hours by the volunteer to simulate use. The zip was opened and closed each time one of the other items within Set 2 was places within the backpack. The drink bottle was handled as per use, with the volunteer's lips pressed over the opening to simulate drinking. The mobile phone was handled as described above and the wallets were handled as previously described in section 2.1.3. After each item was touched, it was placed inside the backpack by the volunteer.

Detection of latent DNA

Only the SIM card, mobile phone and PVC were visualised for the presence of cellular material. The wood and circuit board could not be visualised under fluorescence microscopy due to their background causing complications with the observation of cellular material. In addition to the DinoLite images, collected as described in section 2.1.4, the mobile phone and PVC were visualised on a gross scale using the polilight (490 nm emission) and captured using a Nikon D4300 camera.

Exposure to detonation

For Set 1, samples were placed on the ground 400 mm from the charge, measured from the surface of the charge to the front face of the substrate unit (Figure 6.7 B). For Set 2, the four backpacks were placed immediately next to the charge (Figure 6.7 C).

Collection of DNA from samples

Three workflows were conducted on samples from Set 1, and two were conducted on Set 2. Sampling for each workflow was performed as outlined in 2.1.6. The workflows were: 1) standard direct PCR; 2) sampling for extraction from the samples previously sampled for direct PCR, referred to in this study as the "dual sample" (DS); and 3) sampling followed by extraction. Workflow 2 assesses the viability of performing a second collection followed by an extraction on a sample after sampling for direct PCR has already been performed. For set 1, five underwent workflow 1 and 2, and the other 5 touch replicates underwent workflow 3. For Set 2 all samples were processed by workflows 1 and 2.

6.4.4 Results and Discussion

All negative controls produced profiles with no allelic amplification. DD visualisation allowed reconstitution of post-detonation samples and fragments to their original pre-detonation sample ID for STR analysis processing in all trials performed.

When visualising the cells post-detonation, under the DinoLite, some of the exposed samples exhibited orange fluorescence that was not observed prior to detonation. In most cases, this did not impede the visualisation and scoring of cells (Figure 6.8), however in some cases it completely covered the surface. As this appeared in the exposed samples only, it is proposed that the particles causing the fluorescence are residue arising from the explosion, As the deposit is widespread, it is most likely not a residue of the energetic material in PE4 (i.e., RDX). It is more likely a residue of the non-energetic plasticizer present (petroleum oil) in PE4, which is likely to have native fluorescence emission in the region around 550 nm [426]. However, chemical tests are yet to support this hypothesis.

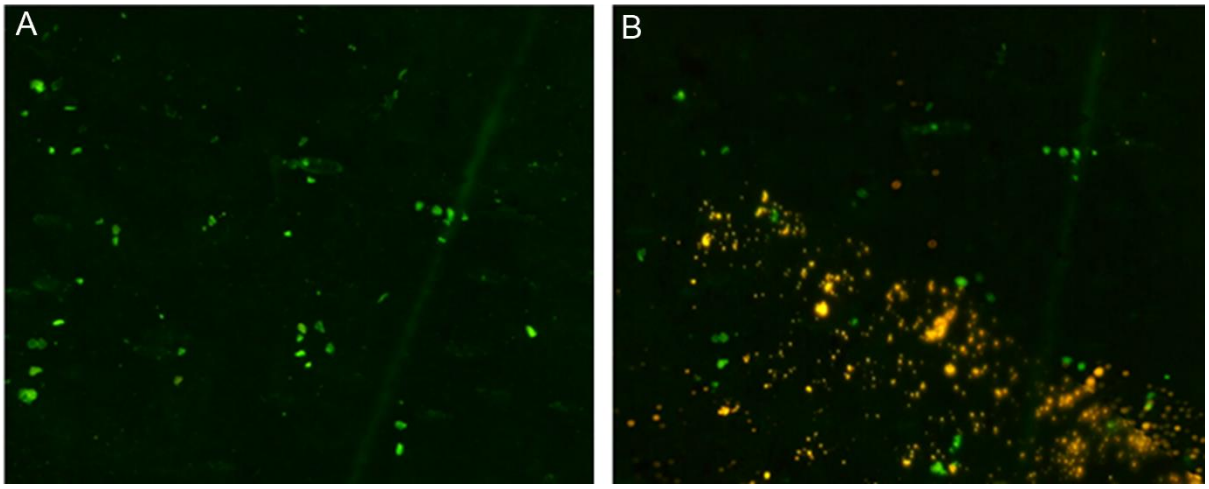


Figure 6.8. Example of pre- (A) and post- (B) detonation images taken at the same location on a battery sample 300 mm from the charge. Orange fluorescence is observed post-detonation. Images were taken at 220x magnification.

Diamond dye fixation assessment:

To address the question of whether the DD solution, utilised for the observation of cellular presence, movement, or loss, caused fixation of the cells to the sample's surface, comparisons were made between the average number of donor alleles called per profile and the average peak height over respective profiles taken for each fingermark pair (stained and unstained) in Trial 2. Considering the dataset entirely, there was no significant difference between the stained and unstained samples post-detonation in the number of donor alleles present; this held when analysing the touch ($p= 0.969$) and saliva controls ($p= 0.833$) separately. The same was true for total RFU ($p= 0.924$; $p= 0.863$; and $p= 0.908$ respectively). The staining of the fingermarks did not change allelic count dependant on substrate either ($p= 0.578$ for ET and $p= 0.601$ for B) (Figure 6.9). This supports the conclusion that DD does not cause fixation of the cellular material prior to detonation, nor inhibit the PCR causing a lowering of STR amplification success. Cellular staining and pre- and post- visualisation is supported as a powerful research tool for the analysis of how extreme treatment conditions affect cell presence, movement, loss rates or PCR template validity.

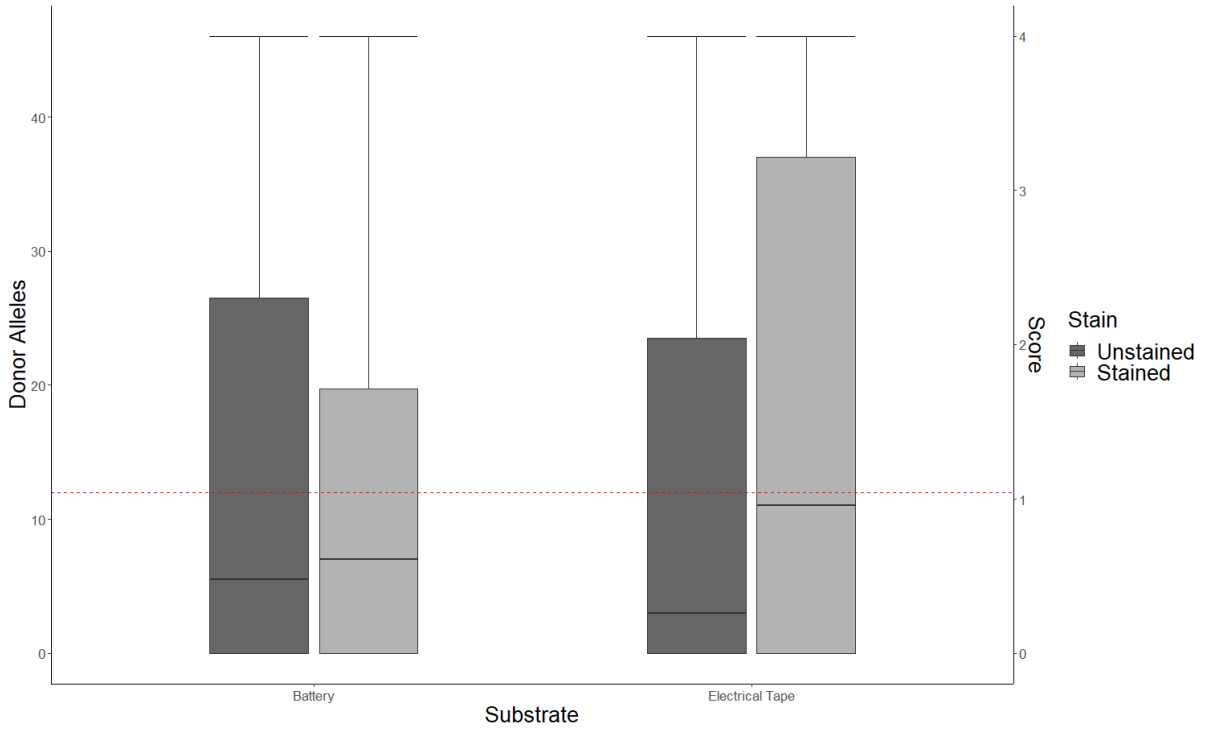


Figure 6.9. Post-detonation battery and electrical tape touch DNA samples, over distances 400 mm – 700 mm from the charge, showing DD stained and unstained samples independently. The horizontal line indicates the 12 autosomal allele threshold. N=96

Trial 1: Indoor and outdoor comparison

As it has been reported that distance from the charge plays a role in cellular retention [313] and there was not an adequate representation of 'indoor' samples at a comparable distance to the 'outdoor' samples, these data are restricted in their analytical power. Samples from the blast chamber trials were selected above 250 mm from the charge to allow the results, as an average over that dataset, to be most comparable to the 'outdoor' 400 mm samples. There were 14 samples (4 aluminium, 6 battery, and 4 electrical tape) within the 'indoor' dataset and 30 samples (10 of each substrate) within the 'outdoor' dataset.

A logistic regression performed between the 'indoor' blast chamber data and the 'outdoor' data showed significance in the number of donor alleles amplified when considering all sample types ($p= 0.00813$). Significance in allelic amplification based on environment was not observed with the batteries ($p= 0.874$) or the electrical tapes ($p= 0.310$), but it was with the aluminium ($p= 0.0162$). A linear regression performed between the 'indoor' blast chamber data and the 'outdoor data showed significance in the difference of the log10(total RFU) per profile ($p= 0.0136$). Significance was not

observed with the batteries (p= 0.596), however it was with the electrical tapes (p= 0.0183) and the aluminium (p= 0.0438).

The proportion of cells remaining post-detonation was calculated as the ratio between the average cell score per sample prior to detonation and the score measured post-detonation. There was no significant difference in cell retention through the detonation process observed between the environmental conditions (p= 0.1882) or the substrate types (p= 0.337) (Supp. Figure 6.9).

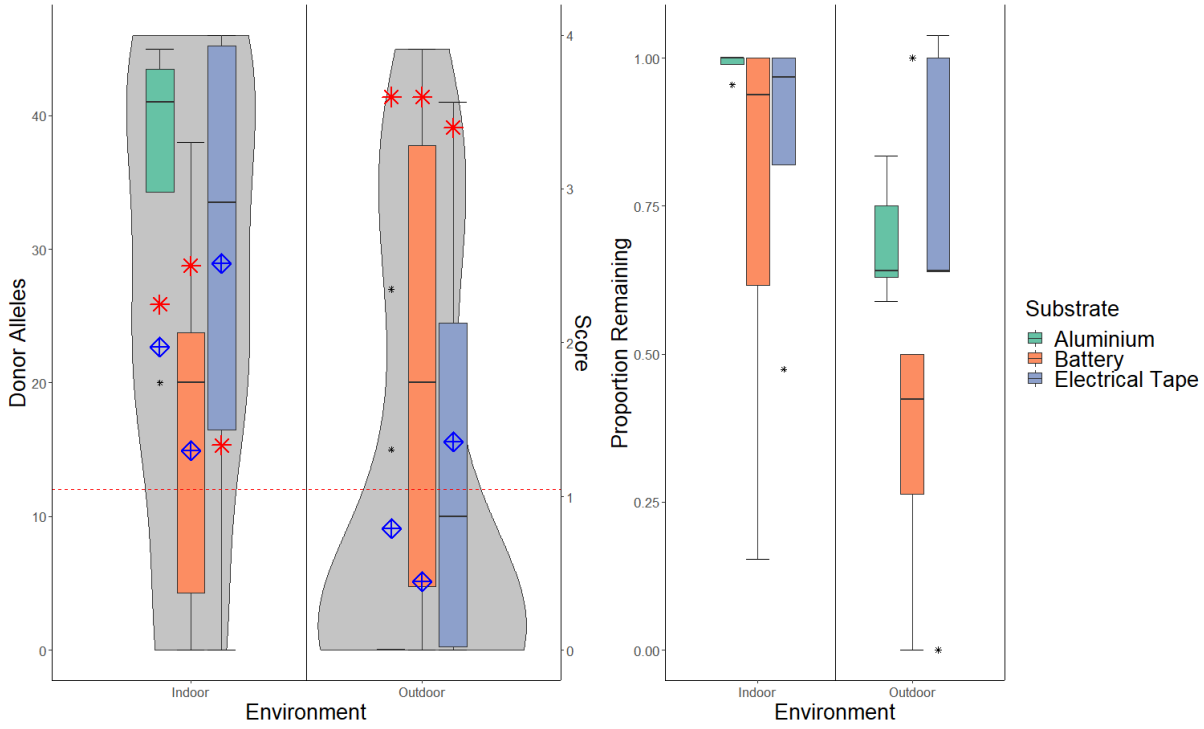


Figure 6.10. Left panel shows the donor alleles amplified, from touch DNA samples post-detonation, comparing aluminium, battery, and electrical tape samples, for both an indoor blast chamber and outdoor, dirty, environment. Average dirt score is overlayed as a red asterisk, and average cell score is overlayed as a blue diamond (score 0-4). Right panel shows the cell proportion remaining post-detonation for the same substrates and environments. N= 14 indoor and N= 30 outdoor.

There was a significant difference observed between the environments with respect to cell score (p= 0.0104 with 'indoor' being higher) and dirt score (p= 8.22×10^{-6} with the outdoor environment being higher) (Figure 6.10). A multiple variable logistic regression including surface type, environment, PCR dirt score, and cell score post detonation was performed and showed that when dirt score and cell score is accounted for within the model, all other variables lose their significance; for example,

the effect of environment for aluminium samples on alleles was significant (p= 0.0162) but when adjusted with dirt score the difference between the environments was no longer significant (p= 0.223). The lack of amplification on aluminium from the outdoor dataset was not expected and there seems to be no reason as to its lack of success; the cell score difference and PCR dirt score were not significantly different to the other substrates (p= 0.172 and p= 0.977 respectively).

The relationship between cell presence and STR results is well established [326], therefore it is expected this would affect the allelic data present in subsequent profiles, and this is what was observed. Dirt, more specifically the humic acid in dirt, is also known to affect PCR efficiency [1, 269] and this effect was observed within this study. Therefore, humic acid, an organic compound prevalent within dirt and natural dust, was suspected to be the main inhibitor to the direct PCR samples within this study due to the high levels of dirt present on the samples post-detonation. Repeating the experiments in an outdoor urban environment, where soil presence may be reduced, would give a greater insight in how well DNA recovery from post-detonation IED components can be performed in different spaces and inform collection and processing teams as to potential successes in different cases. This would also allow the assessment and comparison of research performed and presented in literature to be compared more appropriately.

These data support the conclusion that environment plays a role in the amplification success rate, with respect to the number of donor alleles called and the total donor RFU within the resultant profile, of STR amplification and the proportion of cells retained through the detonation (Figure 6.10) and indicates the importance of analysing more environmental situations in the sphere of post-detonation IED DNA data recovery.

Trial 2: Distance

When considering positive saliva controls there was no difference in the number of alleles observed due to PCR dirt score (p= 0.129), this was expected as the template source was of high quantity and quality. There was, however, a significant difference between the substrates ($p= 3.49x10^{-4}$), with the battery performing better than the electrical tape. With respect to resultant total RFU this trend was also observed whereby the battery produced higher total RFU than the electrical tape (p= 0.00175). Distance was also shown to be significant for allele number (p= 0.00757), but not for total RFU (p= 0.0737). All positive saliva controls returned a profile, with the exception of four electrical tape samples, therefore analysis for touch DNA was supported with this experimental

workflow; in addition, these substrates have been previously shown to allow STR amplification from touch DNA material post-detonation with a direct PCR workflow [313].

The samples placed 600 mm from the charge performed inexplicably poorly. This could be due to ineffective dirt removal by blowing over the samples, as seen by the highest dirt scores for these substrates, excessive physical damage, or deposition with low PCR viable template as observed in the cell score data (Figure 6.11). Because of this, the data from 100 mm and 600 mm were removed from the dataset for further statistical analyses or observational discussion; the difference that distance held over the number of donor alleles changed from $p= 3.27x10^{-8}$ to p= 0.0132 when 100 mm and 600 mm were removed. Data from 100 mm was removed due to severe cellular loss and a complete lack of STR data (Supp. Figure 6.8 and Figure 6.11); any trends within the remaining dataset would have been affected negatively with the retention of these data points.

There was no difference between the substrate types with respect to the donor alleles amplified for the touch DNA template (p= 0.571), unlike with the saliva controls; this was also true for the total RFU (p= 0.6919). When considering touch DNA on both surface types, distance was seen to be significant for the number of alleles amplified (p= 0.0132) and the total RFU within the profiles ($p= 4.01x10^{-4}$) (Figure 6.11). The side of the witness plate on which the substrates were placed also influenced these parameters significantly, with the exposed side producing poorer profiles ($p= 7.335x10^{-5}$, and $p= 2.92x10^{-5}$ respectively) (Figure 6.11) and experiencing a higher PCR dirt score ($p= 2.91x10^{-8}$). Informative profiles were obtained, on average, when ≥300 mm from the charge for covered samples, but this was only experienced when the distance increased to ≥500 mm for the exposed dataset. This was expected as the exposed side experienced shrapnel damage from the charge components and other samples in front of it as well as being exposed to the front of the pressure wave, temperature and chemicals released through the detonation process. The trend between increasing distance and increasing donor allele return is cleaner in the exposed samples compared with the covered samples (Figure 6.11). The covered samples experienced increased amplification closer to the charge than the exposed samples, due to considerations previously described, and this caused the spread of allelic data return to be larger over individual distances for 200 mm - 400 mm and the differences between distances to not be as clear. These data suggest that if a sample is covered from direct exposure from the charge, at a distance of 200 mm, it is possible to produce informative DNA profiles from the viable template that remains.

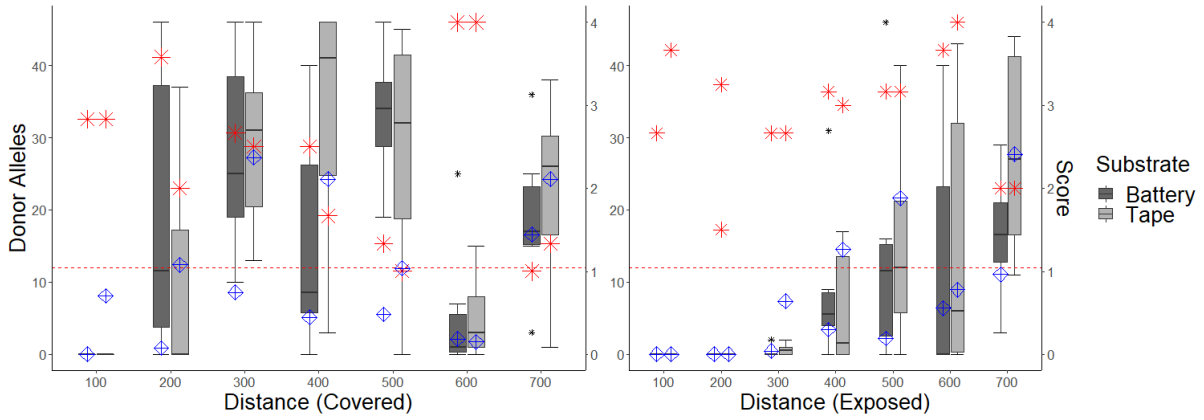


Figure 6.11. Number of donor alleles obtained, from touch DNA, post-detonation for both the covered (left) and exposed (right) sides of the witness plates. Distances are every 100 mm from 100-700 mm. Average dirt score is overlayed as a red asterisk, and average cell score is overlayed as a blue diamond (score 0-4). The horizontal line indicates the 12 autosomal allele threshold. N= 162 (intended as N=168, however six 200 mm samples were unaccounted for).

The effect that cell score and the PCR dirt score had on the number of alleles amplified was inverse to each other as increasing cell score improved the number of alleles observed ($p= 3.519x10^{-5}$), while increasing dirt score decreased allelic return (p= 0.0144); PCR dirt score was also shown to influence the total RFU present within profiles ($p= 2.80x10^{-5}$). To observe whether the interaction between distance, PCR dirt score, and cell score post-detonation affected the number of alleles amplified, a generalised logistic regression was performed with interaction terms; this resulted in significance ($p= 4.22x10^{-5}$). This was also performed with substrate, cell score post-detonation, and PCR dirt score and the same was observed ($p= 3.347x10^{-5}$). This logistical model was plotted and can be seen in Figure 6.12 with the difference that the cell scores post-detonation were converted into a numerical value representational of the quartile of the dataset in which they fell. Only one battery sample fell into the fourth quartile and was therefore removed from the plot to avoid false trends being observed. Figure 6.12 depicts the relationship whereby surface, cell score, and dirt score have a combined effect on the percentage of the profile amplified (100% is 46 alleles). It can be seen that as dirt score increases allelic coverage reduces, while increasing cell score increases the allelic coverage. This is expected and supported by literature, as previously discussed [1, 269].

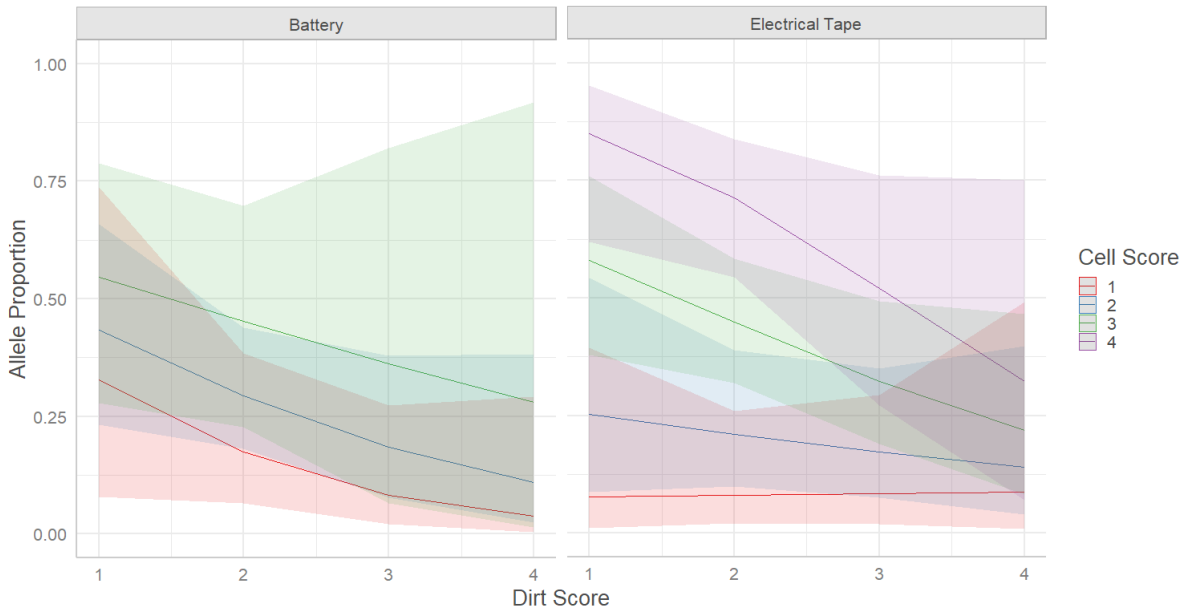


Figure 6.12. Plot of the logistic model observing the interaction between substrate, dirt score, and cell score quartile for their combined influence over the donor allele recovery. Dataset includes distances 200-700, with the removal of 600, and both covered and exposed data. This interaction was shown to be significant on alleles as p= 3.347×10^{-5}.

Cellular loss due to the detonation process was observed through the calculation of the cellular retention, or proportion remaining. This was calculated as the ratio between the cell score pre- and post- detonation. This was found to be affected by the surface type (p= 0.000198), with less cells lost from the electrical tape substrate, and distance (p= 2.32×10^{-9}). The relationship between cell retention, as opposed to cell loss, is visually represented in Figure 6.13. As the distance increases the proportion of cells that remain on the substrate through the detonation process also increases; this is true for both the covered and exposed samples. In addition to this, the average cell retention value was higher per distance for the covered samples than the exposed (Figure 6.13), however this was not significant over the dataset (p= 0.0662). When considering samples ≥400 mm over both conditions, significance is lost in the relationship between distance and the number of alleles (p= 0.452) and the cellular loss (p= 0.186). This highlights that when samples are further than 400 mm from the charge there is no expected impact upon STR profiling due to cellular loss.

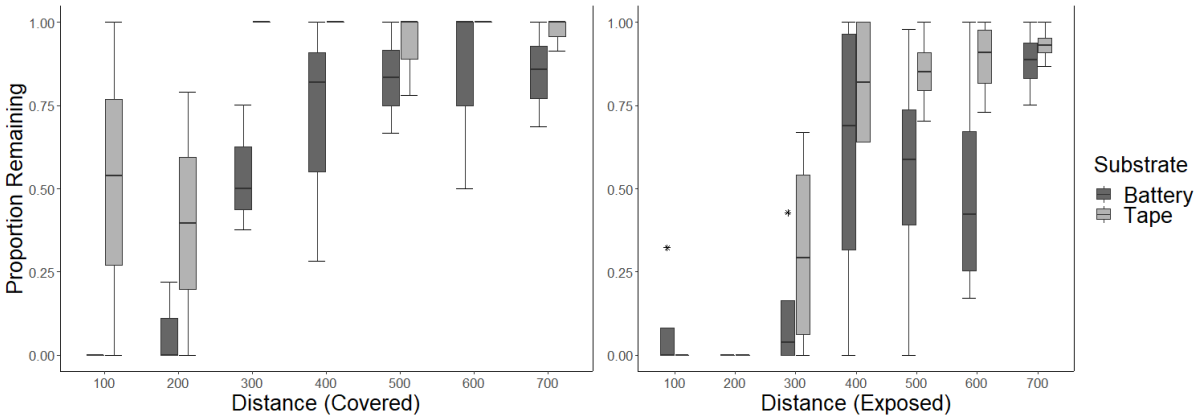


Figure 6.13. Proportion of cells remaining post detonation for both the covered (left) and exposed (right) sides of the witness plates. Distances are every 100 mm from 100 mm-700 mm. Proportion remaining was calculated as the ratio between the cell score pre- and post- detonation taken at the same location on each substrate.

Trial 3: Substrate extension

For the non-detonation controls, allelic amplification success was dependant on substrate (p= 0.048), however this was not observed in the detonated samples. As the non-detonation controls were not stained, there was no adjustment based on cell-score able to be applied to these samples. This control parameter was chosen to observe whether informative profiles could be obtained from these substrate types when utilising touch DNA as template before exposing them to a detonation, and as all samples returned informative profiles, these substrate choices were supported. Workflow, being direct PCR, PCR post-extraction, and PCR post-extraction following direct PCR sampling (DS), did not influence the number of alleles amplified for the control, non-detonated, samples (p= 0.582); this means sufficient template was present on all samples to allow for any process to be performed.

Following detonation, only 4 SIM cards were recovered, therefore due to their small surface area and cellular deposition the decision was made to process all of these through the direct PCR workflow. Considering detonated samples there was no significance in the number of alleles obtained dependant on process (p= 0.192); direct PCR was significantly better than the DS subset (p= 0.0497), but the improvement against the extracted samples was not significant (p= 0.456). Direct PCR offers increased PCR sensitivity with low quality and quantity template [193, 204, 319], however the inhibitor presence due to the dirt somewhat negated this benefit over the extracted samples, where template was reduced through the extraction and no PCR inhibitors were present. There was no significant difference in the number of alleles from different surface types for the entire dataset (p= 0.297); this was true for all processes individually also, for example direct PCR (p= 0.788). The log10(total RFU) of

the resultant profiles was affected by the workflow used (p= 0.0221). Direct PCR amplification resulted in the highest total RFU; the difference was significant when compared to the DS samples (p= 0.00642) but not with the extracted samples (p= 0.127). Informative DNA profiles, a profile with 12 or more autosomal alleles, were obtained from 10/18 extracted samples, 22/34 direct PCR samples and 13/34 DS samples (Figure 6.14). With respect to the proportion of cells retained throughout the detonation process, there was no significant difference observed between the substrate types scored (p= 0.715) (Figure 6.14 and Supp. Figure 6.9).

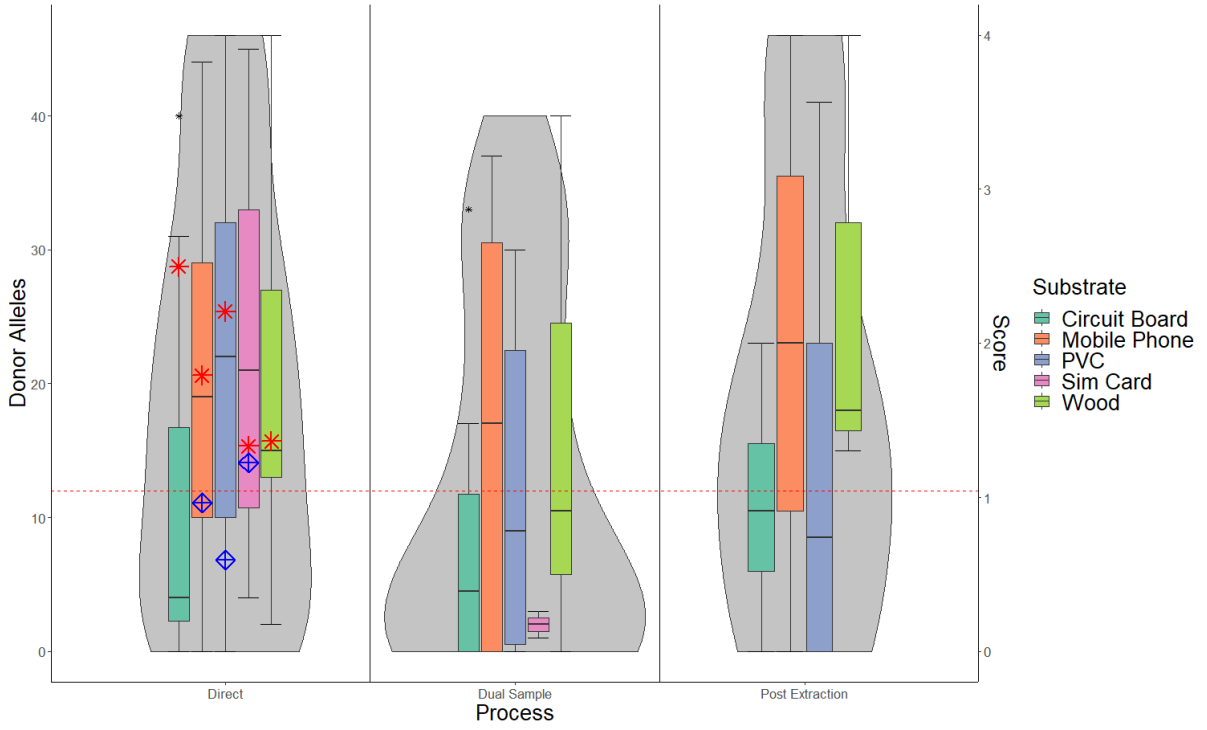


Figure 6.14. Number of donor alleles amplified for each workflow, these being as follows: direct PCR; sampling post-direct PCR sampling followed by extraction (Dual Sample); and PCR post extraction. N=5 per substrate per workflow, with the exception of SIM cards where N=4 for direct and N=0 for post-extraction (the samples in the direct PCR and 'Dual Sample' workflows were the same samples). Average dirt score is overlayed as a red asterisk, and average cell score is overlayed as a blue diamond (score 0-4). The horizontal line indicates the 12 autosomal allele threshold. Cell scoring was not possible on the circuit board or wood samples.

The detonation process caused a severe reduction in the number of alleles observed in resultant profiles ($p= 3.37x10^{-5}$). This difference within this dataset may be due to i) the detonation process itself causing cell loss, ii) the dirt present on the samples causing PCR inhibition, or iii) the substrate's surface causing substrate-specific PCR inhibition as a result of the detonation process. As the number of cells before and after detonation can be visually scored: i) was able to be assessed. Cell loss was found to be caused by the detonation and the reduced post-detonation cell score influenced STR profiling success. The presence of dirt was shown to affect the STR typing results within Trial 2 and this addresses ii); there was not enough data with variance in dirt score or allelic return to observe the effect of dirt on this dataset. Data suggests these substrates, addressing iii), do not pose a direct PCR inhibition threat post-detonation and triage teams can approach the processing of these substrates fairly equally. Although there is no significance observed in the alleles observed or cellular retention due to substrate type, collection and processing of circuit boards should not be prioritised when compared to mobile phones, PVC, SIM cards, or wood (Figure 6.14).

For the post-detonation samples in Set 2, fragments from all four replicates of the mobile phone, leather wallet and plastic bottle could be reconstructed into their original entity. The four canvas bags were very heavily fragmented, therefore the straps of all of the bags were separated into eight portions to produce eight samples in place of four. The canvas bag, mobile phone, leather wallet, and plastic bottle, 18/20 samples failed to produce a profile following direct PCR amplification with the exception of two: a leather wallet sample (17 alleles) and a mobile phone sample (21 alleles). Due to the close proximity to the charge, lack of staining data, dirt presence and surface area differences compared to other trials, no conclusion as to direct PCR substrate specific inhibition from these substrates can be made. All post-detonation substrates were processed with a secondary swab or tapelift taken for extraction processing following the direct PCR amplification for DS processing. This resulted in informative profiles, on average, for the canvas bag, leather wallet, and plastic bottle; of the 20 DE samples, 13 were informative (Figure 6.15). A QQ plot showed data of the relationship between the quantification data and the number of alleles observed in subsequent profiles fell within 95% CI of the linear equation built to represent the residuals and theoretical quantiles relationship (Supp. Figure 6.10). This is expected and show that inhibitors were removed during the extraction process and did not affect subsequent PCR. There was no significant difference observed in the number of donor alleles amplified between the substrates ($p= 0.762$) (Figure 6.15). The large spread in allelic data observed from the canvas bag strap is likely due to the range in sample surface area size and sample integrity. Some of the bag strap fragments were highly frayed, melted, or were broken

into very small pieces (Supp. Figure 6.11); each of these will affect the template available in respect to quality and quantity. In some cases, multiple smaller fragments were targeted as a single sample as the donor was known to be the same. This may not be possible in all casework; therefore, some caution must be taken when translating expected results in a casework setting. The mobile phone exhibited the smallest surface area of the substrates tested, and this may be an influential factor in the lower allelic yield observed (Figure 6.15).

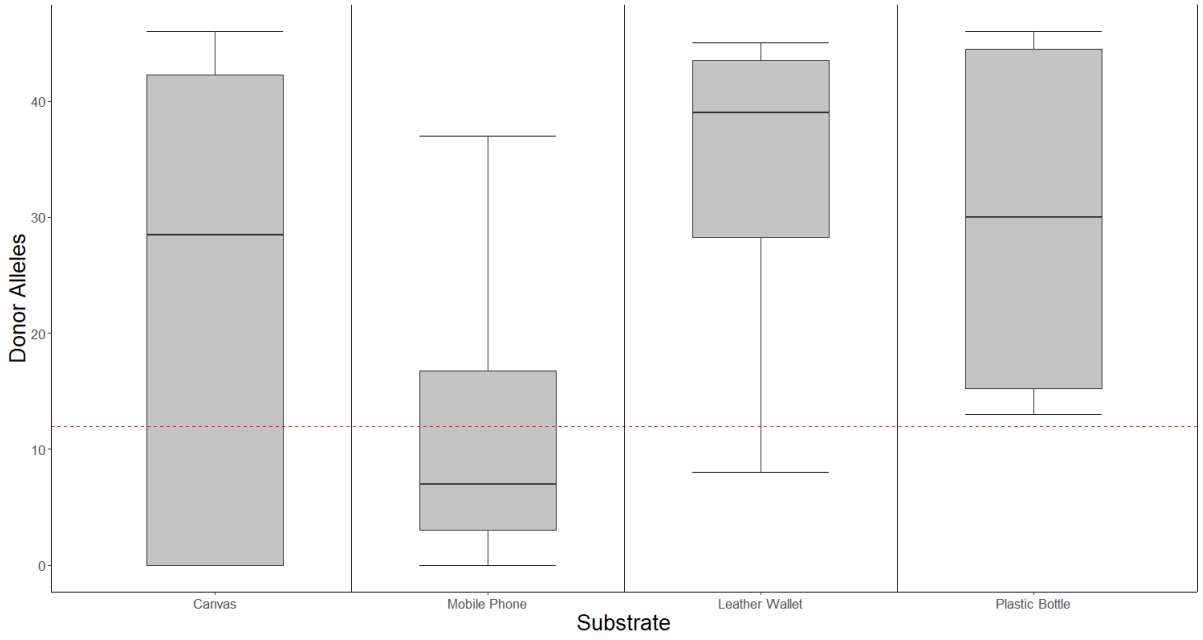


Figure 6.15. Donor alleles obtained following the 'Dual Sample' workflow on touch DNA samples post-detonation. The horizontal line indicates the 12 autosomal allele threshold. N=20.

As a larger surface area was targeted for these sample types than with the other trials, it is expected that there was a higher proportion of inhibitors to trace DNA template present on the swab-head/tape fraction for this study than previously observed. This would have a detrimental effect on the direct PCR process, reducing the likelihood of obtaining an informative DNA profile. It is proposed that the larger surface area targeted with the swabs or tapelifts for extraction facilitated increased cellular collection, while the extraction process successfully removed most PCR inhibitors allowing the DS workflow to be preferred.

6.4.5 Conclusion

Data presented support the use of DD for experimental design to facilitate triage and cell loss assessment as there was no evidence that its application affected cellular retention through the detonation process or within the subsequent PCR.

Although a limiting factor of the analysis between the indoor blast chamber and outdoor environments was the absence of similar distances from the charge between the datasets, there were trends that were observed, although their strength is constrained by this limitation. Increased dirt score and decreased cell score post-detonation were observed in the outdoor dataset. Amplification post-detonation of touch DNA from aluminium samples failed, but there was no significant decrease for the battery and electrical tape. Dirt score was shown to affect the STR data obtained post-amplification with direct PCR, therefore understanding inhibitors present within collected post-detonation samples is important for workflow. The environment in which the detonation took place did not affect the amount of cellular loss observed due to the detonation process.

As substrates were moved further from the detonation, the number of alleles and total RFU within a profile, and cellular retention on the substrates increased. This trend was observed more clearly in the exposed dataset, where the samples were directly exposed to the pressure wave, heat, UV light, and deposition of explosive residue from the charge; the covered samples presented more variance in the spread of profile data. Samples placed ≥400 mm from the charge experienced no significant difference with respect to the number of alleles present in subsequent profiles or the cell loss experienced due to the detonation. Therefore, cell loss is closely linked to the distance from the charge in post-detonation samples. It was observed that the relationship between PCR dirtiness and cell score had inverse effects on allelic data return for both substrates. These data support previous work that discusses PCR inhibition due to dirt and the link between cellular abundance and STR profiling success. These data suggest that processing of samples ≥400 mm from the charge when exposed to the charge and ≥200 mm when covered from the charge is able to provide informative DNA profiles using a direct PCR workflow; the caveat to this is the surface area and substrate of the targeted sample.

Although it has been previously observed that certain substrates can cause substrate-specific PCR inhibition post-detonation, none of the samples analysed within this work showed this. Because of this, it is indicated that triage teams can collect mobile phone components, PVC pipe, SIM cards, and wooden samples with equal preference and circuit boards secondary to these. It was also possible

to produce DNA profiles following an extraction when sampled after an initial direct PCR sample was taken from a sample; this did result in lower donor alleles called than the direct PCR counterparts, however for intelligence purposes this workflow is also viable. When comparing the substrate units following direct PCR and PCR post extraction, with no previous sampling to the surface, there was no difference observed with respect to the donor alleles present within subsequent profiles. Therefore, either workflow is advantageous when detonation is experienced outdoors; direct PCR offers increased sensitivity but is affected by inhibitors within the dirt and charge, while PCR post extraction is not as sensitive but no inhibition to PCR ensues. There are no additional collection triaging guidelines provided by this work.

It was observed that when samples with a large surface area were analysed, direct PCR failed to allow amplification of STR data, while PCR post extraction did. This is most likely due to the low template/high inhibitor presence and the lack of direct PCRs robustness against these inhibitors in such high quantities. When a larger surface is sampled, template collection is unlikely to be proportionate to the inhibitor collection due to the overall surface presence of each.

It is advised that, for collection and processing triage, considerations of distance from the charge, whether the sample is likely to have been exposed to or covered from the charge, the substrate surface area and surface type, and inhibitor presence on the sample surface should be made.

6.4.6 Conflict of Interest

The authors declare no conflict of interest.

6.4.7 Funding and Acknowledgments

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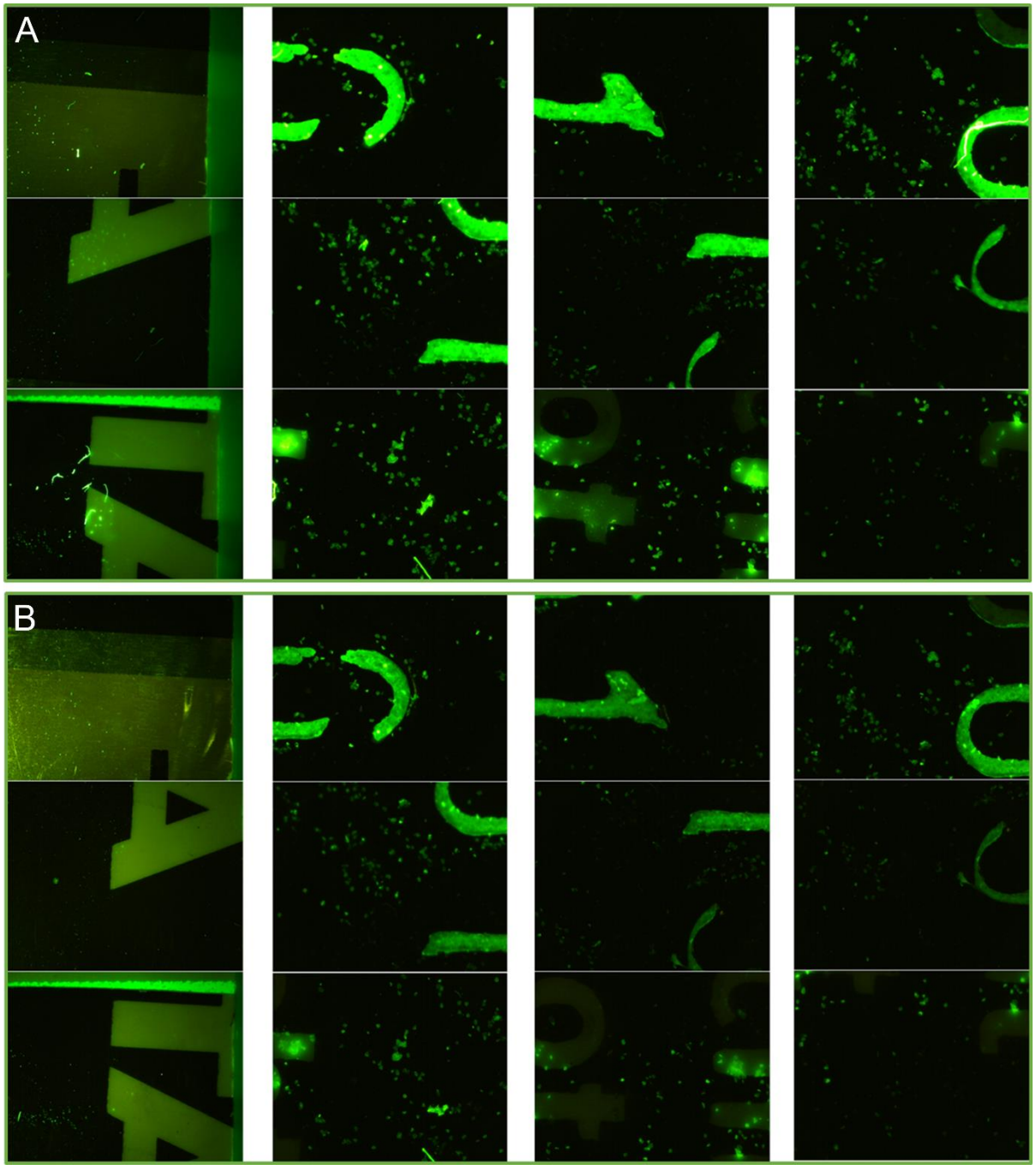
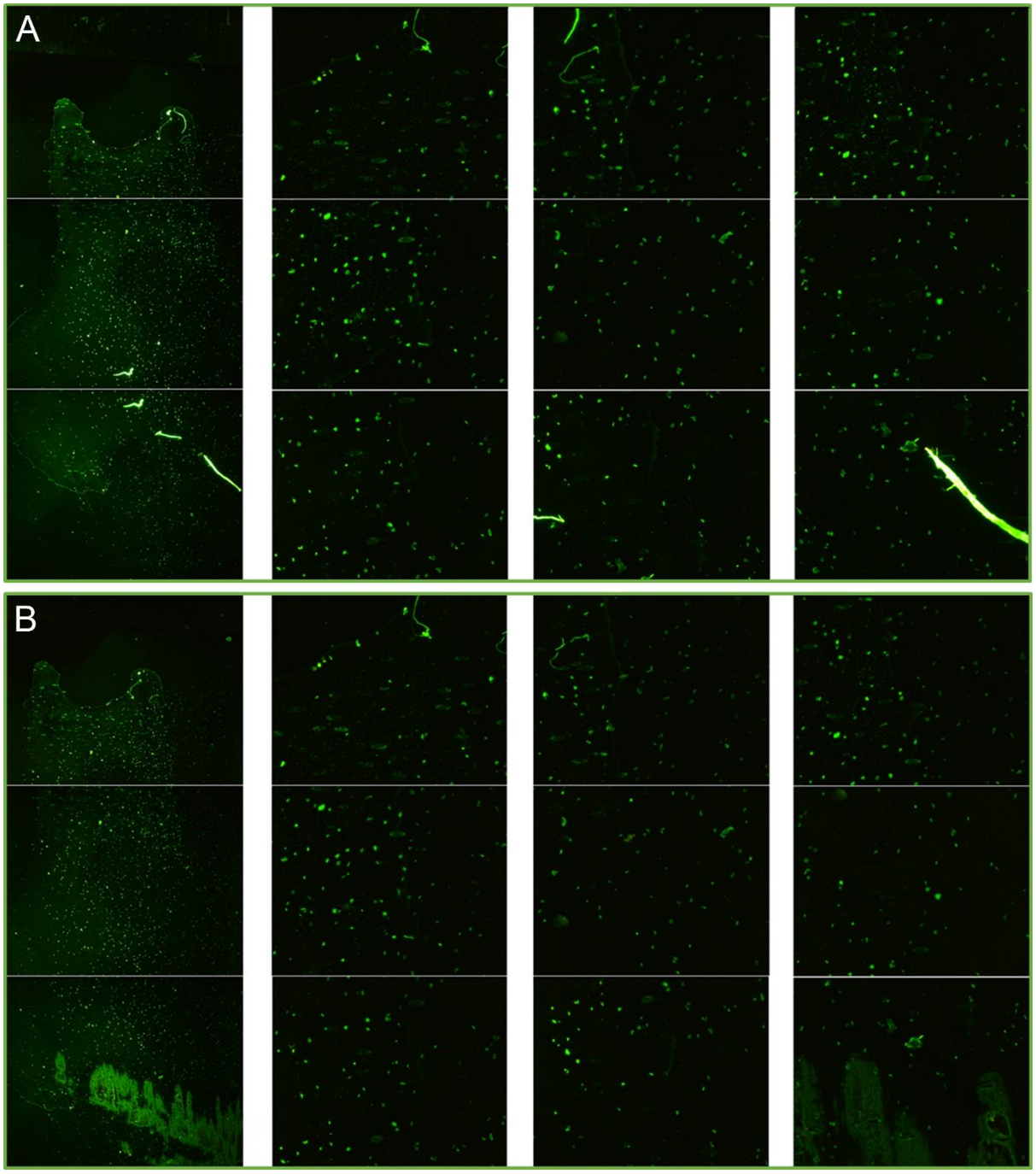
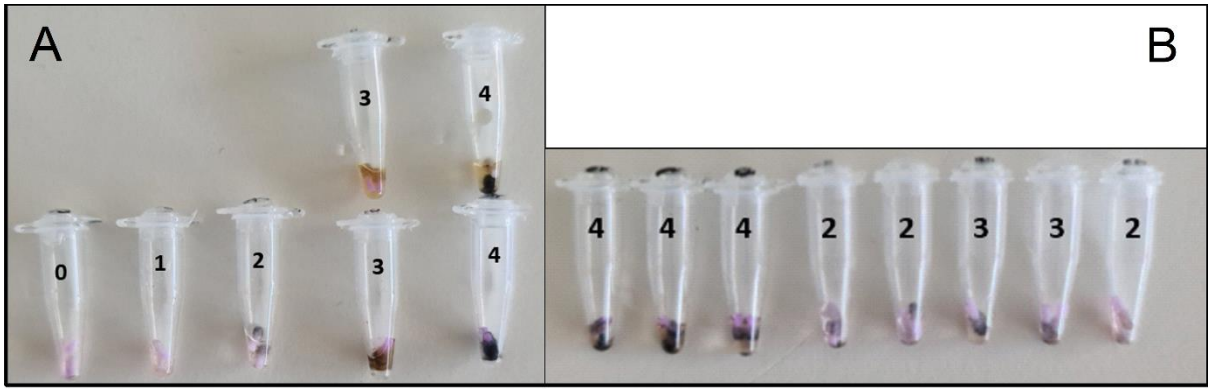
6.4.8 Supplementary Material



Supp. Figure 6.4. Example of pre- (A) and post- (B) images taken from the same locations on a battery sample placed 700 mm from the charge. Left vertical panels are at 50x magnification and the other three vertical panels are at 220x magnification.

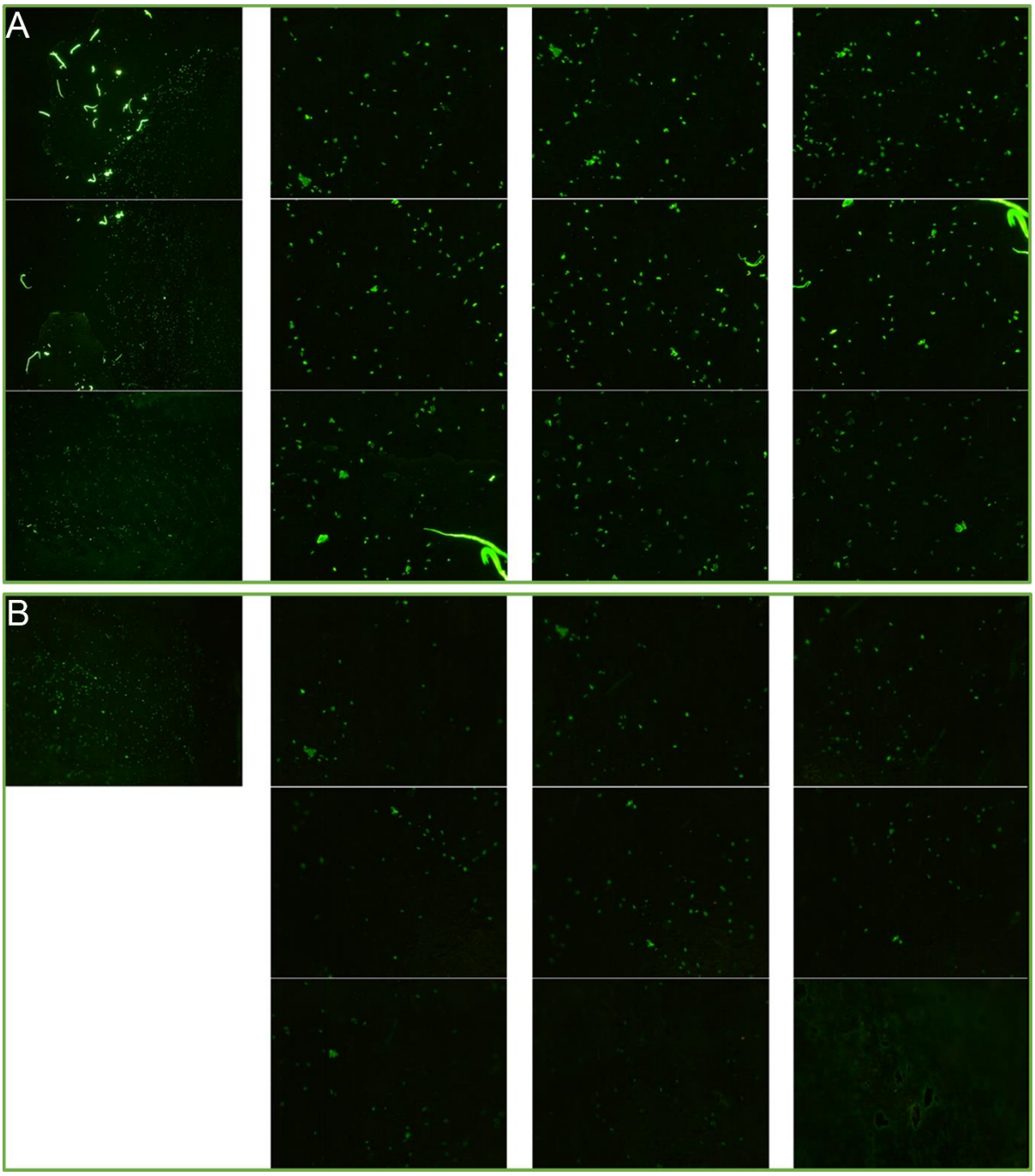


Supp. Figure 6.5. Example of pre- and post- images taken from the same locations on an electrical tape sample placed 700 mm from the charge. Left vertical panels are at 50x magnification and the other three vertical panels are at 220x magnification.

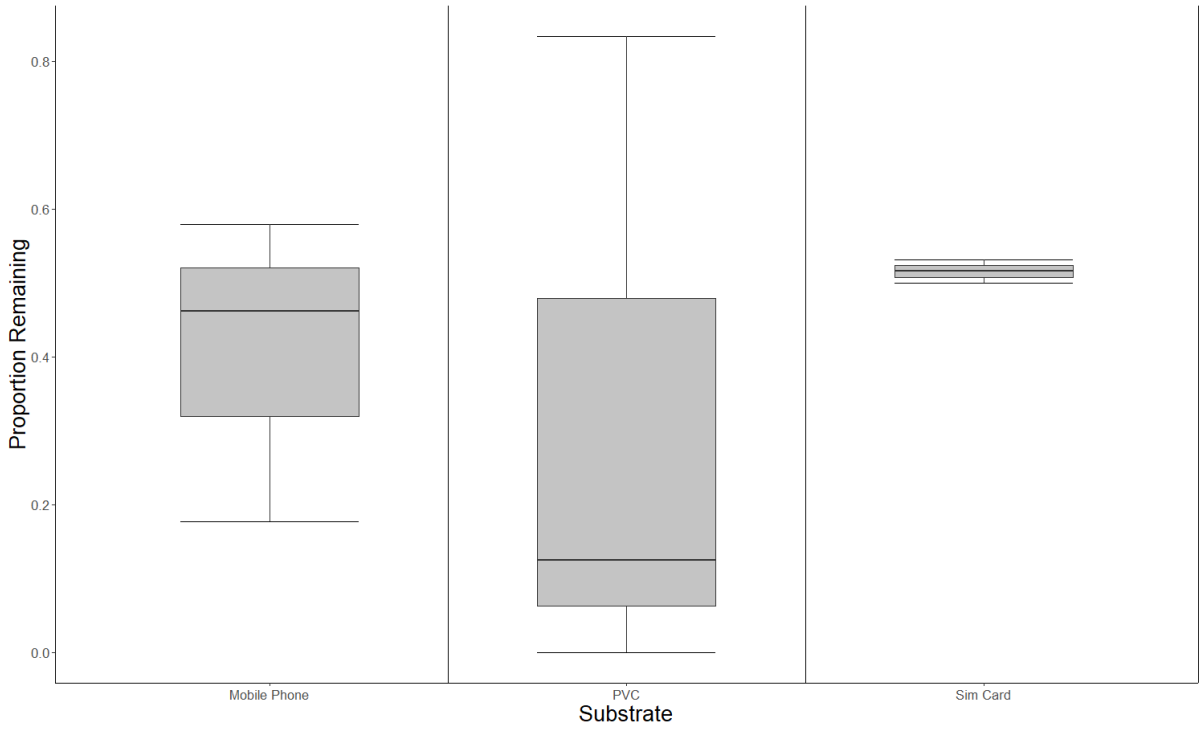


Supp. Figure 6.6. Panel A is a visual representation of PCR tubes within each dirt score rank. PCR grading standards: 0 – no dirt, or discolouration, 1 – slight dirt, or slight discolouration, 2 – moderate dirt, or moderate discolouration, 3 – lots of dirt, or moderate dirt and high discolouration, 4 – high amounts of dirt and/or high amounts of discolouration. Panel B is an example of PCR score assessment at 100 mm form the charge.

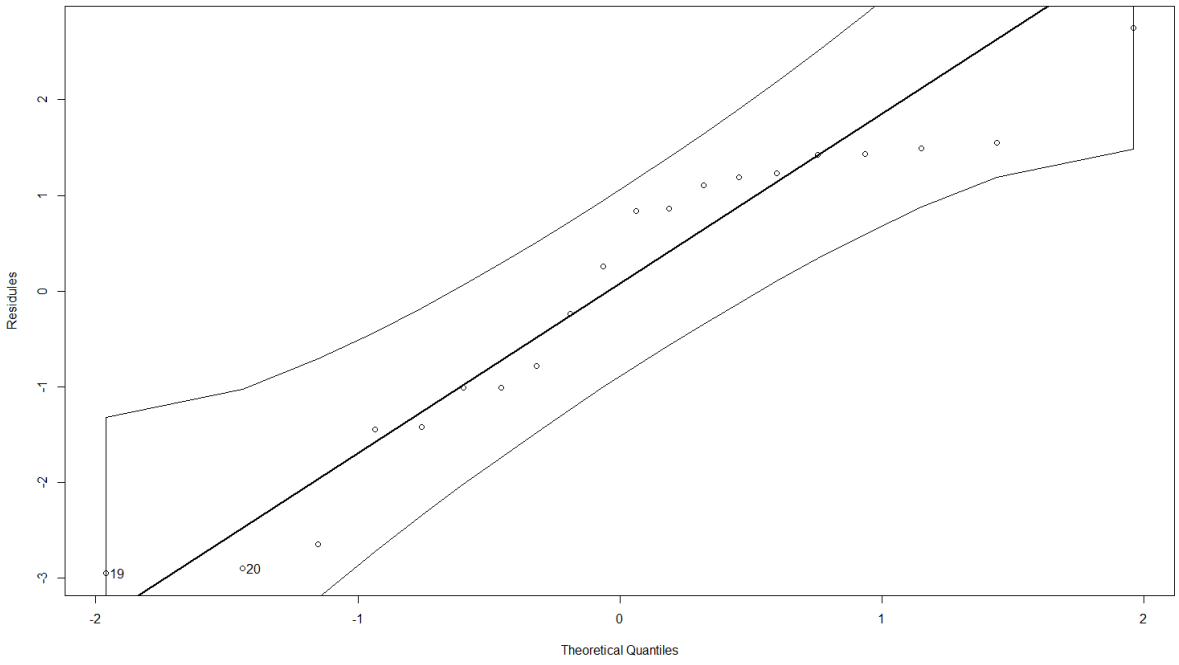
Supp. Figure 6.7. Sample units for Trial 1 (left), Trial 2 (middle), and Trial 3 (right). Sample units are comprised of the following: a peeled battery mounted on aluminium sheeting, aluminium sheeting, and electrical tape for Trail 1; a peeled battery mounted on aluminium sheeting and electrical tape for Trial 2; and a mobile phone, SIM card, pine wood, circuit board, and PVC for Trial 3. Each sample within the sample unit contains reference ID information.



Supp. Figure 6.8. Example of cell loss between the pre-detonation (A) and post-detonation (B) images taken from the same locations on an electrical tape sample placed 100 mm from the charge. Left vertical panels are at 50x magnification and the other three vertical panels are at 220x magnification.



Supp. Figure 6.9. Proportion of cells remaining post detonation for the substrate extension samples. Proportion remaining was calculated as the ratio between the cell score pre- and post- detonation taken at the same location on each substrate.



Supp. Figure 6.10. QQ plot of the log10(human quant) and alleles relationship.

Supp. Figure 6.11. Post-detonation canvas bag fragments from the straps tapelifted for STR analysis. Bags were placed immediately next to the charge.

6.5 CONCLUDING COMMENTS

Results discussed within this chapter report the highest success of post-detonation touch DNA STR profiling success, with respect to number of alleles, in the literature. The charge detonated in these studies is substantial, but not as large as can be found [427]. These studies examine multiple variables that influence amplification; namely distance from the charge, cellular deposition, dirt inhibitor presence, substrate, and surface area sampled.

The data presented in 6.3 supports the introduction of direct PCR amplification for the analysis of touch DNA from most post-detonation samples. This work, conducted in a blast chamber, identified the importance of analysing many sample types as it was found that post-detonation some samples caused substrate-specific inhibition to direct PCR methods. It is advised that future work investigates plastic samples more closely and determine what component of the substrate is causing PCR inhibition. There is also scope for charge size increase, altering charge type, and including different types of IED mock-units. A relationship between the sample's distance from the charge and the number of alleles obtained post-processing was also observed. Samples closer than 100 mm experienced cell loss due to detonation that was significant and profiling success was limited. Cell loss was insignificant at greater than 250 mm. Samples at a distance from the charge greater than 750 mm showed no difference in profile attributes when compared to non-detonation controls. The aim "*to investigate the potential for direct PCR to provide informative STR data from post-detonation IED samples*" has been addressed within this work.

The data presented in 6.4 supports the introduction of direct PCR amplification for samples with small surface area, while for larger items PCR post-extraction is preferable. An in-depth analysis of how multiple variables interact to influence STR profiling success was undertaken successfully, and the data produced and outlined is valuable for collection and processing teams in counterterrorism teams. The transition to a rural detonation site introduced humic acid inhibition to the direct PCR workflow, however this did not cause an absence of STR data; up-loadable profiles were still obtained. Although eight additional substrates were investigated, none of these posed substrate-specific inhibition post-detonation. The power and value of direct PCR in the niche of IED analysis is shown and supported. The aim "*to investigate the difference an outdoor, dirty, environment has on direct PCRs capability to provide informative STR data from post-detonation IED samples*" has been addressed within this work.

CHAPTER VII:

CONCLUDING REMARKS AND FURTHER APPLICATION

7.1 PREFACE

Chapter VII summarises the findings of each chapter and emphasises the significant data and new knowledge now available to the forensic community due to this thesis. The impact on the research and operational forensic community is briefly mentioned (Figure 7.1), as well as a discussion around future direction made possible by this work (Figure 7.2).

Touch DNA as an evidence type is extremely prevalent in forensic casework load; both in standard operational facilities and specialised counterterrorism operations. It is an invaluable source of information for investigative and justice systems, however due to its constituents STR profiling results are often poor. This can be due to many factors including poor template quantity and quality, sampling inefficiently, or loss through the extraction process. Direct PCR mitigates the requirement for an extraction and introduces all collected template into the PCR for amplification. As it involves less analytical steps there is a reduction in processing time and financial requirements. Direct PCR is therefore an ideal workflow to analyse deeply for the processing of trace DNA sources such as touch DNA.

Direct PCR with respect to an incubation or lysate step, that still facilitates a sample pool, is well founded in the literature. The direct PCR discussed within this work, whereby no pre-treatment of the native or collected sample is conducted, was not previously well defined. There was an understanding that hair, nails, and other whole substrates could be placed into the PCR directly with increased profiling sensitivity, and that touch DNA could be processed from samples such as fabrics or glass with results being superior to their post-extraction counterparts. The analysis of touch DNA on samples of forensic relevance was scarce, as was clear boundaries or considerations as to when or if this workflow was valuable; as extraction and STR profiling kits' efficiency increased the limitations of direct PCR did not outweigh the benefits of time and cost for operational facilities especially given the lack of understanding around the workflow as a whole. The work within this thesis was designed to clearly answer questions about limitations, operational advantages, and niche application value of the direct PCR workflow and well test and define its boundaries.

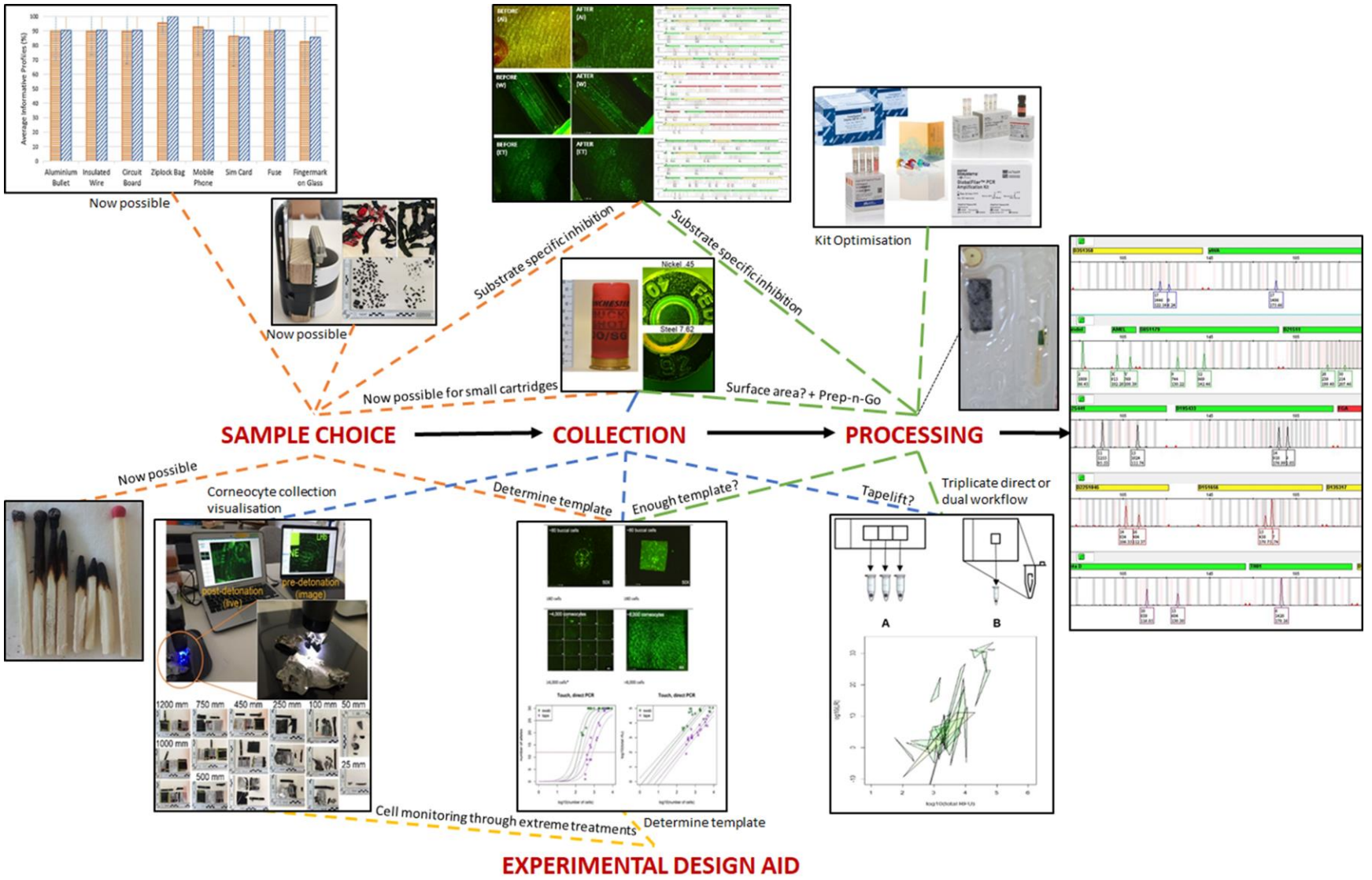


Figure 7.1. Graphical conclusion summarising the major connection between each project and the direct PCR forensic workflow.

7.2 CHAPTER SUMMARY, AND FUTURE DIRECTIVES

Chapter II was centred around improving the forensic community's understanding about the 'processing' capabilities, limitations, and benefits of utilising the direct PCR workflow (Figure 7.1). This has been effectively done with recommendations and considerations clearly stated. The data presented represents the first comparison of commercially available STR kits when applied to a direct PCR workflow. It is extensive and informative as considerations of number of alleles, RFU and comments on additional baseline or artefact presence allow an invaluable resource to research laboratories and operational facilities for assessment of current practise or planning for future changes given their individual requirements. With the exception of when additional STR kits are released there is no explicit need for additional research to be performed in this space publicly; individual laboratories may want to compare personally used STR kits.

Chapter III encompassed assessments of each stage of the forensic DNA typing workflow (Figure 7.1). An extensive set of surface types has been analysed by direct PCR encompassing translational properties to many samples likely to be touched and processed as a forensic exhibit; this adds to the 'sample' possibilities of the forensic workflow. These include the following: mobile phone, SIM card, fuse, glass slide, unfired aluminium cartridge case, insulated wire, circuit board, and a ziplock bag. Processing of touch DNA from these surface types by direct PCR fills a previously observed gap in tested substrates through this workflow. When considering this work, in addition to the surface types sampled in subsequent chapters and previous literature, there is very little with respect to surface properties that have not been discussed in relation to direct PCR amplification capabilities.

The well-established premise that individuals have different propensities to deposit cellular template, in the form of the touch DNA complex, for subsequent collection and STR analysis was supported by an investigation into the development of a shedder classification technique. Individuals were shown to fall into one of three shedder status categories, and their deposition plateaued after 60 mins post handwashing. This classification was translated to EPG quality, for example high shedders produced increased numbers of alleles and/or RFU to intermediate and low shedders. The methods presented describe a quick and inexpensive method to determine someone's deposition potential, and by extension how likely it is to amplify a DNA profile post direct PCR processing. This research falls before the forensic workflow described in Figure 7.1 in that it addresses deposition onto a sample rather than its processing methods. Extension to this work includes the assessment of more donors, as well as how seasonal changes, distinct activities, substrate handling, personal pressures

(i.e. pregnancy, stress) affect these classification categories, or intra-personal classification; will the three categories become a continuum or remain discrete?

Following on from the observation of a relationship between shedder status and DNA profile data definitive visualised cellular material thresholds have been determined for both standard extraction-based PCR and direct PCR workflows; this adds strength and understanding to the 'collection' and 'processing' portions of the forensic workflow, as well as provides guidance for an informative method for experimental design and data analysis tools (Figure 7.1). The parameters examined encompass every workflow possibility: for a full Identifiler® Plus STR profile direct PCR requires ≥40 collected buccal cells with either collection medium, ≥800 collected corneocytes when swabbed, or ≥4,000 collected corneocytes when tapelifted; PCR post extraction requires ≥80 buccal cells with either collection medium, ≥4,000 collected corneocytes when swabbed, or ≥8,000 collected corneocytes when tapelifted. This work also supports the use of DD for triaging purposes on forensic samples or on tapelifts through staining, visualising, and determining the number of cells, and therefore template, present. The methods shown can be utilised in research to better compare sample treatment variables and assess the strength of their results in light of expected outcomes given these thresholds. This study addresses the sentence in Chapter 1.5 "*The amount of cellular material deposited by touch, including corneocytes, keratinocytes, cell-free and exogenous, required for successful STR amplification is currently unknown.*" in full. There is future research potential using the methods and analytical procedures described here by applying inhibitors to the PCR process directly or by sample surface treatment, by assessing other profiling technologies, or by examining additional cell types (Figure 7.2).

As the previous work suggested it was possible to obtain DNA profiles post-extraction from ≥4,000 or ≥8,000 collected corneocytes, dependent on collection media, it was determined that the analysis of touch DNA should be trialled using rapid DNA technology. This was conducted on the ThermoFisher Scientific RapidHIT ID. It was determined that the RapidHIT did not facilitate up-loadable DNA profiles from single fingermarks, as was possible with a standard extraction workflow with sufficient template. Even by increasing the template to sample handling with multiple fingers DNA profiling success was unpredictable and limited. Although this did not utilise a direct PCR workflow, as the template source was from touch deposits its analysis remained in the remit of this thesis and fits into the 'processing' sector of the overarching forensic DNA analysis workflow (Figure 7.1). It was also found that some samples require swabbing or tapelifting rather than direct insertion, even when they

fit within the reservoir, due to the absorbency of the substrate causing an error in lysate flow through the instrument. In its current form, the RapidHIT is not suitable for the analysis of forensic samples from which touch DNA would be the amplification template. Further analysis of this technology should incorporate the methods undertaken in Chapter 3.5 to better identify template requirements. In addition to this, modifications to cartridges or an increased ability to control specific portions of the instrument's workflow should be monitored for future application readiness examinations (Figure 7.2).

Chapter IV, as a major part of the work within this thesis, was designed to address one of the foremost limitations of direct PCR: the inability to re-test a sample. This has been a limiting factor in the use of direct PCR for forensic casework samples in many operational laboratories due to legislative, accreditation, or internal requirements for an eluate to re-test or analyse for a different purpose. This study encompasses template 'collection' and workflow 'processing' choices in unique ways (Figure 7.1). Tapelifts were utilised for porous and non-porous substrates for the amplification of multiple PCRs. In one approach a single tapelift facilitates three direct PCRs and these were shown to be congruent with one another with respect to donor allele numbers, RFU, and the LR value of a donor match. The proposal for these three sub-samples to be considered technical replicates of one another was supported. This allows, for the first time, multiple direct amplification reactions to be performed from a single sample. This could be in the form of the same test or different analyses, for example phenotypic and genotypic. With the premise that the tapelift remnants are usually discarded following direct PCR sub-sample removal, the assessment of tapelifts enabling dual workflow processing was examined. This second approach found donor allele concordance between the direct PCR and PCR post-extraction portions of the same tapelift; each portion was also compared to each other in a way that produced a likelihood of a match and the values further supported the conclusion that sub-sampling a single tapelift is an acceptable approach for STR data production. Amplification through both workflows will allow a 'best case' amplification to be performed whether this is through direct PCR due to increased sensitivity or through extraction due to inhibitor presence.

With the tapelift use methods described here the ability to use direct PCR in a casework setting will become more easily accepted, as when these methods can be applied a major limitation is made moot. The incorporation of these methods is not necessary for a vast number of forensic samples but where touch DNA is suspected to be the template source, and this may be limited in quantity, the

benefits of direct PCR are obvious. The techniques can be approached with confidence as profile concordance was observed between sub-samples for both processing proposals.

Referring to a sentence from Chapter 1.5.1 "*Although there are studies that have compared collection methods from various substrates utilising extraction pathways, there is little research that compares methods with respect to the direct PCR pathway*", Chapter IV also allowed this to be superficially analysed. Due to the small surface area of the samples and the knowledge of touch deposit presence these data are not completely comparable or relevant to forensic casework sample practice. Swabs and tapelifts were compared, using the direct PCR amplification pathway, for six substrate types of various porosities; these included a ziplock bag, rubber mallet handle, electrical tape mounted on a glass slide, circuit board, twine, and cotton fabric. Swabs were observed to facilitate increased STR amplification, however it was not significant for all substrates, and this is understandable in light of the conclusions raised in Chapter III.

The data presented in Chapter V is heavily centred around testing the limits for STR amplification from touch DNA by examining sub-optimal sample types; these samples are not 'sterile' from potential inhibitors and the touch deposition has the potential to be altered in quantity or quality by the production of the sample. Neither matchsticks nor fired cartridge cases are known to allow the production of good quality profiles from touch DNA. The DNA on matchsticks can be lost due to the loss of stick length or may be affected by the heat of the flame. This work represents the only assessment of direct PCR to matchsticks, with no increase to cycling parameters required to produce up-loadable DNA profiles. It has allowed for this sample type to be considered for amplification where a matchstick may be the source of ignition in an arson event. It adds to our knowledge about what 'samples' can be processed with direct PCR (Figure 7.1). There is no basis for future research with this one sample type.

The DNA on ammunition casings has the potential to be damaged due to heat, as well as pressure and chemical depositions through firing. Loading ammunition into a magazine can cause different levels of deposition prior to firing due to the number of bullets loaded systematically, and the pressure and technique required to do so; the template present on these casings is trace in nature. The data within Chapter 5.4 presents the direct PCR amplification of touch DNA from fired casings ('samples') as a viable 'processing' option (Figure 7.1). Although the processing of smaller calibre bullets by direct PCR is advised, 12 G casings should be analysed through a standard extraction-based workflow. It was noted that a simple addition of Prep-n-Go™ to the direct PCR amplification increased

the PCRs robustness against any inhibitors present due to the primer or GSR post-firing (Figure 7.1). This methodological find is important and valuable for the wider forensic community where direct PCR may be applied to sub-optimal sample types. Following on from these studies, the optimisation of direct PCR in the presence of inhibitors, specifically through additives rather than cycling parameter alterations, would be valuable for the forensic community (Figure 7.2). The observation of artefacts within the post-firing sample profiles generated following direct PCR is important to note. As these appear to be consistent, future research into categorising these, in terms of location and morphology, more acutely is important (Figure 7.2). By using the methods outlined in Chapter VI, with respect to pre- and post- treatment cell visualisation, the firing process could be monitored for cell presence, movement, or loss and add important insight to the conclusions drawn about the GSR or primer presence's role in STR profiling or how the firing process itself affects the template quality; PCR inhibitor identification is imperative if true optimisation is desired (Figure 7.2).

The work presented in Chapter VI represents the only study to detonate touch DNA samples with a reasonable charge size and obtain STR profiles that were considered up-loadable to a DNA database. The boundaries of what was considered possible have been expanded, and the data presented supports the STR typing of post-detonation IED components. These research papers examine an extensive range of variables and have resulted in the successful amplification of STRs from touch DNA from fragments as close as 10 mm from the charge. Recommendations have been made to refrain from collecting twin core wire and polypropylene plastic sheet due to the inhibitory effects these substrates cause to direct PCR following detonation. It has been noted that sample surface area, dirt presence, exposure to the charge, distance from the charge, surface type, and cellular deposition quantity all play a role in how the forensic workflow should be approached. These data address 'sample choice' through assessment of the aforementioned variables, template 'collection' methods and triaging through DD visualisation, and 'processing' choice with respect to the sample's surface area or the prospect of substrate specific inhibition (Figure 7.1).

There is much that can be done in the sphere of IED genetic profiling following the discovery that STR profiling is possible from touch DNA template on post-detonation substrates (Figure 7.2). This includes increasing the charge size, changing the charge type, initiating mock IED production lines to investigate different construction methods, performing detonations in different environmental locations, and altering the genetic targets or technology utilised.

In addition to the research suggested above, the application and optimisation of direct PCR to MPS technology and CE-based SNP panels would also be of interest and could be aided by the results obtained or methods described within this work. This would give analysts additional tools for human identification in a casework or investigative setting.

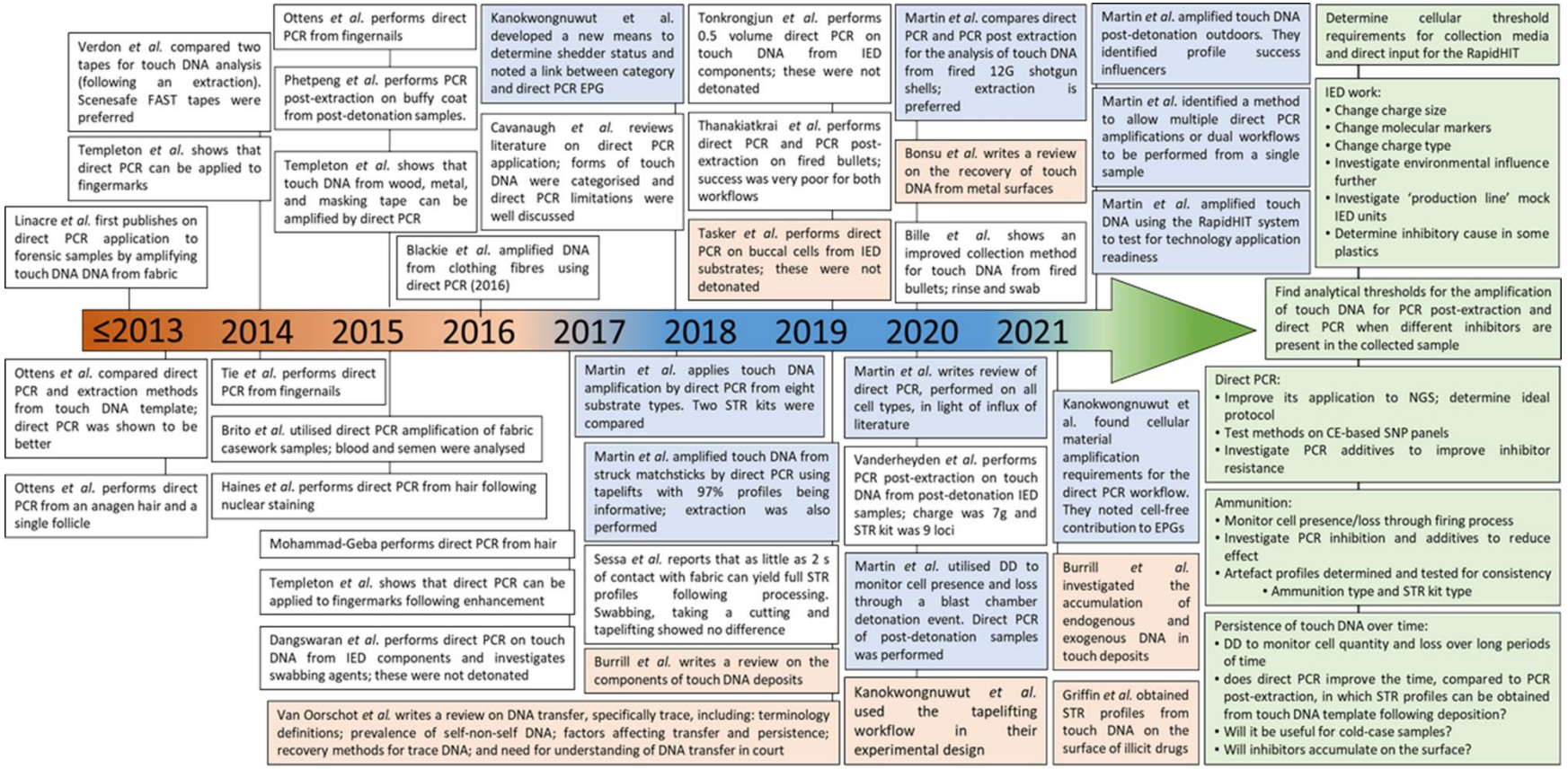


Figure 7.2. Flow diagram containing some of the key research papers around touch DNA and/or direct PCR, in the form utilised in this thesis. The blue boxes contain work within this thesis, the salmon boxes are work that referenced work presented in this thesis in a meaningful way, and the green boxes contain possible future research ideas either suggested or made possible by the work within this thesis.

7.2 FINAL STATEMENT

Before the commencement of the work within this thesis, the boundaries of direct PCRs application to sample processing were limited in their framework. The literature on touch DNA analysis was sparse, with most of the work conducted being from touch DNA deposition on glass or on fabric. It was well understood that direct PCR improved the sensitivity of touch DNA amplification and improves EPG results, however guidance to forensic laboratories as to when it should and should not be applied to their workflows was not sufficient. The benefit of increased sensitivity did not outweigh the inability to retain a portion of the sample for subsequent testing or the unknown parameters of what substrates benefit from the workflow choice or how inhibitor presence can be managed or addressed. This thesis aimed to bring a fuller description of the limitations, and potential mitigations to these, direct PCR has to inform the forensic community about when its niche application would be valuable to their research or operational outcomes.

The key requirements for considering direct PCR for an analytical workflow are:

- The presence of low-level template in quantity or quality, such as touch DNA. A requirement of ≥4,000 recently deposited cells are required when swabbing for full STR profiling generation.
- A sample with a relatively small surface area or targeted swabbing capacity
- Low to medium inhibitor presence on the surface of the sample
- Prep-n-Go™ addition to increase stability in the PCR against inhibitors
- That swabs be used in preference to other sampling devices

In addition, the purpose of the genetic testing must be considered as these have different requirements within the direct PCR workflow. For intelligence-based work the main restriction to the use of direct PCR would be too much template. Where potential template is considered low this method can be applied to collected material from any surface type. In a forensic casework setting care must be taken around assertions of activity from the produced EPGs due to the sensitivity of the method and that the rate of latent DNA transfer via vectors is known to be high. The requirement for requirement for re-testability, retention, or quantification data will lead the choice of whether a tapelift or swab is required for sampling, and the processing method can be chosen according to the scope of the case (Figure 4.1). There is no need for sample retention, consideration of the above requirements are still vital.

Direct PCR, in the form discussed within this thesis, should not be applied to samples with high template material such as blood or large quantities of saliva; only ≥40 buccal cells are required for a full profile through direct methods. The analyst should refrain from this workflow if there are high levels of inhibitors present on the surface that could be collected and placed into the PCR; if these surface inhibitors can be removed, such as through blowing compressed air over it, then this can be attempted. If a standard extraction pathway can be performed from the sample with STR result confidence, and the benefit of reduced financial strain or processing time is not of importance then this workflow remains ideal.

The developments of touch DNA analysis research that are accelerated, enabled, and suggested by the work within this thesis, from the data presented and methods described, are exciting for the human identification potential of forensic samples. Both local crime and counterterrorism operational laboratories can utilise the findings within this work to advise on workflow options, with respect to sample choice, collection of template, and processing choices far more conclusively than previously possible.

CHAPTER VIII:

APPENDIX

Manuscripts enclosed:

Young, J., **Martin, B**., Linacre, A., (2019). "*Evaluation of the QIAGEN 140-SNP forensic identification multiplex from latent DNA using massively parallel sequencing*." Australian Journal of Forensic Sciences

Young, J., **Martin, B**., Kanokwongnuwut, P., Linacre, A (2019) "*Detection of forensic identification and intelligence SNP data from latent DNA using three commercial MPS panels.*" Forensic Science International: Genetics Supplement Series

8.1 ADDITIONAL MANUSCRIPT I:

8.1 Evaluation of the QIAGEN 140-SNP forensic identification multiplex from latent DNA using massively parallel sequencing

Manuscript published: *Australian Journal of Forensic Sciences*, 2019

Author	Participation
Jennifer Young	Assisted with experimental design, carried out laboratory work, and wrote the manuscript.
Belinda Martin (Candidate)	Assisted with experimental design and carried out laboratory work
Adrian Linacre	Helped edit the manuscript

8.1.1 Abstract

Direct PCR can be used to successfully generate full STR profiles from latent DNA. However, some substrates have been shown to be more problematic and in some cases only partial profiles are recovered. As latent DNA is present on the surface of objects in very low quantity, and potentially low quality, the fragment lengths targeted by STR typing may be too large to successfully amplify all markers. As an alternative, QIAGEN have developed a 140-SNP multiplex that targets much shorter amplicons and generates extremely low probabilities of any two unrelated individuals having identical genotypes. Here, we present the first forensic identification SNP data from latent DNA using massively parallel sequencing. We applied the QIAGEN 140-SNP forensic identification multiplex to swabs collected from multiple substrates (including mobile phone, fingerprint, wire, zip-lock bag and SIM card) and multiple donors.

8.1.2 Introduction

MPS is an emerging field in forensic science, which offers the ability to detect and analyse low quantity and low quality DNA[1–3]. Previous studies have shown the application of MPS to generate STR profiles[4,5], mitochondrial genomes[6,7] and SNP data[8,9] for human identification. DNA extraction from surface swabs results in a significant loss of DNA, thus an insufficient template is available at the PCR step to generate informative (i.e. 'up-loadable' to the Australian NCIDD) STR DNA profiles. Direct PCR (which eliminates the DNA extraction step) is an effective method for successfully generating STR profiles from touched surfaces[10,11]. To further improve the recovery and detection of latent DNA, often a more sensitive technique is required. Here, MPS technology is coupled with direct PCR of the QIAGEN 140-SNP Forensic Identification Panel to generate genetic profiles from items touched by known

individuals. MPS enables multiple samples to be sequenced in parallel, more DNA markers to be typed per sample, and thus offers a high discriminatory power between individuals from trace levels of DNA.

8.1.3 Methods

A total of 83 samples were analysed using the QIAGEN 140-SNP Forensic Identification Panel using MPS. Samples consisted of five reference samples (one buccal swab per individual), 75 touch samples (five individuals × five items × three replicates), and three no template controls (NTC) where no swab or DNA extract was added to the PCR. A buccal swab was collected for five participating individuals and DNA was extracted using the QIAGEN DNA Mini Kit. The DNA concentration was quantified and normalized to 1 ng/µL and 1 µL of DNA extract was added to the 140-SNP PCR multiplex. Reference samples were processed separately from all touch DNA samples until after the incorporation of sample-specific unique index sequences at the library preparation step. Each individual touched five substrates (see Figure 8.1 (a)) for 15 s and, for replication, each item was repeated three times (A, B and C) by all individuals. DNA was recovered from each item using a sterile nylon swab pre-moistened with 2 µL of 0.1% Triton™ X-100. The swab tip was then cut directly into a 0.2 mL PCR tube. The GeneRead DNAseq Targeted Panel V2 library preparation workflow (QIAGEN) was used (with the PCR modified to include 2 µL of PREP and GO for the touch DNA samples and NTCs) and sequenced on the Illumina MiniSeq Platform. Raw sequences were de-multiplexed using the Illumina Software and the QIAGEN Biomedical Genomics Workbench 5.0.1 'Qiagen GeneRead Panel Analysis' workflow was used to map sequences to the Human Genome reference, quality filter and call variants. R was used to visually compare genotypes.

8.1.4 Results and Discussion

The average number of SNPs detected from the reference samples and touch DNA samples was 81 and 75 respectively. The number of SNPs detected was not influenced by the individual or the item swabbed (Figure 8.1). The average number of SNPs detected in the no template controls was 44, which was significantly lower than that detected from both the reference and touch DNA samples.

Of the 75 touch DNA samples included in this analysis, 64 samples were correctly classified to the respective donor (Figure 8.2). For nine of the samples that were misclassified, fewer than 50 SNPs were detected. These samples formed a sixth cluster due to the high level of missing data. A minimum threshold of 50 SNPs was required to accurately identify a match, with the exception of three samples (4-5A, 4-5B and 2-2B – highlighted in red in Figure 8.2) which were wrongly classified in this analysis

despite detection of more than 50 SNPs. By applying a minimum SNP threshold of 50 to call a reliable result, negative control samples were excluded as all three controls fell below this threshold.

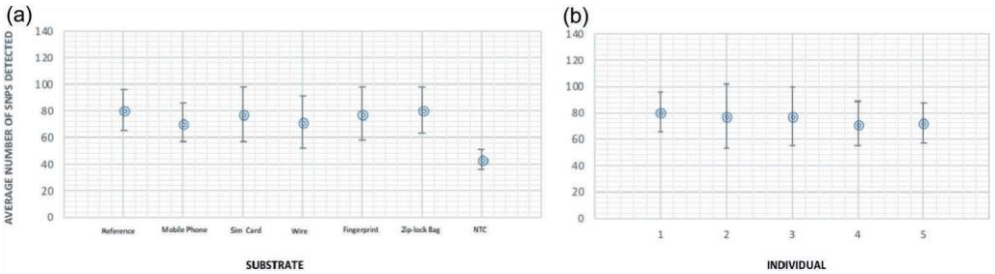


Figure 8.1. Average number of SNPs detected from (a) reference samples, touch DNA samples (shown per item) and no template controls, and (b) each individual.

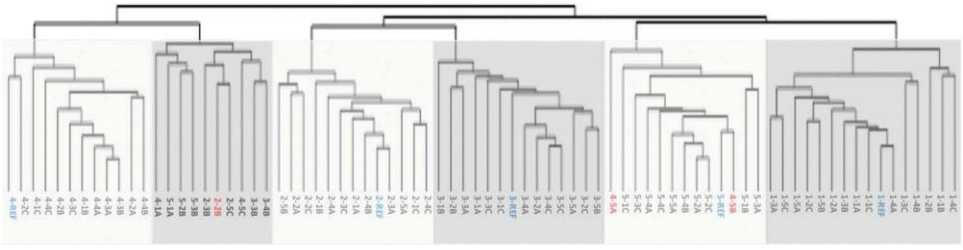


Figure 8.2. Cluster dendrogram showing the similarity of genotypes between all reference and touch DNA samples.

8.1.5 Conclusion

This study demonstrates that informative SNP data can be obtained from trace amounts of DNA transferred to items touched for a short period of time (only 30 s). It highlights the importance of including negative control samples and the potential issues in recovering profiles from items handled by multiple individuals. It indicates minimum thresholds are required to confidently call a profile due to the increased sensitivity of the technology.

8.1.6 Conflict of Interest

No potential conflict of interest was reported by the authors.

8.1.7 Funding and Acknowledgements

Funding provided by the DSTG. The authors wish to thank Ivonne Petermann (QIAGEN)

8.1.8 References

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8.2 ADDITIONAL MANUSCRIPT II:

8.2 Detection of forensic identification and intelligence SNP data from latent DNA using three commercial MPS panels

Manuscript published: Forensic Science International: Genetics Supplement Series, 2019

Author	Participation
Jennifer Young	Assisted with experimental design, carried out laboratory work, wrote the manuscript
Belinda Martin (Candidate)	Carried out laboratory work and edited the manuscript
Piyamas Kanokwongnuwut	Carried out laboratory work and edited the manuscript
Adrian Linacre	Helped edit the manuscript

8.2.1 Abstract

MPS offers the ability to detect and analyse low quantity and quality DNA present on touched items. Here, we present MPS identity and intelligence SNP data generated from latent DNA using direct PCR and three different forensic panels: (1) the QIAGEN 140-SNP forensic identification multiplex (2) the 24 SNP HIrisplex System and (3) the 165 SNP Precision ID Ancestry Panel. The effect of individual and substrate type (wire, glass and zip-lock bag) on the recovery of SNPs and prediction accuracy was examined and compared across the three panels.

8.2.2 Introduction

Latent DNA is transferred to items via touch and is often the only source of DNA present on casework exhibits. However, it is well documented that STR profiling success from handled items can be less than 20% due to significant DNA loss during sampling and DNA extraction [1,2]. This is a major issue for forensic laboratories as it not only adds significant financial loss and decreases productivity, but ultimately prevents the ability to resolve cases and bring justice to grieving families. To overcome this issue, direct PCR has been explored as an effective means to bypass the DNA extraction step and generate usable profiles [3]. Furthermore, STRs are not useful when no reference profile is available for comparison so instead intelligence data are required. Our previous study demonstrated the success of obtaining identity SNPs from latent DNA using direct PCR coupled with MPS [4]. Here we expand this by including and comparing the recovery of intelligence SNP data from latent DNA using two additional forensic MPS-SNP panels.

8.2.3 Methods

Three individuals were used to assess the recovery of latent DNA using three different forensic MPS-SNP panels (1) QIAGEN140-SNP Identification Multiplex (herein called 140 SNP-plex), (2) Precision ID Ancestry Panel (herein referred to as Ancestry), and (3) Ion AmpliSeq HID Phenotyping Panel using the HIrisplex System (herein referred to as HIrisplex). Each individual touched three substrate types (wire, glass and zip-lock bag) for 15 s. Substrates were swabbed and subject to direct PCR using one of the three MPS panels (following manufacturer's instructions). All sampling and swabbing, and the 140 SNP-plex library preparation (using the GeneRead DNAseq Targeted Panel V2 library preparation workflow) were done in-house at Flinders University. PCR amplification and library preparation for the HIrisplex and Ancestry panels were done at ThermoFisher Scientific, Melbourne. For the 140 SNP-plex, raw sequences were de-multiplexed using the Illumina Software and the QIAGEN Biomedical Genomics Workbench 5.0.1 'QIAGEN GeneRead Panel Analysis' workflow was used to map sequences to the Human Genome reference, quality filter and call variants. The HIrisplex and Ancestry libraries were prepared using the Ion Chef and sequenced on the Ion GeneStudio S5 System. Phenotype predictions for the HIrisplex were determined using https://hirisplex. erasmusmc.nl and the ancestry predictions (to major continental groups: Europe, Oceania, East Asia, Africa, South Asia, America) were obtained using HID SNP Genotyper software. For each panel a buccal swab from each individual was analysed as a reference sample.

8.2.4 Results and Discussion

Of the three panels tested, the 140 SNP-plex showed the lowest % SNP detection (Figure 8.3 A and B). 100% of SNPs were detected from all three reference samples using the HIrisplex and Ancestry panels, whereas only 64%, 56% and 40% of SNPs were detected with the 140SNP-plex for individuals 1, 2 and 3, respectively. Similarly, the 140 SNP-plex showed the lowest % SNP detection from the touch samples with an average of 39.0% compared to 84% and 65% for the HIrisplex and Ancestry Panel respectively. Although the 140 SNP-plex showed lowest SNP detection, the average % SNP concordance was comparable to the other panels (92.9%, 77.6% and 89.5% for the 140 SNP-plex, HIrisplex and Ancestry Panel, respectively) and the prediction accuracy was consistent across the three panels.

For all three panels, the average % of SNPs detected correlated with individual (Figure 8.3 A).The effect of individual was most prevalent in the ancestry panel which showed an average decrease in % SNPs of 47.5% from individual 1 to individual 3 (compared to only 18.5% and

15.3% for the 140 SNP-plex and HIrisplex panels, respectively). This suggests that the impact of individual on SNP detection may be related to the total number of SNPs targeted in the panel; the more SNPs included in the panel, the greater influence of individual. The effect of individual was also evident in the prediction accuracy for all three panels (Figure 8.3 C), which is unsurprising as a decrease in SNP detection will limit the ability to correctly assign identity or phenotypic characteristics.

There were no consistent trends observed between panels based on substrate type (Figure 8.3 B). For the HIrisplex, the wire showed the lowest average % SNP detection and concordance, whereas for the ancestry panel the glass substrate had lowest success; this was mainly due to five glass slide samples which failed during either library preparation or sequencing and thus generated no genotype data. In contrast, all three substrates were comparable for the 140 SNP-plex.

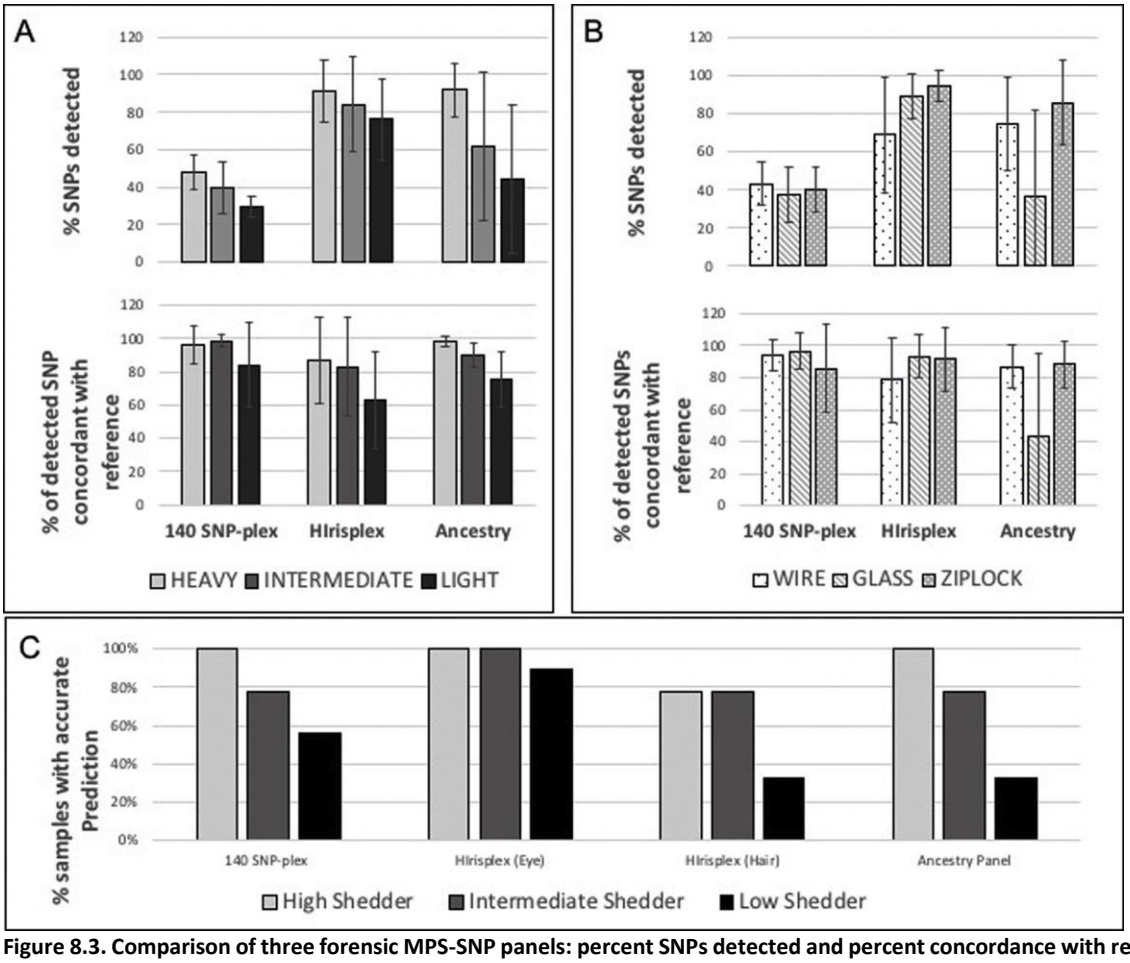

Figure 8.3. Comparison of three forensic MPS-SNP panels: percent SNPs detected and percent concordance with reference per individual (A) and substrate type (B), and the percent samples with accurate identity, phenotypic and ancestry predictions.

8.2.5 Conclusion

This study demonstrates the increased success rate of generating useable genetic data from latent DNA using direct PCR coupled with MPS for either identity or intelligence. For identity alone (140 SNPplex), we demonstrate a 78% success compared to < 20% success reported previously for STR profiling [1,2]. For all three panels combined, accurate predictions were obtained from 70% of the samples. Within each panel, SNP detection, and prediction accuracy correlated with the individual. Overall, the 140 SNP-plex detected the lowest percent SNPs from both reference and touch samples, however, this did not result in the poorest prediction success. This suggests that panels with a higher number of target SNPs can tolerate a higher rate of missing data to obtain accurate predictions, further supporting the use of SNPs and MPS for low quality, low quantity latent DNA samples.

8.2.6 Conflict of Interest

None.

8.2.7 Funding and Acknowledgements

Funding for this project was from Defence Science and Technology Group and National Institute of Forensic Sciences.

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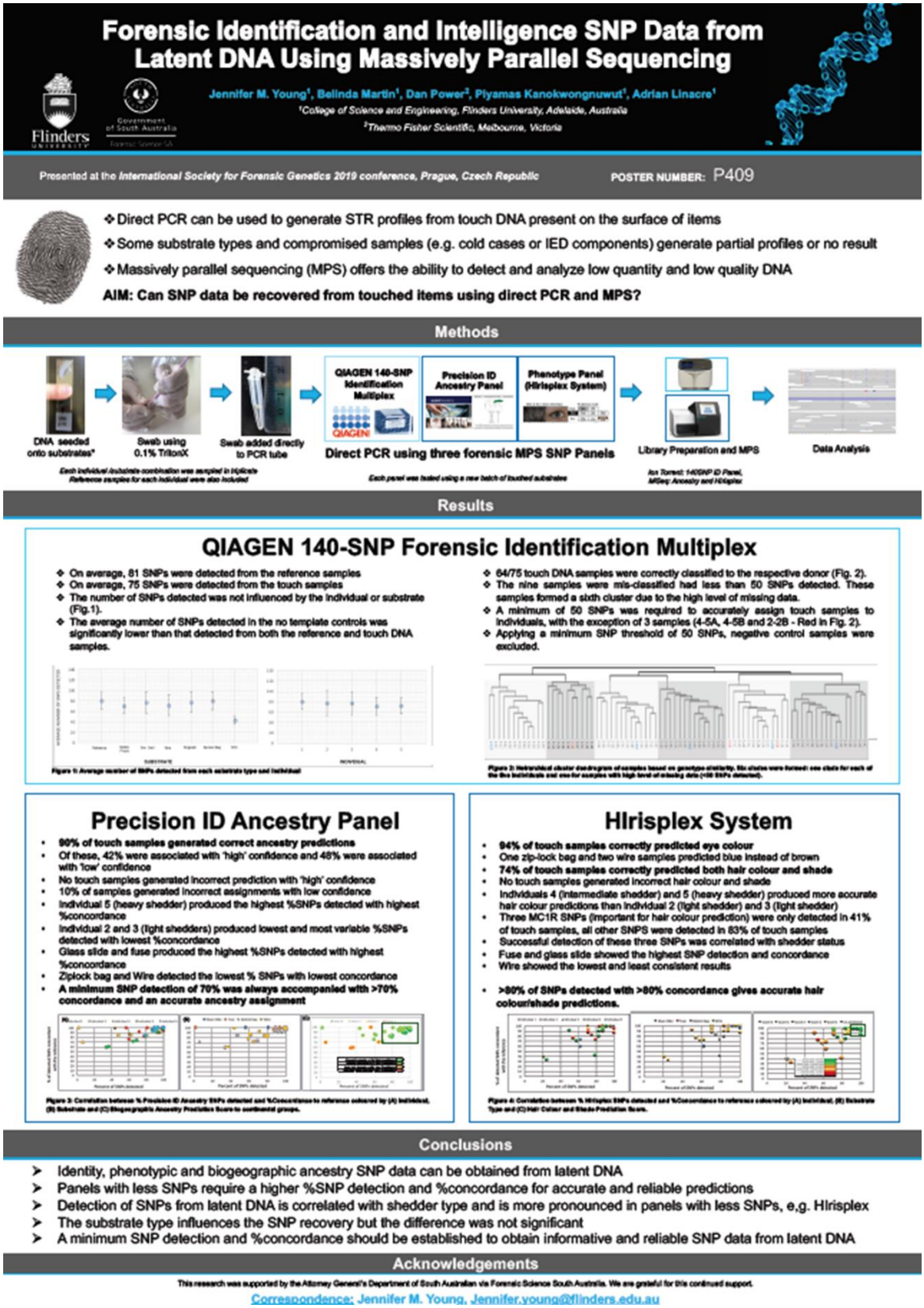

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28 July 2021

Belinda Kate Martin
Flinders University
Sturt Road
Bedford Park SA 5042

Dear Belinda,

APR.Intern Internship Completion Record | DST/Flinders (Belinda Martin) INT - 0996

Congratulations on completing your APR internship!

This letter is to confirm that you have successfully completed the above-listed internship facilitated by APR.Intern, which is part of the Australian Mathematical Sciences Institute. The internship is supported by the Australian Government Department of Education and you have met the Intern requirements of the National Research Internships Program for an industry rebate.

APR.Intern Reference: INT - 0996
Location: SA
Industry Partner: Defence Science and Technology
Project Title: InFoDust: Recovery of dust microbiomes from personal belongings for counter-terrorism intelligence.
Student Institution: Flinders University
Academic Mentor: Dr Jennifer Young
Duration of Project: 3 months
Study Load: Full-Time

If you require any more information, please contact us by emailing contact@aprintern.org.au or phoning 03 8344 1785.

Yours sincerely,

LISA FARRAR *National Program Manager* APR.INTERN

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8.3 APR INTERNSHIP FINAL REPORT

This report is an amended version of the document submitted as part of the APR scheme completed during the candidature period, in July 2021. It has been approved by the Defence Science Technology Group DSTG in its current format for inclusion in the Appendix Chapter of this thesis.

8.3.1 Project background/summary of project

Environmental DNA (eDNA) has been used extensively in environmental science from historical reconstructions and monitoring to species detection and has even been applied to human health assessment. The ability to take an environmental sample, such as water or soil, and accurately determine the microbial community contained within could have substantial benefits to the field of forensic science. Identifying key microbial communities that are unique to environmental samples from specific locations would enable provenance estimates to be made for samples from unknown locations. The applications of this would be numerous in the field of forensic science, however, eDNA has not gained the same momentum in forensic science as it has in other disciplines. This is partially due to the cost of validated Next Generation Sequencing (NGS) kits, lack of research with a specific forensic application utilising eDNA, and the absence of individuals within the field with the specific knowledgebase to perform the required data analysis. As additional studies are performed that bring NGS into a forensic framework, some of these issues can be addressed such that eDNA may become integrated into forensic science applications.

One matrix that is encountered routinely in casework, both locally and internationally, is soil; specifically dust on soil-derived items. Within the soil matrix diversity in both organic and inorganic materials can be found. This includes the minerology of the soil, determined using X-Ray diffraction (XRD), giving it characteristics that allow soils from similar locations to be grouped. The combination of a soil's minerology, colour, density, particle size, pH, and texture make up the physical properties of the sample and can act as an identification matrix for forensic characterisation or sample interpretation. Until recently this was the extent of forensic analysis, with respect to soils, however recent application of the fungal internal transcribed specer1 (ITS), prokaryotic 16S rRNA and eukaryotic 18S rRNA NGS markers have shown that they have spatial prediction capabilities when applied to bulk samples. The signals present within bulk samples taken at ten South Australian sites were shown to be more similar within sites than between them. Although this presents the promise of forensic soil linking between sites or samples the actual relationship between the bulk sample and the sample collected from evidence is unknown. In fact, the ability to collect dust fractions from samples for biological analyses and our subsequent ability to amplify these markers is unknown. This work aims to devise a collection method that facilitates the extraction and amplification (ITS1) of DNA

within a collected dust sample from a range of substrate types typical of those experienced in counterterrorism operations. In addition to this, the soil extraction method will be modified to allow adequate DNA extraction and the retention of the dust sample for XRD analysis. The projection of sequencing results onto a database of the soils collected through this study, as well as those previously collected around South Australia, will provide information as to the most useful taxa for spatial prediction. This will be analysed to identify what collection method yields the most predictively useful data to advise technicians in the future.

Results will be utilised in a DIP grant for simulated casework and additional studies, such as a pipeline and AI training system to perform spatial prediction based on multiple NGS markers and XRD results, to move this into a workflow that can be implemented for casework. When completed, these techniques have the potential to provide additional tools to address a person of interest's claims of movement or origin in cases of national security.

8.3.2 Summary of research

8.3.2.1 Research Method

Three bulk soils were chosen around SA with different classifications, clayey sand, loam and sandy loam, were chosen with all being non-calcareous. These are outlined below in Table 8.1.

Table 8.1: Bulk soil samples used within this body of work.

Site	Type	Colour when Moist	pH	Effervescence	Location
FLB0002	Clayey sand	5YR 4/3	5.41	Non-calcareous	Mount Crawford/Williamstown (North)
KAN0001	Loam	10YR 5/4	5.87	Non-calcareous	Cape Jarvis (South)
KAN0002	Sandy loam	10YR 4/4	5.13	Non-calcareous	McLaren Vale (South)

Substrates onto which dust would be distributed and collected were chosen to represent commonly encountered exhibits in counterterrorism border-control cases. These were 70 x 150 mm substrates as follows: plastic mobile phone case; fabric (to simulate clothing or a suitcase); faux leather case (to simulate the outer surface of a passport). A dust chamber, designed to simulate dust deposition on those items, along with all equipment and substrates were cleaned with a soap solution, followed by 2% sodium hypochlorite bleach solution and finally 70% ethanol. Negative controls were taken by running the dust chamber with empty Eppendorf tubes within it, of the Sellotape, swab and

fabric used within the trial, as well as surface controls of each substrate sampled with a swab or tapelift. Triplicate dust controls were collected by leaving three Eppendorf tubes open within the sample compartment of the dust chamber for the duration of that soil's trial. The dust chamber was run for two increments of 30s per substrate before the substrate was removed and sampled. The bulk soil was accessed and redistributed within the base chamber after every third distribution event to ensure a consistent distribution of dust over the collection trials.

Following distribution over the surface areas, collection via different mechanical means ensued. Swabbing and tapelifting was performed for the plastic and faux leather substrates, while tapelifting or a cutting was taken from the fabric swatch. The substrate was halved width wise, by drawing a line, to allow for both collection mediums to be used on each sample; this was performed to reduce intra-sample distribution variation. Swabbing was performed with a puartin cotton swab pre-moistened with 60 µL 0.1% Triton™ X-100 following the written SOP and the head of the swab removed into a 1.5 mL Eppendorf tube. Tapelifting was performed using a 5 x 2.5 cm segment of Sellotape® following the written SOP. The tape was rolled, with the adhesive side facing inward, and placed into the base of a 1.5 mL Eppendorf tube with sterile tweezers. For the fabric cutting samples, one 3 x 3 cm square of fabric was removed and placed into a 1.5 mL Eppendorf tube. *SOPs have been removed for this report's inclusion into the intern's PhD thesis' Appendix chapter.*

A modified extraction method was performed using the reagents from the DNeasy® PowerSoil® Pro (QIAGEN, Hilden, Germany), zirconium beads, and Casework Spin Baskets (Promega Corporation, Sydney, AUS). This method is detained in the SOP presented to the APR partner; briefly, 600 µL of C1 was added to the swabs, tapelifts, or cutting and incubated for 10 mins at 65 °C and 650 rpm. The collection medium was then placed into a spin basket, within the same tube, centrifuged and disposed of. This solution was resuspended and placed into tubes appropriate for bead beating with zirconium beads. From here, the method was as outlined in the manufacturer's protocol. Extraction blanks were performed with each extraction batch to monitor the background DNA levels in the extraction reagents. Pellets were kept for further analyses to observe whether XRD could be obtained from the same sample as the DNA sequencing data.

Extracts were quantified using a QUBIT™ system, nut no normalisation was performed for dust samples based on these results. Extracts were amplified using metabarcoding markers for the internal transcribed spacer 1 (ITS1) for fungal communities. All primers were modified to include dual barcodes for each extract and Illumina sequencing adapters. PCR amplification was performed in 12.5 µL comprised of 2 mM $MgSO_4$, 0.6 mM dNTPs, 0.4 µM of each primer, 0.3 U Platinum™ Taq DNA Polymerase High Fidelity in 10x reaction buffer (Introgen™, Carlsbad, CA, USA),

and 1.25 µL DNA extract. For bulk soil samples a 1:10 extract dilution was performed for FLB0002, while 1:5 was performed for KAN0001/0002 due to their DNA concentration values. PCR conditions are seen below.

94 °C	94 °C	52 °C	68 °C	68 °C
1 min	30 s	30 s	30 s	7 min
		X35		

PCR amplifications were performed in triplicate, and pooled post-amplification, to minimise the effect of PCR bias on results. In addition, non-template controls (NTC) were included in each PCR set-up to monitor background DNA levels present in the PCR reagents. PCR products (3 µL) were subjected to 1.5% (w/v) agarose gel electrophoresis at 85V for 30 mins to allow pooling into four solutions (each comprised 21 samples) based on band fluorescence once visualised and compared. The samples put into each pool were distributed evenly among the four with respect to their identity (negative/bulk/sample) and band fluorescence so each pool was roughly equal with respect to PCR product input. These pools were subjected to purification using the 0.8x Agencourt AMPure XP PCR Purifiction process (Beckman Coulter Genomics, NSW). The purified products were quantified using the Aglient 2200 TapeStation (Agilent Technologies, Santa Clara, CA, USA), pooled into a single library to equimolar concentration and were quantified again using the TapeStation to obtain a library concentration. An aliquot of the library, 10 µL, measuring 88.1 nM was sent for sequencing at SAHMRI using a 300 cycle Illumina MiSeq.

8.3.2.2 Intern's Contribution

The intern performed initial trials on a custom dust chamber to determine distribution patterns and quantities of the dust fraction of soil, to ensure project's feasibility and to optimise the chamber's performance. Dust distribution events were conducted for 27 samples comprising three different bulk soil samples and three substrate types. Following distribution, the collection of dust from these 27 substrates was performed using either a swab, tapelift, or cutting. Control dust samples, representative of the distribution within the dust chamber for diversity comparison against samples post collection, and bulk soil samples were also taken. Following collection, an extraction method was devised and protocols were altered to facilitate extraction from these collection mediums. Post extraction eluates underwent triplicate ITS1 target PCR amplification and were later collated. These were performed on each soil, dust and collected dust sample; each had a unique combination of primer barcodes to allow for demultiplexing downstream. The intern performed gel electrophoresis to determine approximate relative concentrations of each sample to allow for appropriate pooling, and performed the pooling. Only 1 replicate of each negative control was pooled

to ensure the library was not overly dilute. The intern performed a 0.8x Ampure purification to remove primer dimer within the pooled samples; this was performed on four pools comprised of 21 initial PCR samples. After quantifying the four pools, they were combined into a single library with equimolar representation. This was quantified a final time to find the molarity of the library before sequencing was performed.

At the date of APR completion, the sequencing data had not been obtained. Therefore, the intern performed analysis based on the gel fluorescence and initial extract QUBIT results to observe any trends. The intern will analyse the demultiplexed data received from SAHMRI using QIIME2 and present an updated report directly to the funding provider outside of the APR scheme.

SOP documentation was written for the collection of dust from non-porous items by swab and porous items by tapelift, as well as for the altered extraction method required for this analysis. *These have been removed for this report's inclusion into the intern's PhD thesis' Appendix chapter.*

8.3.2.3 Research Results and Outcomes

SOPs were written and are presented to the APR partner. *These have been removed for this report's inclusion into the intern's PhD thesis' Appendix chapter.* The proposed extraction method variations proved successful in the production of a pellet, able to be retained, and DNA extract with an appropriate concentration for downstream NGS amplification.

The table below (Table 8.1) indicates the extract concentration, determined by QUBIT, and the relative concentration of each PCR product, based on gel electrophoresis results. This, in the absence of sequencing data, provides an indication as to the success of extraction and subsequent ITS1 amplification for each sample. As the quantification method is not specific for ITS DNA, rather all DNA within a sample, this is not entirely informative as to what the PCR success may be; this can be due to no target DNA within the sample or inhibitors remaining through the extraction. The bulk soils produced high DNA concentrations, however, their PCRs were heavily inhibited as indicated by the lack of product bands within the gel, considering the much lower quants of the dust samples and collected samples. Soils are as follows: A is FLB0001; B is KAN0001; C is KAN0002.

Although the difference was not always significant, trends appeared within the dataset when analysing both the DNA extracted and the subsequent PCR efficacy (Table 8.3). When considering these response variables, the tapelift out-performed the cutting taken from the fabric, while the swab produced higher quants and subsequent PCR product than the tapelift from the other two surface types, with the exception of Soil C from the leather. The distributions of all collected samples are shown in Figure 8.4.

Table 8.2: Sample ID, extract quant, and PCR concentration relative to the highest sample (8). Each letter corresponds to a soil sample (A/B/C), sample type (F/L/P), or collection method (T/S/C).

Tube#	Sample ID	Quant	PCR concentration	Tube#	Sample ID	Quant	PCR concentration	Tube#	Sample ID	Quant	PCR concentration
1	AF_C_1	0	0.33	33	BL_T_2	0.0796	0.33	65	CD_1	0.124	0.33
2	AF_C_2	0	0.25	34	BL_T_3	0.796	0.33	66	CD_2	0.124	0.25
3	AF_C_3	0	0.25	35	BP_S_1	0.295	0.40	67	CD_3	0.218	0.20
4	AF_T_1	0	0.33	36	BP_S_2	0.968	0.50	68	EXT B3 NEG	0	0.20
5	AF_T_2	0.02	0.22	37	BP_S_3	0.652	0.33	69	ABS 1	10.5	0.20
6	AF_T_3	0.028	0.29	38	BP_T_1	0.086	0.25	70	ABS 2	17.1	0.20
7	AL_S_1	0.05	0.29	39	BP_T_2	0.105	0.20	71	ABS 3	12.2	0.20
8	AL_S_2	1.53	1.00	40	BP_T_3	0.0804	0.20	72	BBS 1	too high	0.20
9	AL_S_3	0.1	0.50	41	BD_1	0.786	0.20	73	BBS 2	too high	0.20
10	AL_T_1	0.069	0.25	42	BD_2	0.7	0.20	74	BBS 3	too high	0.20
11	AL_T_2	0	0.25	43	BD_3	2.23	0.20	75	CBS 1	13.6	0.20
12	AL_T_3	0	0.25	44	EXT B1 NEG	0	0.20	76	CBS 2	23.6	0.20
13	AP_S_1	0.35	0.33	45	EXT B2 NEG	0	0.20	77	CBS 3	23.2	0.20
14	AP_S_2	2.8	0.33	46	CF_C_1	0	0.20	78	BULK EXT B5 NEG	0	0.20
15	AP_S_3	0.83	0.33	47	CF_C_2	0	0.20	79	EXT B4 NEG	0	0.20
16	AP_T_1	0	0.22	48	CF_C_3	0.0652	0.25	80	NEG T 1	0	0.20
17	AP_T_2	0	0.22	49	CF_T_1	0.0732	0.25	81	NEG T 2	0	0.20
18	AP_T_3	0.56	0.20	50	CF_T_2	0.516	0.22	82	NEG T 3	0	0.20
19	AD_1	0.148	0.29	51	CF_T_3	0.6	0.22	83	NEG F C 1	0	0.20
20	AD_2	0.256	0.29	52	CL_S_1	2.18	0.25	84	NEG F C 2	0	0.20
21	AD_3	0.178	0.25	53	CL_S_2	1.4	0.20	85	NEG F C 3	0	0.20
22	BF_C_1	0.064	0.22	54	CL_S_3	0	0.20	86	NEG L S 1	0	0.20
23	BF_C_2	0	0.22	55	CL_T_1	0.366	0.25	87	NEG L S 2	0	0.20
24	BF_C_3	0	0.29	56	CL_T_2	0.227	0.25	88	NEG L S 3	0	0.20
25	BF_T_1	0.392	0.29	57	CL_T_3	0	0.22	89	NEG MP S 1	0	0.20
26	BF_T_2	2.51	0.29	58	CP_S_1	2.92	0.20	90	NEG MP S 2	0	0.20
27	BF_T_3	0.369	0.29	59	CP_S_2	0.365	0.25	91	NEG MP S 3	0	0.20
28	BL_S_1	0.752	0.67	60	CP_S_3	0.44	0.29	92	NEG F TL 1	0	0.20
29	BL_S_2	0.341	0.50	61	CP_T_1	2.58	0.20	93	NEG F TL 2	0	0.20
30	BL_S_3	0.992	0.67	62	CP_T_2	0.0428	0.20	94	NEG_F_TL_3	0	0.20
31	BL_T_1	0.347	0.33	63	PCR Neg 2	0	0.20	95	CHAMBER NEG	0	0.20
32	PCR Neg 1	0	0.20	64	CP_T_3	0.024	0.22	96	PCR Neg 3	0	0.20

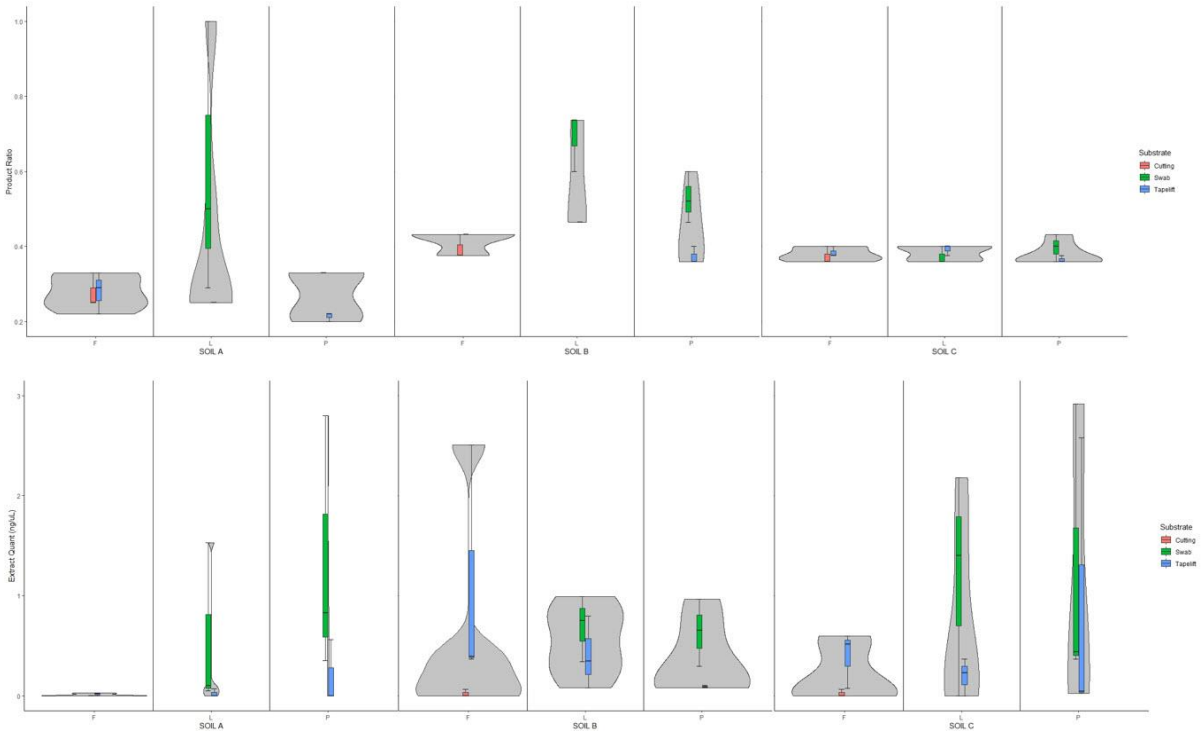


Figure 8.4. Violin plots showing the distribution of all data within the data-subset (in grey) including overlayed boxplots showing the breakdown of each component within the violin. Upper is the PCR efficacy, indicated through gel electrophoresis, calculated as a ratio based on the highest fluorescent sample. Lower is the QUBIT quant result from the DNA extracts in ng/µL. Each is separated by soil type and by substrate type.

Table 8.3: Linear regression p-values with the collection medium producing the higher average in brackets. Significant differences are highlighted in yellow. Collection media were tapelifts (T), swabs (S) and cutting (C). Substrates are fabric (F), from which a C/T was performed, Leather (L), from which a T/S was taken, and Plastic (P), from which a T/S was taken.

PCR			
Substrate/Soil	A	B	C
F	0.94 (T)	0.116 (T)	0.53 (T)
L	0.175 (S)	0.0075 (S)	0.296 (T)
P	$6.62e^{-5}$ (S)	0.021 (S)	0.211(S)
Quant			
Substrate/Soil	A	B	C
F	0.127 (T)	0.207 (T)	0.0855 (T)
L	0.331 (S)	0.366 (S)	0.198 (S)
P	0.214 (S)	0.048 (S)	0.778 (S)

Despite the conclusions drawn here, until the sequencing data is obtained recommendations as to what collection method provides data most reflective to its bulk sample cannot be made. Additional considerations into XRD success/similarity from the extract pellet, due to different collection media, will also need to be made if dual workflows are to be performed from a single sample. Tapelifts appeared to reduce the available pellet as most of the dust sample was retained on the tapelift. If this can be used for XRD analysis, the pellet size may not be of concern.

This proof-of-concept study provides support to the notion that dust can be collected from a range of substrate types, and DNA from this sample can be successfully extracted and amplified for ITS1 sequencing.

8.3.3 Summary of professional development as a researcher

This project has enabled me to be exposed to completely different analytical techniques and sample types to what I have previously been involved with. The exposure to different literature material has been fantastic for my critical reading and thinking skills. I have learned concepts behind data analysis techniques completely new to me and had the opportunity to.

Involvement with communications with the Terrestrial Ecosystem Research Network (TERN), Geoscience Australia, and individuals within the AFP has given me great resources for knowledge to learn material quickly and be made aware of some fascinating research I would never have investigated previously. These individuals are highly responsive to questions and my interdisciplinary and interpersonal communication skills have vastly increased through this experience. Networking with individuals from the Flinders Accelerator for Microbiome Exploration (FAME) group has also been incredibly valuable to practice this skill and increase personal access to experts in this space.

Induction into the Advanced DNA, Identification and Forensic Facility (ADIFF) laboratories has given me exposure to different workflows and environments than the laboratories I have previously worked in. Training around their cleaning and DNA management has been informative and valuable.

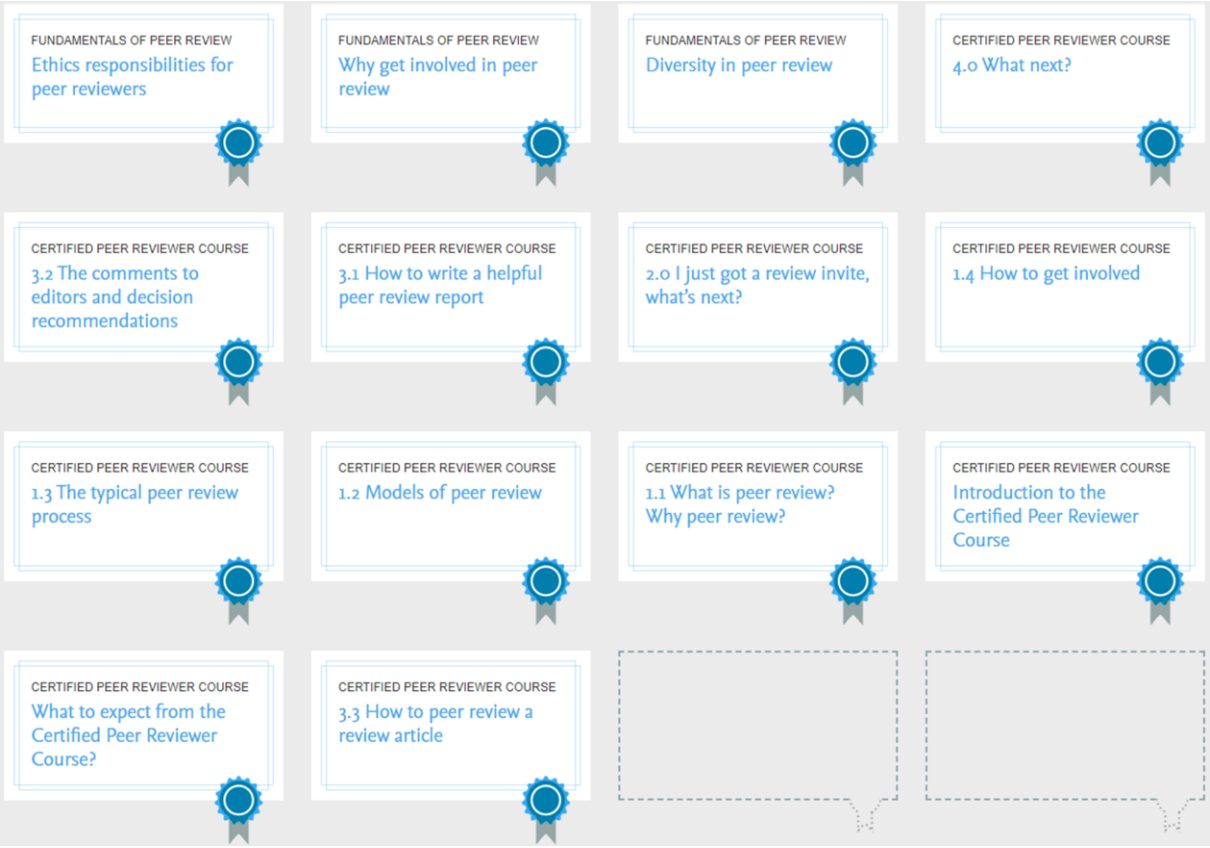
The chance to modify current methodology and develop these alterations to facilitate the extraction of our samples has involved critical thinking skills, verbal and written communication, and tested my practical abilities.

8.3.4 Funding recognition

This research is supported by the Defence Science and Technology Group (DSTG) of the Australian Department of Defence though the APR program. Financial support by DSTG does not constitute an express implied endorsement of the results or conclusions of the research by either DSTG or its respective Departments.

8.4 CERTIFICATES

8.4.1 Evidence of peer reviewer course completion



8.4.2 Evidence of reviews conducted for journals

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CHAPTER IIX

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