

Novel Fingerprint Detection Methods Using Biomolecular Recognition

by

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Certificate of Authorship and Originality

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Rolanda Lam

February 17, 2018

Dedicated to Somebody I Loved Dearly –
You will forever be in my thoughts.

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List of Abbreviations

5-SSA	5-sulphosalicylic acid
7EG	Heptaethylene glycol
AB	Amido Black
ACE-V	Analysis, Comparison, Evaluation – Verification
AgNCs	Silver nanoclusters
aq	Aqueous
Au	Gold
AuNPs	Gold nanoparticles
AY7	Acid Yellow 7
BSA	Bovine serum albumin
BY40	Basic Yellow 40
CA	Cyanoacrylate
CaCl ₂	Calcium chloride
CAST	Centre for Applied Science & Technology
CE	Capillary electrophoresis
CMSC	Carleton Mass Spectrometry Centre
CO ₂	Carbon dioxide
DCM	Dichloromethane
DFO	1,8-Diazafluoren-9-one
DI	Deionised
DNA	Deoxyribonucleic acid
ECF	Ethyl chloroformate
EDC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide
EDTA	Ethylenediaminetetraacetic acid
EG	Ethylene glycol
EGA	Estimated gestational age
ELISA	Enzyme-linked immunosorbent assay
ESI	Electronic supplementary information
EtOH	Ethanol
FLS	Forensic light source
GC-MS	Gas chromatography-mass spectrometry

GYRO	Green-Yellow-Red-Orange
HDPE	High-density polyethylene
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High performance liquid chromatography
HTS	High throughput sequencing
IFRG	International Fingerprint Research Group
iMMD	Immunological multi-metal deposition
IND-Zn	1,2-Indanedione-zinc chloride
KCl	Potassium chloride
LADDER	Laboratory for Aptamer Discovery and Development of Emerging Research
LDPE	Low-density polyethylene
MeOH	Methanol
MES	2-(N-morpholino)ethanesulfonic acid
MgCl ₂	Magnesium chloride
MgCl ₂ ·6H ₂ O	Magnesium chloride hexahydrate
MMD	Multi-metal deposition
NaCl	Sodium chloride
NaOAc	Sodium acetate
NHS	N-hydroxysuccinimide
NIN	Ninhydrin
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Physical developer
PE	Polyethylene
PES	Polyethersulfone
PET	Polyethylene terephthalate
PiAnoS	Picture Annotation System
pNTP	<i>p</i> -Nitrothiophenol
PVDF	Polyvinylidene fluoride
R6G	Rhodamine 6G
RBC	Red blood cell

RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SELEX	Systematic Evolution of Ligands by EXponential enrichment
SERS	Surface-enhanced Raman scattering
SiO ₂	Silicon dioxide
ssDNA	Single-stranded deoxyribonucleic acid
SWGFAST	Scientific Working Group on Friction Ridge Analysis, Study and Technology
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
THF	Tetrahydrofuran
TNT	Trinitrotoluene
TTBS	Tris-buffered saline with Tween® 20
UC	University of Canberra
UCNPs	Upconversion nanoparticles
UTS	University of Technology Sydney
UV-vis	Ultraviolet-visible
VMD	Vacuum metal deposition

Abstract

Over the past decade, there has been a resurgence of interest to design fingerprint enhancement reagents capable of biomolecular recognition; such reagents would offer high selectivity and sensitivity, two areas where some believe improvement is desired with current fingerprint detection methods. In addition to these, a high degree of adaptability for visualisation can be achieved with biomolecular recognition probes, such as antibodies and aptamers, allowing for the selection of the most appropriate visualisation wavelength for a particular luminescent probe or substrate without the need for sophisticated instrumentation or imaging systems. However, the major hurdle to overcome is the balance between sensitivity and selectivity. Single-target biomolecular recognition may be highly selective, purported to have better detection limits than chemical reactions or stains, and can provide information about identity and/or activity, but often results in incomplete ridge pattern development because only a fraction of the fingerprint residue is being specifically targeted.

Consequently, the development and evaluation of multi-target biomolecular reagents for fingerprint enhancement was investigated, with the focus on endogenous eccrine secretions. A variety of parameters (i.e., processing time, fixing and working solution conditions) were optimised on a wide range of non-porous and semi-porous substrates representative of casework materials to assess the suitability of the biomolecular reagents for potential operational use. The relative performance of biomolecular reagents was compared to that of routine methods applied to latent and body fluid-contaminated fingerprints. The incorporation of these novel reagents into routine technique sequences was also investigated. The experimental results indicated that the multi-target biomolecular reagents were not a suitable alternative to routine detection methods, did not provide any significant enhancement when included in routine sequences; however, they may still have potential for a niche application yet to be identified.

While a larger fraction of the fingerprint was being targeted by multi-target reagents, the resulting development seemed to be influenced by inter-donor variability; it was unknown which combination of biomolecular recognition probes would be the most

“universal”. The focus of this research shifted to aptamers due to their many advantageous features over antibodies, one being their versatile *in vitro* selection process called Systematic Evolution of Ligands by EXponential enrichment or SELEX. Up to sixteen fingerprint donors deposited variously aged natural fingerprints onto two realistic substrates (i.e., pooled target approach), which were then subjected to a novel SELEX variation termed fingerprint-SELEX. Select DNA aptamer candidates, developed specifically against genuine fingerprint residues, were subsequently incorporated into a fingerprint enhancement reagent. The proof-of-concept work demonstrated this novel reagent’s ability to successfully develop friction ridge detail on non-porous substrates. Its relative performance was superior to that of single-target and multi-target biomolecular reagents previously designed within the same research group. This study has further opened up the possibilities of incorporating biomolecular recognition into fingerprint detection methods by recognising and tapping into the potential of SELEX and resulting aptamer candidates in this forensic discipline.

Publications and Presentations

PEER-REVIEWED PUBLICATIONS

1. **Lam, R.**; Hofstetter, O.; Lennard, C.; Roux, C.; Spindler, X. (2016) Evaluation of Multi-Target Immunogenic Reagents for the Detection of Latent and Body Fluid-Contaminated Fingermarks. *For. Sci. Int.* 264, 168-175.

ORAL PRESENTATIONS (Presenter = Underlined)

1. **Lam, R.** Novel Fingerprint Detection Methods Using Biomolecular Recognition. University of Technology Sydney Stage 3 Seminar, July 21, 2017, Broadway, NSW, Australia.
2. Spindler, X.; **Lam, R.**; Sullivan-Davenport, K.; Dilag, J.; Hofstetter, O.; Lennard, C.; Roux, C. Possibilities and Challenges in Using Biomolecular Recognition for Latent Fingerprint Detection. Australian and New Zealand Forensic Science Society 23rd International Symposium on the Forensic Sciences, September 22, 2016, Auckland, New Zealand.
3. **Lam, R.** Universal Immunogenic Reagents for the Detection of Latent Fingermarks. University of Technology Sydney Stage 2 Seminar, July 8, 2016, Broadway, NSW, Australia.
4. **Lam, R.**; Ruscito, A.; DeRosa, M.C.; Spindler, X.; Lennard, C.; Roux, C. Fingerprint-SELEX: A Novel Approach to Develop DNA Aptamers for Fingerprint Detection. 60th Annual Conference of the Canadian Society of Forensic Sciences, May 19, 2016, Montreal, QC, Canada.

5. Spindler, X.; **Lam, R.**; Dilag, J.; Sullivan, K.; Hofstetter, O.; Lennard, C.; Roux, C. Optimisation and Evaluation of Multi-Target Biomolecular Reagents for Latent and Bloody Fingerprint Detection: Latest Developments. International Fingerprint Research Group Meeting, October 23, 2015, Patiala, India.
6. Spindler, X.; **Lam, R.**; Dilag, J.; Lennard, C.; Roux, C. Next-Generation Fingerprint Reagents: Molecular Recognition, Multispectral Imaging and Mapping. 7th European Academy of Forensic Science Conference, September 8, 2015, Prague, Czech Republic.
7. **Lam, R.**; Spindler, X.; Lennard, C.; Roux, C. Optimisation of Multi-Target Immunogenic Reagents and Comparison to Routine Detection Methods for Latent and Body Fluid-Contaminated Fingerprints. 7th European Academy of Forensic Science Conference, September 8, 2015, Prague, Czech Republic.
8. **Lam, R.**; Spindler, X.; Lennard, C.; Roux, C. Optimisation of Multi-Target Immunogenic Reagents and Comparison to Routine Detection Methods for Latent and Body Fluid-Contaminated Fingerprints. 4th Doctoral School of the École des Sciences Criminelles, August 26, 2015, Les Diablerets, Switzerland.
9. **Lam, R.** Optimisation of Multi-Target Immunogenic Reagents and Comparison to Routine Detection Methods for Latent and Body Fluid-Contaminated Fingerprints. UTS-UWS Forensic Science Research Student Forum, June 30, 2015, Penrith, NSW, Australia.

POSTER PRESENTATIONS (Presenter = Underlined)

1. **Lam, R.**; Ruscito, A.; DeRosa, M.C.; Spindler, X.; Lennard, C.; Roux, C. Fingerprint-SELEX: A Novel Approach to Develop DNA Aptamers for Fingerprint Detection. 21st Triennial Meeting of the International Association of Forensic Sciences 2017, August 24, 2017, Toronto, ON, Canada.

2. **Lam, R.**; Ruscito, A.; DeRosa, M.C.; Roux, C. “Fingerprint” Aptamers: From Random Oligonucleotide Library to Fingerprint Detection Reagent. International Association for Identification’s 102nd International Forensic Educational Conference, August 8, 2017, Atlanta, GA, USA.

3. **Lam, R.**; Ruscito, A.; DeRosa, M.C.; Spindler, X.; Lennard, C.; Roux, C. Fingerprint-SELEX: A Novel Approach to Developing Immunogenic Reagents for Fingerprint Detection. Australian and New Zealand Forensic Science Society 23rd International Symposium on the Forensic Sciences, September 22, 2016, Auckland, New Zealand.

Chapter 1: Introduction

Chapter 1 Introduction

1.1 Fingerprints

In everyday life, people touch a multitude of objects. Whether it is a cup of coffee, a car door, or a work document, traces – remnants of a source or an activity [1] such as fingermarks or DNA – will be left behind. Nowadays, fingerprints are used in a variety of ways; from verifying an individual's identity through biometric security [2] into restricted areas or even mobile phones, to identifying amnesia victims or unknown deceased [3]. Fingerprints are especially important in criminal situations whereby providing a possible link between an individual and a crime scene. However, long before fingerprints were used in the field of forensic identification that we know today, they were used for identification purposes during the Qin Dynasty (221 to 206 B.C.) in China [4, 5]. Clay seals were used to show authorship of documents and to prove that the document had not been tampered with prior to reaching the intended recipient. Both the author's name and fingerprint were impressed on the stamp, the latter being evidence of intentional reproduction as a means of identification [4-6]. Other Asian nations, such as Japan and India, also used friction ridge skin impressions as signatures prior to their discovery in European and North American countries [5, 6].

1.1.1 Identification

Fingerprints are one of the most reliable traces used for forensic identification and have been used for over 100 years. Dr Henry Faulds was the first person to publish the value of friction ridge skin for identification, especially its use as evidence [5]. Identification, according to the Scientific Working Group on Friction Ridge Analysis, Study, and Technology (SWGFAST), can be used interchangeably with the term individualisation; they refer to the determination that there is sufficient quality and minutiae in agreement to conclude that two friction ridge impressions originated from the same source [7]. However, in many disciplines, identification and individualisation have two different meanings. The former refers to the classification of an entity to a group, while the latter is a type of identification specifically where the group size is

one [8]. Champod et al. recommend that fingerprint experts avoid using the term individualisation due to what is at stake in terms of evidential power [8].

The first scientific system used to identify criminals was created by Alphonse Bertillon in the late 1800s and involved the use of anthropometry, the study of body measurements [5, 6]. Bertillonage, as the system became to be known, was used throughout most of the world until the early 1900s. Unfortunately, it soon became apparent that the system's application was inadequate; the primary problem being that measurements of an individual taken by different officers were not always in agreement, or that measurements of two individuals were so similar that they were identified as the same person [9]. As friction ridge skin identification became more prevalent, fingerprints were added to the anthropometric records.

The first practical use of fingerprints by law enforcement personnel has been credited to Juan Vucetich, who started to devise his own classification system in 1891 [5, 6, 10]. However, it was Sir Edward Henry's classification system, based on publications by Dr Johannes Evangelist Purkinje in 1823 and by Sir Frances Galton in 1892, that prevailed [5, 6, 10]. Purkinje had classified fingerprint patterns into nine categories [5, 9], while Galton was the first to define specific minutiae within a fingerprint [5]. In 1900, it was recommended that all criminal identification records be classified with the Henry classification system [5].

The evidential value of fingerprint evidence is based on fingerprints being highly polymorphic and immutable. Galton's book, *Finger Prints*, was the first to state that friction ridge skin was unique and persistent [5, 11]. Uniqueness refers to the idea that no two friction ridge skin patterns are the same, a statement that is no longer sufficient to be the sole argument for an identification conclusion [8] and that fingerprint examiners have been moving away from over recent years. Friction ridge skin was first described in detail by Dr Nehemiah Grew in 1684 [5, 6]. However, it was not until 1788 when the uniqueness hallmark of fingerprints was recognised by Johann Christoph Andreas Mayer [5, 6]. Persistence or permanence refers to the idea that one's friction ridge skin does not change from before birth until decomposition after death [12, 13]. Sir William James Herschel is credited as the first person to study persistence of friction ridge skin. In 1877, as Magistrate and Collector at Hooghly in

India, Hershel instituted a fingerprint system for criminal and government matters [5, 6]. For a more complete historical compilation covering the development of fingerprinting around the world, it is recommended to read Robert Heindl's *System und Praxis der Daktyloskopie* [14].

The dominant “scientific” method used by fingerprint examiners involves four steps: analysis, comparison, evaluation, and verification (ACE-V) [6, 8, 15]. During this process, examiners will look for three different levels of detail [6, 8, 15] within the unknown fingermark (i.e., friction ridge impression recovered from a crime scene or exhibit). Should there be sufficient detail in the unknown fingermark, it is compared to a known fingerprint (i.e., friction ridge impression from a reference or criminal record). The examiner will then come to a conclusion as to whether the two impressions originated from a common source, and the whole process is repeated, or verified, by a second fingerprint examiner.

First level detail refers to the overall pattern formed by the flow of ridges on the fingerprint surface [8] and is insufficient for identifications. Most fingerprints fall into one of three main pattern types: loops, arches, or whorls (Figure 1-1). Loops have the ridges flowing from one side of the impression towards the centre or core and then recurving back towards the same side they entered. Arches have the ridges flowing in from one side of the impression to the other with a rise in the centre. Whorls form concentric patterns and have at least two deltas [7]. Second level detail refers to the specific friction ridge deviations or minutiae, commonly known as Galton details. All minutiae are either ridge endings, bifurcations, dots, or combinations of these (Figure 1-2). Third level detail refers to the morphology of the ridges, including pore locations and edges [6-8].



Figure 1-1 Three main overall patterns (Reproduced from Champod et al. [8]).



Figure 1-2 Illustrations of second level detail (Reproduced from Champod et al. [8]).

Since the release of publications like the National Research Council report [16], the fingerprint field has come under scrutiny. To address some of these concerns, the “scientific” method has had to evolve; improved documentation tools like Picture Annotation System (PiAnoS) [8] or the Green-Yellow-Red-Orange (GYRO) system [17] have been adopted during the analysis phase, and SWGFAST has created a sufficiency graph [18] that takes into account both the quality and quantity of minutiae during the comparison and evaluation phases. The most significant change, while gradual, has been the introduction of statistical models, such as likelihood ratios, into the friction ridge identification process. By including probabilities, it helps to minimise the confusion associated with terms used to discuss differences between the fingermark and the fingerprint. While these probabilistic tools are a means to provide support to the expert’s opinion, aiding with uncertainties and decision-making [8], that has not stopped the United States Army Criminal Investigation Laboratory from moving, earlier last year, solely to probabilistic reporting based on a statistical model it developed [19].

1.1.2 Formation of Friction Ridge Skin

As the largest organ in the human body, skin has many functions ranging from regulating the body’s temperature to protecting it from viruses and bacteria. The skin is composed of two anatomical layers. The outermost layer, or epidermis, is composed of five sublayers, the two extremes being the basal generating layer and the cornified layer [8]. The innermost layer, or dermis, is fifteen to forty times thicker than the epidermis [8] and supports it with various connective tissues. It is in this layer that the ridges and furrows of friction ridge skin are rooted [20].

Friction ridge skin begins to develop during the early weeks of the gestational process. Around 5 to 6 weeks of estimated gestational age (EGA), the hands start to develop, followed by the fingers a week or two later. Volar pads, which are transient swellings of mesenchymal tissue [8], first appear on the palms and then on each finger at 7 to 8 weeks EGA. Major development of the friction ridge skin occurs during weeks 11 to 20 EGA [8]. As the hands continue to grow, the volar pads start to regress and are no longer visible by week 16 EGA. Minutiae are formed completely before birth.

1.2 Fingerprint Detection Methods

There is a plethora of fingerprint detection methods currently available to practitioners [21, 22]. While a fingerprint detection method can be used independently, it has been shown that sequential processing can be beneficial and increase the chances of fingerprint recovery [21, 23-25]. When used in sequence, the techniques should be used from least to most destructive. This means that optical methods tend to be used first. Physical methods tend to be used next, as they are generally liquid-free and reduce the risk of washing away fingerprint residues. If a chemical solution needs to be used, organic solvent-based reagents should be used before water-based ones, as the latter is more detrimental [21]. Deciding which technique(s) to use depends on a number of variables such as fingerprint type, fingerprint composition, substrate type, condition of the substrate, etc.

1.2.1 Types of Fingerprints

Fingerprints can be divided into two general categories: visible and latent. Visible fingerprints can be seen with the naked eye without the need of any sort of chemical treatment. They may appear positive if a contaminating material like paint is being transferred on the substrate surface, or they may appear negative where the ridges have removed a contaminating material such as dust off of the surface. When friction ridge skin comes in contact with a malleable substrate like wax, it will leave behind a three-dimensional impression, also known as an indented fingerprint. Often, optical techniques may be required to enhance the contrast between the fingerprint and the

background. A staining agent may still be used to increase the amount and quality of ridge detail of a visible blood-contaminated fingerprint.

Latent fingerprints are the most common type of fingerprint and are challenging because they are invisible to the naked eye. Some means of enhancement, be it optical, physical, or chemical, is required to visualise the ridge detail [26]. Knowledge of the fingerprint composition, the substrate on which the fingerprint was deposited, and the environment in which the substrate was stored is paramount and needs to be considered when selecting the appropriate latent detection technique or sequence.

1.2.1.1 Fingerprint Residue Composition

Of the three major glands responsible for the skin's natural secretions [22, 27, 28], eccrine glands are the only type found on the palms and soles of the hands and feet, respectively [20]. Eccrine secretions are mostly water, with the remainder comprised of organic and inorganic compounds (Table 1-1). Amino acids are important targets for latent detection methods. Secretions from sebaceous glands, which are all over the body except on the palms of the hands and soles of the feet, are also targeted with fingerprint detection methods. The primary compounds present in sebaceous secretions are lipids. The final glands located in the groin and armpit areas are apocrine glands [22]. Their secretions are typically mixed with those of sebaceous glands.

Table 1-1 Main chemical constituents of the glandular secretions (Adapted from Champod et al. [8]; Lee et al. [27]; Knowles [28]).

Source	Constituents	
	Inorganic	Organic
Eccrine glands	Chlorides	Amino acids
	Metal ions (Na^+ , K^+ , Ca^{2+})	Proteins
	Sulfates	Urea
	Phosphates	Uric acid
	Ammonia	Lactic acid
	Water (> 98%)	Sugars
		Creatinine
		Choline
Sebaceous glands		Glycerides (30-40%)
		Fatty acid (15-25%)
		Squalene (10-12%)
		Sterol esters (2-3%)
		Sterols (1-3%)
Apocrine glands	Iron	Proteins
	Water (> 98%)	Carbohydrates
		Sterols

1.2.1.2 Substrate Characteristics

Substrates are presently classified into three general categories based on the interactions between the surface and the fingerprint residues. Porous substrates like paper, cardboard, and some fabrics absorb the water-soluble components of fingerprint residues easily and quickly. The non-water-soluble components stay on top of the surface for a longer period of time before migrating into the substrate [8].

Non-porous substrates such as plastic, glass, and metal, will not allow fingerprint residues to penetrate into the surface. Therefore, both the water-soluble and non-water-soluble fingerprint residue components stay on the surface for a very long time [8]. Unfortunately, a consequence of this is that the fingerprint residues are more

prone to being rubbed off the substrate. Care must be taken to avoid damage due to friction especially during transportation and storage of an exhibit [21].

Lastly, any surface that does not fit well into porous or non-porous categories is deemed semi-porous. The water-soluble components of the fingerprint residues do absorb into the surface, but not as quickly as with porous substrates. The non-water-soluble components stay on the surface longer than they would with porous substrates, but some diffusion into the surface does eventually occur [8]. Semi-porous substrates have characteristics of both porous and non-porous substrates. As such, routine techniques should be used in combination in a logical order to maximise fingerprint recovery on what may be considered a difficult substrate such as polymer banknotes.

Substrates cannot always be easily classified into these three categories, as the interactions between fingerprint residues and the surface may vary a great deal. Therefore, current research is investigating the possibility of a more accurate and comprehensive model [29].

1.2.1.3 Factors Affecting Fingerprint Deposition and Composition

Numerous factors can influence the presence and quality of a fingerprint (Figure 1-3) [30] and can be divided into three categories. Pre-transfer conditions refer to the condition of the friction ridge skin and the type of residues present on the finger. These are affected by such things like age, occupation, disease, and previously touched items before deposition. Transfer conditions will dictate whether a fingerprint or impression will be left behind. Many substrate properties, such as texture, elasticity, rigidity, size, shape, and condition, can affect fingerprint deposition. Likewise, many aspects of the deposition action can also influence transfer, such as contact pressure, angle, and duration. Post-transfer conditions relate to the environmental factors that affect the quality and persistence of the fingerprint after deposition [31-33]. These factors can include the substrate coming into contact with another object, exposure to water, humidity, or heat.

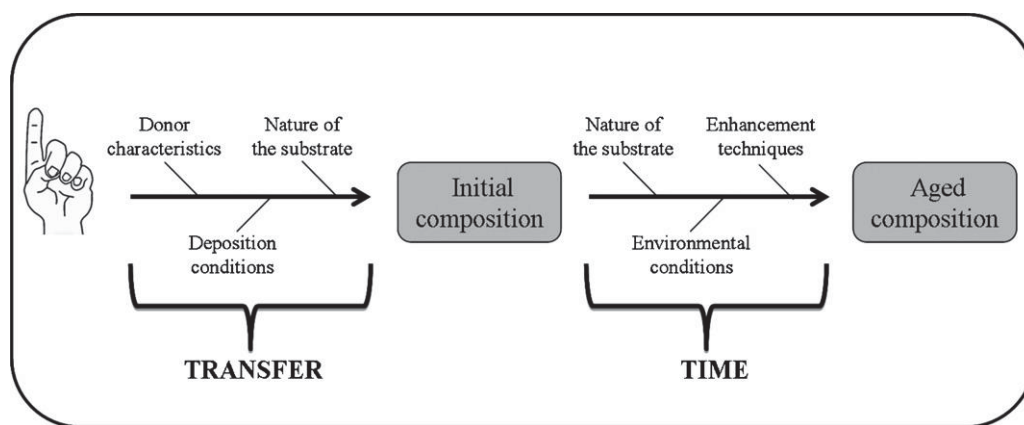


Figure 1-3 Schematic representation of factors influencing fingerprint composition
(Reproduced from Girod et al. [30]).

1.2.2 Current Latent Fingerprint Detection Methods

The most common enhancement reagents for the detection of latent fingerprints on dry porous substrates (e.g., paper, cardboard) are amino acid-sensitive reagents, such as 1,8-diazafluoren-9-one (DFO), 1,2-indanedione-zinc chloride (IND-Zn), and ninhydrin (NIN). They are effective at developing fingerprints because not only is there an abundance of amino acids in eccrine secretions, but the amino acid distribution within such secretions is relatively constant (Table 1-2). These reagents do not react with a single amino acid, but rather an array, to produce the desired characteristic colour. Amino acids get absorbed into the porous substrates, and due to their affinity to cellulose, they do not migrate too much, making them good targets for fingerprint detection. Both DFO and IND-Zn are considered dual reagents, as they not only produce a visible product (light purple and light pink, respectively), but a fluorescent one as well [34]; NIN produces a dark purple product visible to the naked eye. Spotty development – the result of the eccrine secretions excreting and accumulating at the pore locations – can affect the clarity of ridge detail observed with DFO, IND-Zn, and most noticeably NIN [35]. All three of these routine amino acid-sensitive reagents are commercially available as sprays, the preferred application method for *in situ* processing by Australian industry partners, but one issue with these products is that the working solutions do have limited shelf-lives and the unused reagents may have to be discarded if expired. When used in sequence, it has been shown that NIN can further

enhance weakly developed and additional fingerprints after DFO or IND-Zn [23, 25]. Likewise, physical developer (PD) can increase fingerprint recovery after NIN [24]. PD is the technique of choice for wetted porous substrates, as it is thought to target the water-insoluble components of fingerprint residues, or a mixture containing sufficient water insoluble components to protect the other fingerprint constituents [36]. Processing dry or wetted porous substrates results in dark ridge development. One major drawback of this fingerprint enhancement technique is that it is laborious and it tends to work better with aged fingerprints.

Table 1-2 Summary of relative abundance (serine ratio) of amino acids in fingerprint deposits (Reproduced from Ramotowski [27]).

Amino Acid	Hamilton [37]	Hadorn et al. [38]	Oro and Skewes [39]
Serine	100	100	100
Glycine	67	54	59
Ornithine	32	45	45
(Ornithine, lysine)	42	47	45
Alanine	27	35	28
Aspartic acid	22	11	22
Threonine	17	9	18
Histidine	17	13	14
Valine	12	10	9
Leucine	10	7	10
Isoleucine	8	6	8
Glutamic acid	8	12	5
Lysine	10	5	-
Phenylalanine	7	5	5
Tyrosine	6	3	5

If fingerprints are fresh, simply dusting non-porous substrates with fingerprint powders can reveal friction ridge detail; little experience is necessary to obtain satisfactory results. Fingerprint powders are portable and ideal to process immovable objects from crime scenes. However, they are known to lose their effectiveness as fingerprints age. Powder can also be incorporated into a detergent solution, creating a thin powder suspension (i.e., small particle reagent) that works well on wetted non-porous substrates as a spray or immersion technique, or a thick suspension (e.g., sticky side powder) used to treat the adhesive sides of tape [8] and other difficult non- and semi-porous substrates like leatherette [21]. The most widely used chemical technique

for processing non-porous substrates in a laboratory setting is cyanoacrylate (CA) fuming. Monomers of CA esters polymerise forming hard three-dimensional ridges. If development is faint, a luminescent dyestain can be applied to improve enhancement. A highly sensitive technique that can develop fingermarks previously undetected by all other techniques is vacuum metal deposition (VMD) [8, 40]. Traditionally, the process is multi-metal deposition of gold and zinc, but single-metal deposition is also possible (e.g., silver), and sometimes even preferred on some porous substrates like fabric.

1.2.3 Current Blood-Contaminated Fingermark Detection Methods

Enhancement techniques used in the detection of fingermarks in blood must not be detrimental to subsequent DNA analyses. Some blood enhancement techniques do not develop or may even ruin latent fingermarks, and therefore, it is important to sequence techniques to preserve fingerprint evidence [41]. Blood-contaminated fingermarks should first be fixed with either methanol (MeOH) or 5-sulfosalicylic acid (5-SSA) solutions to prevent the blood from diffusing upon application of a protein stain. A protein stain that works well on both porous and non-porous substrates is amido black (AB). It results in visible dark blue ridges and even after multiple wash de-staining solutions, the background may appear a light blue or colourless. If an item is dark, non-luminescent, and non-porous, AB will not be as effective as a luminescent protein stain such as acid yellow 7 (AY7). This stain should not be used for porous substrates.

Other blood enhancement reagents are haem-reactive to produce visible or chemiluminescent products. Protein stains are likely to be more sensitive than these haem-reactive reagents, but not very selective; they not only react with proteins, but with the globular protein in haemoglobin as well [42]. Conversely, haem-reactive reagents are more selective to blood, but with decreased sensitivity [42]. Haem-reactive reagents are not as commonly used as protein stains due to associated health concerns. Leuco crystal violet produces a purple product and is less detrimental to fingermarks compared to luminol, which is known to produce blue chemiluminescence [21]. Leuco crystal violet is more effective than diaminobenzidine, which forms a dark brown insoluble product [8], and

tetramethylbezindine that forms a blue/green product. The observed colour of leuco crystal violet development is also more intense than that of leuco malachite green, which forms a green/blue product. Fluorescein also reacts with the haem group of blood, but its performance offers no added benefits compared to other haem-reactive reagents [21].

1.2.4 Current Limitations

Fingermarks are one of the most common traces used for identification purposes. Unfortunately, routine detection methods currently employed by practitioners may be limited by factors such as insufficient sensitivity or selectivity [43-45], resulting in a substantial proportion of fingermarks not being detected [46-49]. Practitioners may have to attend or process exhibits from a challenging crime scene that was contaminated or exposed to extreme environmental conditions like arson. As such, the chemical reactivity of fingermark residue constituents may be reduced, leading to less effective detection methods [50]. The exhibit itself may have a challenging surface composition; for example, thermal paper that has a heat-sensitive layer; this prevents the use of traditional methods that require a heat treatment [51]. According to Champod et al. [52], an ideal detection method should have the following desirable features: increased sensitivity; portability to crime scenes; compatibility with other fingermark detection methods and forensic analyses (e.g., DNA profiling) [53]; process simplicity; and reduction in cost and use of hazardous chemicals. Furthermore, the ability to develop fingermarks that remain undetected, or partially detected, by current methods is a highly desirable goal [52, 54].

1.3 Project Aim and Objectives

The overall aim of this research is to further explore the potential use of biomolecular recognition to address some of the existing issues related to the sensitivity and selectivity of current fingermark detection methods. The two main biomolecular recognition probes of interest are antibodies (see Chapter 2) and aptamers (see Chapters 3 and 4).

Antibodies have long since been considered the standard for biomolecular recognition. As such, incorporating them into a fingerprint detection reagent seemed logical. Research has already been published on the use of single-target antibodies for fingerprint detection; however, no other research group has published work on multi-target reagents. These types of reagents would theoretically be less affected by the inherent intra- and inter-donor variability known to be associated with fingerprints than single-target reagents. Chapter 2 involved the optimisation and validation of such reagents containing predominantly antibodies. Investigations into whether these multi-target reagents for latent and body fluid-contaminated fingerprints are suitable for operational use and their compatibility with routine technique sequences were performed on realistic substrates.

Aptamers, also known as “synthetic antibodies”, are gaining more interest in the forensic science community, especially in forensic toxicology. To date, all aptamer-based fingerprint reagents have used previously published oligonucleotide sequences. These aptamers were developed through the *in vitro* selection process called Systematic Evolution of Ligand by EXponential enrichment (SELEX). Chapter 3 considered the possibility of designing a novel SELEX variation with a pooled/untargeted approach (i.e., there is not one specific target), to develop aptamers against genuine fingerprint residues. Chapter 4 demonstrated the proof-of-concept of using the resulting “fingerprint” aptamer candidates to develop latent fingerprints. Preliminary comparisons to a routine fingerprint technique sequence and to single- and multi-target biomolecular reagents were also completed. Finally, a general discussion, recommendations, and overall conclusions based on the research presented in this thesis can be found in Chapter 5.

***Chapter 2: Optimisation and
Validation of Multi-Target
Biomolecular Reagents***

Chapter 2 Optimisation and Validation of Multi-Target Biomolecular Reagents

2.1 Introduction

When reviewing the current limitations associated with conventional fingerprint detection methods, there are a few avenues that could be taken to overcome them, one of which is to develop novel methods. Most of the time, proof-of-concept studies are academically focused. Researchers demonstrate that their new compound or method works with various applications or items; frequently, the novelty of their research outweighs its practicality. While some may address current issues (e.g., sensitivity, difficult substrates), they may not necessarily take operational requirements into consideration [55]. The research idea is not often thoroughly investigated for implementation into casework.

Antibodies, or immunoglobulins, are glycoproteins composed of polypeptide chains with amino acids and carbohydrate moieties [56]. They are typically found in the γ -fraction of blood serum [57] and are produced as a response to antigens, which are macromolecules that the immune system recognises as being foreign [58]. Antibodies are highly selective to antigens; the former having binding sites that are specific to their corresponding antigens. The portion of the antigen that specifically binds is known as the antigenic determinant or epitope. The analogue of a lock and key is often used to describe the close fit between the epitope and antibody [59].

Antibodies are widely recognised in disease diagnostic and therapeutic applications [60-62], for example cancer treatments, due to their high sensitivity and selectivity. They are incorporated into tools such as western blotting and enzyme-linked immunosorbent assays (ELISA) [63-65] to identify and quantify molecules not visible by the naked eye. Arruebo et al. reviewed publications about antibody-conjugated nanoparticles for biomedical applications [66]. The conjugation of antibodies to gold nanoparticles (AuNPs) allows for products to have the combined properties of selectivity from the antibodies and the versatility of nanoparticle characteristics [66].

2.2 Immunodetection

2.2.1 Antibodies in Forensic Science

Modern forensic serology depends on two main techniques: immunoassays and DNA analysis. Immunoassays are relatively rapid, they require little sample manipulation, and have satisfactory sensitivity. Species of origin determinations are tested using immunological methods. The main concept behind precipitin tests is to bring antigens and antibodies into contact with one another in solution; a positive result indicated by the formation of a precipitate [67]. These tests have evolved to quick lateral flow immunoassays, similar to commercially available pregnancy tests or roadside drug tests.

Being able to screen for a particular target analyte is of great interest to those dealing with illicit substances. Affinity biosensors, or immunosensors, have emerged as important tools for the forensic analysis of illicit substances like drugs and explosives. They resolve issues such as lack of selectivity, limited sample selection, and the need for confirmatory analyses associated with traditionally used immunoassays. Biosensors are an ideal alternative to laboratory chromatographic methods for the detection of illicit drugs [68] and can be developed for both screening and quantification purposes. Yáñez-Sedeño et al. critically review the various detection strategies that have been developed for forensic toxicology and weapons of a chemical and biological nature [69].

2.2.1.1 *Antibodies in Fingerprint Detection*

To address some of the issues raised with current fingerprint detection methods, investigation began into the use of biomolecular recognition, due to the high sensitivity and selectivity achieved by antibodies, lectins, and enzymes [45, 70-79]. There have been a few recent review papers describing emerging trends in fingerprint detection [55, 80, 81], but Wood et al.'s focuses purely on the progress of immunogenic reagents as a fingerprint detection method [43]. The first known use of immunology in fingerprint detection was in 1977 by Ishiyama et al. [72], who were able to use

antibodies and lectins to blood type fingermarks, in addition to observing some usable ridge detail. Their reagents were able to enhance fingermarks previously treated with NIN on porous substrates.

Pounds and Hussain mentioned the existence of limitations to detection methods, many of which were listed in the previous chapter (Subsection 1.2.4). Therefore, they decided to further investigate the use of antibodies, lectins, and enzymes to determine the blood group and enzymatic types of body fluid stains [45, 82]. Initially starting with commercially available anti-A and -B sera, they were unable to detect fingermarks from donors with blood type O due to the lack of an anti-H serum. Once anti-H sera were synthesised, fingermarks were developed regardless of the blood type [45]. Pounds and Hussain proposed that if there were more antibodies available to major constituents in fingermark deposits, detection may increase. It was anticipated that as more antibodies became available, they would be able to be used in fingermark detection while conjugated to fluorescent, radioactive, or coloured particles. Research by Cecka et al. was also able to determine the blood type from saliva, semen, and fingermarks, but did not discuss the visible ridge detail present [83].

Although promising, the idea of incorporating immunology into fingermark detection went dormant for many years. It started to resurge in 2007 when Russell and co-workers were able to create a forensic “information” gathering fingermark detection method. In their initial work, they were able to determine the presence of nicotine and its metabolite, cotinine, using antibody-nanoparticle conjugates [75]. Gold nanoparticles, synthesised by the Turkevich et al. method [84], were functionalised with multiple anti-cotinine antibodies and fluorescently tagged. Developed fingermarks contained all three levels of detail. They were also able to determine whether the fingermark donor was a smoker or not. Other work included the use of antibody-magnetic particle conjugates to target different drugs and their metabolites [76, 77, 85]. To visualise development, bright field and fluorescence microscopy were utilised. As the concentration of metabolites decreased in the sweat deposited, the intensity of the images also decreased. They also found that the use of a magnet made it easier to remove the excess unbound particles. Fluorescently tagged antibody-magnetic particle conjugates were also used to detect cotinine in fingermarks which were aged in varying light and temperature conditions [77]. Unfortunately, these

“information” gathering approaches (Figure 2-1) are limited, as such compounds are not universal in fingerprint deposits. Russell and colleagues believed that if the nanoparticles were functionalised with other antibodies, additional specific detection would occur with the various antigens within fingerprints.

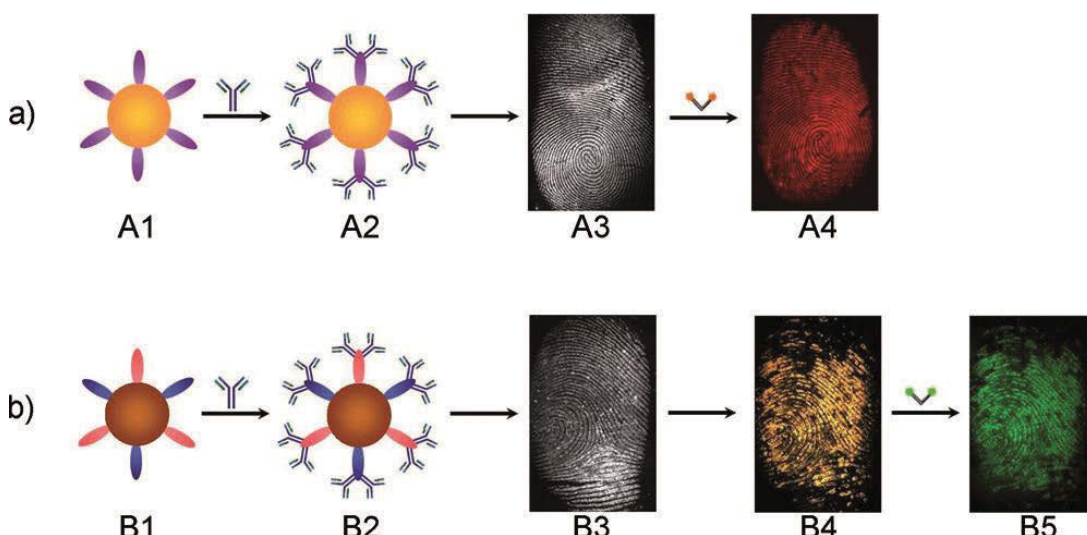


Figure 2-1 Schematic representation of the detection of drugs and/or drug metabolites in latent fingerprints using a) gold nanoparticles and b) magnetic particles (Reproduced from Hazarika and Russell [86]).

As there are many organic and inorganic constituents in secretions, a range of possible targets is available to choose from for fingerprint detection. In 2008, Reinholz [78] targeted albumin, a protein in eccrine secretions that constitutes about 60% of the serum-protein content [87]. To enhance development, Reinholz incorporated the use of fluorescein isothiocyanate. It was compatible with routine development techniques for porous substrates, DFO and PD, but not NIN [78].

Since there are over 400 known polypeptides to be present in fingerprint residue [88], Drapel et al. [71] decided to target the most abundant proteins involved in the desquamation phase of skin regeneration process, which were keratins 1 and 10, cathepsin D, and dermcidin. The keratins were identified by immunodetection on the western blot and cathepsin D was identified by immunodetection. Detection of dermcidin was not possible on the western blot, but was attempted by

immunodetection with the anti-dermcidin antibody. It was found that antigens from the desquamation process (keratins, cathepsin D) gave very precise ridge edges, while antigens from sweat (cathepsin D, dermcidin) gave dotted pattern from the pores (Figure 2-2).

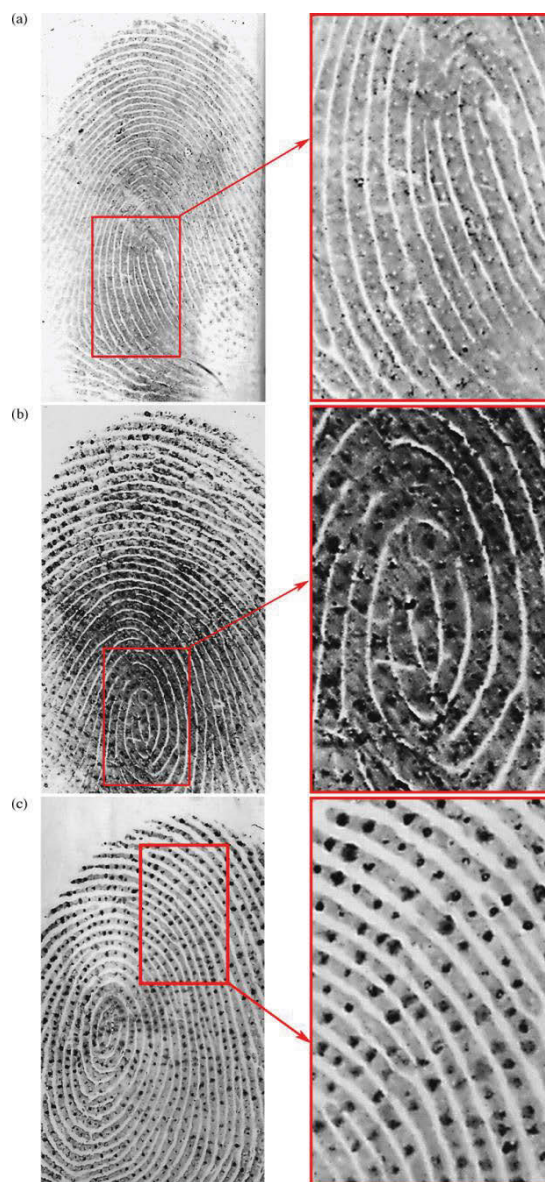


Figure 2-2 Fingermarks developed on polyvinylidene fluoride (PVDF) after being immunodetected with (a) anti-keratin 1/10, (b) anti-cathepsin-D, and (c) anti-dermcidin (Reproduced from Drapel et al. [71]).

Research by van Dam et al. [79] also showed that dermcidin was able to provide information on pore location in an attempt to improve the quality of poorly developed

fingermarks. Van Dam et al. first demonstrated the possibility of developing natural one-day-old fingermarks on nitrocellulose membrane and glass slides by targeting dermcidin and albumin simultaneously [89]. Next, their immunolabelling method was trialed on a variety of realistic substrates with natural fingermarks aged for either one hour or one day, with dermcidin, albumin, and keratin as the targets [90]. While the substrates may have been forensically relevant, older fingermarks should have been tested. Immunolabelling was also used following NIN detection; results were inconsistent as some fingermarks showed improved contrast, while others did not (Figure 2-3). Van Dam et al. only focused on their immunolabelling method as a secondary or tertiary technique; it was not evaluated in terms of compatibility prior to IND-Zn; the sequential processing of IND-Zn followed by NIN; PD; and various cyanoacrylate products [91].

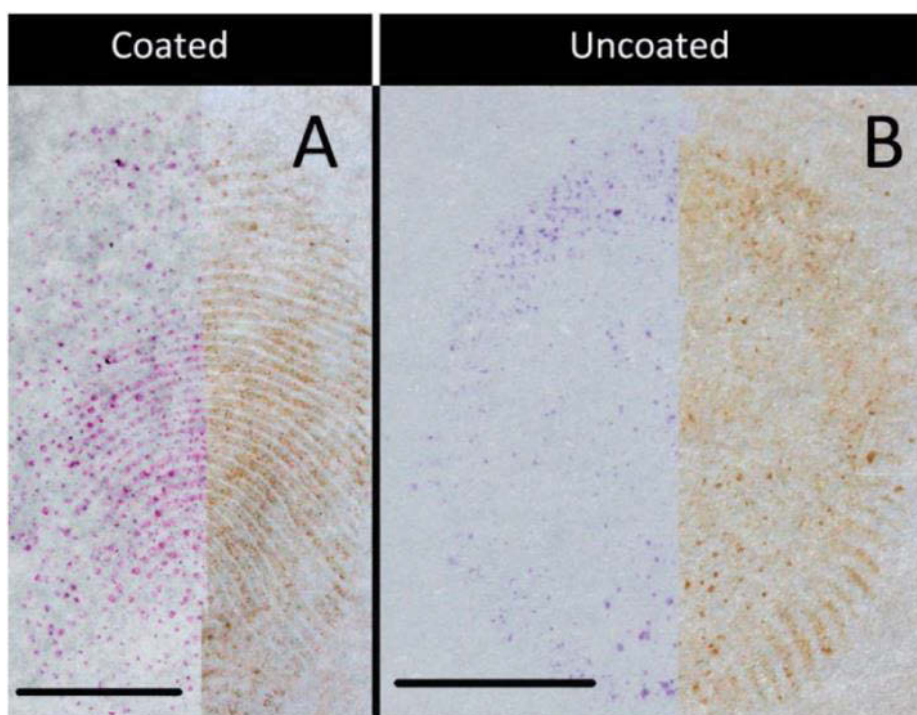


Figure 2-3 Fingermarks deposited on thermal paper first developed with ninhydrin (left side of each image), and then further developed with the immunolabeling method (anti-dermcidin, anti-albumin and antikeratin) (right side of each image) (Reproduced from van Dam et al. [90]).

Spindler et al. [70] used AuNPs conjugated to antibodies in order to detect free amino acids from fingermarks on aluminium foil and glass slides. The anti-L-amino acid

antibodies were raised in rabbits [92] and then attached directly or via thiol linkers to the AuNPs (Figure 2-4). After the working solution was applied to the fingermarks, and rinsed off after an incubation period, the bound conjugates were detected via fluorescently tagged secondary antibodies. Successful development of fingermarks on the non-porous substrates was observed. This was a great achievement since amino acids are present in eccrine secretions, which are to some degree in all fingermarks at time of deposition, and are not normally targeted on non-porous substrates. It was noted that this reagent worked better on aged or degraded fingermarks compared to a couple of conventional detection methods. Its effectiveness did not seem affected by fingermark age, which means it could potentially be a new sensitive reagent for exhibits of unknown ages.

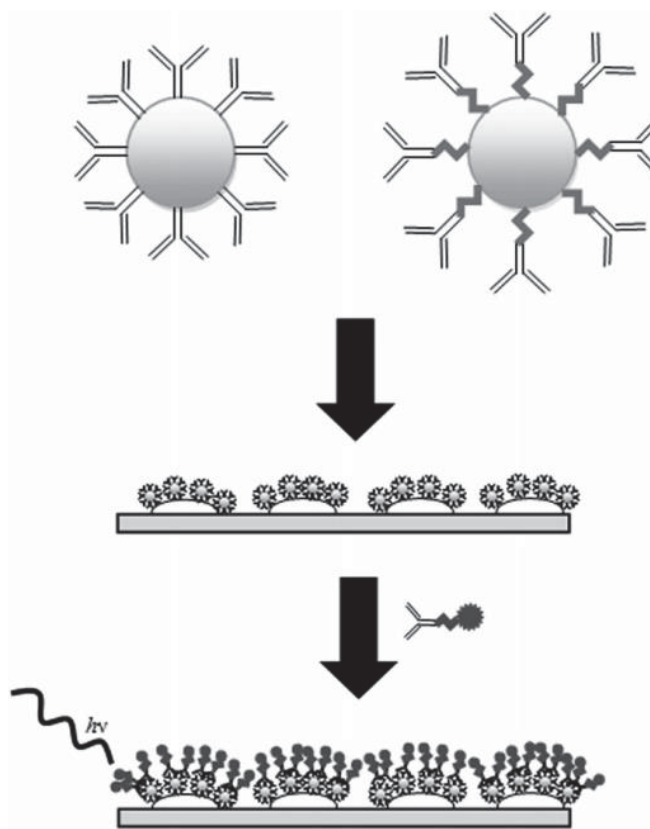


Figure 2-4 Schematic diagram for the application of two conjugated nanoparticle systems – directly-bound antibodies (top left) and alkyl-thiol-linked antibodies (top right) – to latent fingermarks (Reproduced from Spindler et al. [70]).

Conventional multi-metal deposition (MMD) was coupled with antibody-conjugated AuNPs – termed immunological MMD (iMMD) – to detect IgG-spiked fingermarks

and eccrine-rich fingermarks on forensic lifting tape as the substrate with epidermal growth factor and lysozyme as the targets of interest [91]. A few chemical spot tests were performed to demonstrate that development was due to the antibody-AuNP conjugates. However, without the antibodies, development was still likely to occur as AuNPs are attracted to proteins and some peptides in fingerprint ridges; the AuNPs act as nucleation sites for silver deposition [21]. Nonetheless, the iMMD method did simplify the MMD process down to three steps and no forensic light source was required for visualisation, as the ridge detail was visible to the naked eye.

Researchers have recently been trying to combine immunoassays with portable and efficient imaging techniques [81]. Xu et al. used electrochemiluminescence imaging to visualise IgG-spiked fingermarks, as well as eccrine-rich and natural fingermarks by targeting epidermal growth factor, lysozyme, and dermcidin, with biotinylated secondary antibodies and horse radish peroxidase-labelled streptavidin [93]. The fingermarks were in contact with a gold electrode surface for up to one minute before a hydrophobic barrier was created to contain the working solution. The authors' initial primary antibody results were of poor quality due to the low concentration of the targets, requiring exposure times of four minutes.

Surface-enhanced Raman scattering (SERS) spectroscopy is a non-destructive technique used in biological detection and imaging. Attempts of utilising SERS tags for fingerprint detection were first made by Song et al. [94] (Figure 2-5). They designed antibody-silver nanoparticle-Raman probe conjugates to develop IgG-spiked fingermarks from a single donor on pre-treated glass slides. It was observed that the higher the concentration of IgG, the more intense the SERS signals became. Zhang et al. were able to visualise fingermarks by targeting lysozyme, IgG, and cotinine with antibodies conjugated to superbright SERS tags [95]. Spiked fingermarks were placed onto aldehyde-functionalised glass slides and incubated for two hours before their images were merged. Both of these studies failed to explore whether their methods would be successful with natural fingermarks on realistic substrates.

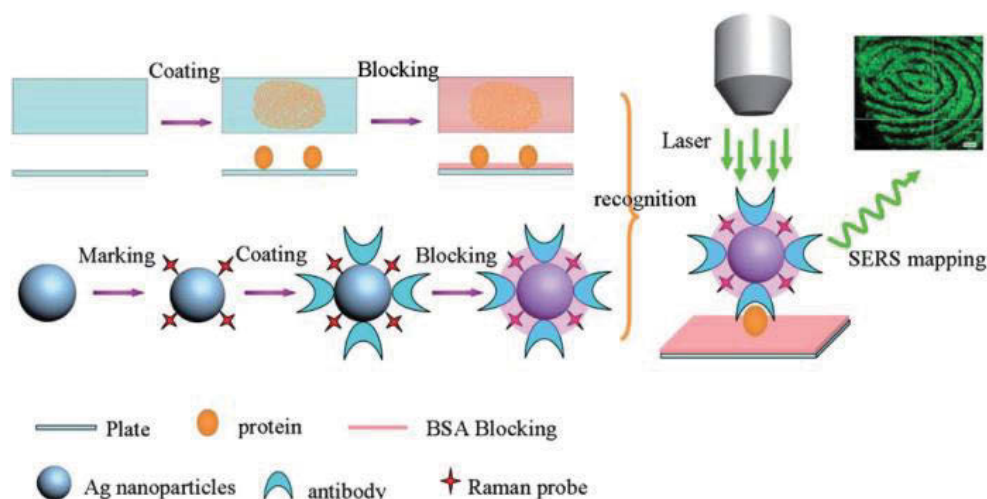


Figure 2-5 Schematic representation of the detection of proteins within latent fingerprints using SERS imaging technique (Reproduced from Song et al. [94]).

In summary, most of the published work to date has focused on immunodetection methods for one to three targets only and has rarely considered inherent intra- and inter-donor variability of fingerprint residues. Very little research included direct comparisons with routine methods or the effects of incorporating the immunodetection method within a routine sequence. These limitations prompted the current research objectives.

2.3 Objectives

Due to the inherent intra- and inter-donor variability of fingerprint residues, there is a need to create a more “universal” multi-target immunogenic fingerprint enhancement reagent. This reagent should target endogenous secretions in latent fingerprints, rather than drug metabolites or exogenous residues. Immunodetection methods should also be assessed in terms of compatibility with existing technique sequences.

With the continuing interest into immunogenic reagents for fingerprint detection, the first objective was to optimise relevant parameters for the enhancement of latent and body fluid-contaminated fingerprints deposited on a wider range of substrates commonly encountered in casework. Also, in order to evaluate these multi-target

reagents for potential operational use, the relative performances of the antibody- and aptamer-based reagents were compared to those of established latent and blood detection methods. Investigations into the compatibility of the optimised multi-target biomolecular reagents with existing routine technique sequences were also performed. Experiments were executed to determine if they were viable alternatives to routine techniques or complementary techniques in routine sequences.

2.4 Experimental Design

According to the International Fingerprint Research Group (IFRG) guidelines [96], relevant parameters (e.g., formulation, development conditions) should first be optimised. Then, the relative performance of the novel method should be compared to those of routine fingerprint detection methods in terms of sensitivity (i.e., capacity to develop weaker fingerprints) and selectivity (i.e., ability to target components of the fingerprint preferentially over the substrate [96], where specificity equates to 100% selectivity). A part of validation studies, which are performed to assess the suitability of a novel method for operational use, is to assess its performance on its own on realistic substrates, but also to determine its position within established detection sequences. In compliance with the IFRG guidelines, the research conducted in this chapter followed the key experiment design aspects for optimisation, comparison, and validation studies, such as collecting fingerprints from up to 12 donors; processing depletion series of fingerprints (i.e., successive impressions from the same digit, providing a sequential reduction in fingerprint residue); using over three realistic locally sourced substrates; using quantitative absolute and comparative scales to assess quality of fingerprint development; and assessing the multi-target reagents in terms of individual performance as well as in sequence with routine methods [96].

Antibodies were primarily used in the research presented in this chapter, which is building on from Spindler and colleagues' experimental work on multi-target immunogenic reagents or multiplex solutions [97-100]. Some aptamers were also incorporated into the multiplex solutions after Wood developed an aptamer only-based detection reagent [101]. Further investigations focusing on aptamers will be presented in later chapters.

Figure 2-6 illustrates the overall approach of this chapter. Fingermarks were deposited on a range of non-porous and semi-porous substrates and processed as detailed in the coming subsections for three main research areas: optimisation, direct comparisons, and technique sequencing. These substrates were chosen, as porous substrates were problematic in Spindler et al.'s previous research [99]. While the main focus of this PhD research was latent fingermark detection, different multiplex solutions and relevant parameters were not only optimised for latent fingermarks, but also three types of body fluid-contaminated fingermarks (blood, saliva, semen). Optimisation studies focused on relevant parameters such as processing times, the number antibodies and/or aptamers within the reagents, and different combinations of fixing and working solutions to potentially indicate whether these biomolecular reagents were practical. Only latent and blood-contaminated fingermarks were used to compare the relative performances of the optimised multi-target reagents to those of routine techniques, as well as positioning them within established sequences to determine utility for investigative casework.

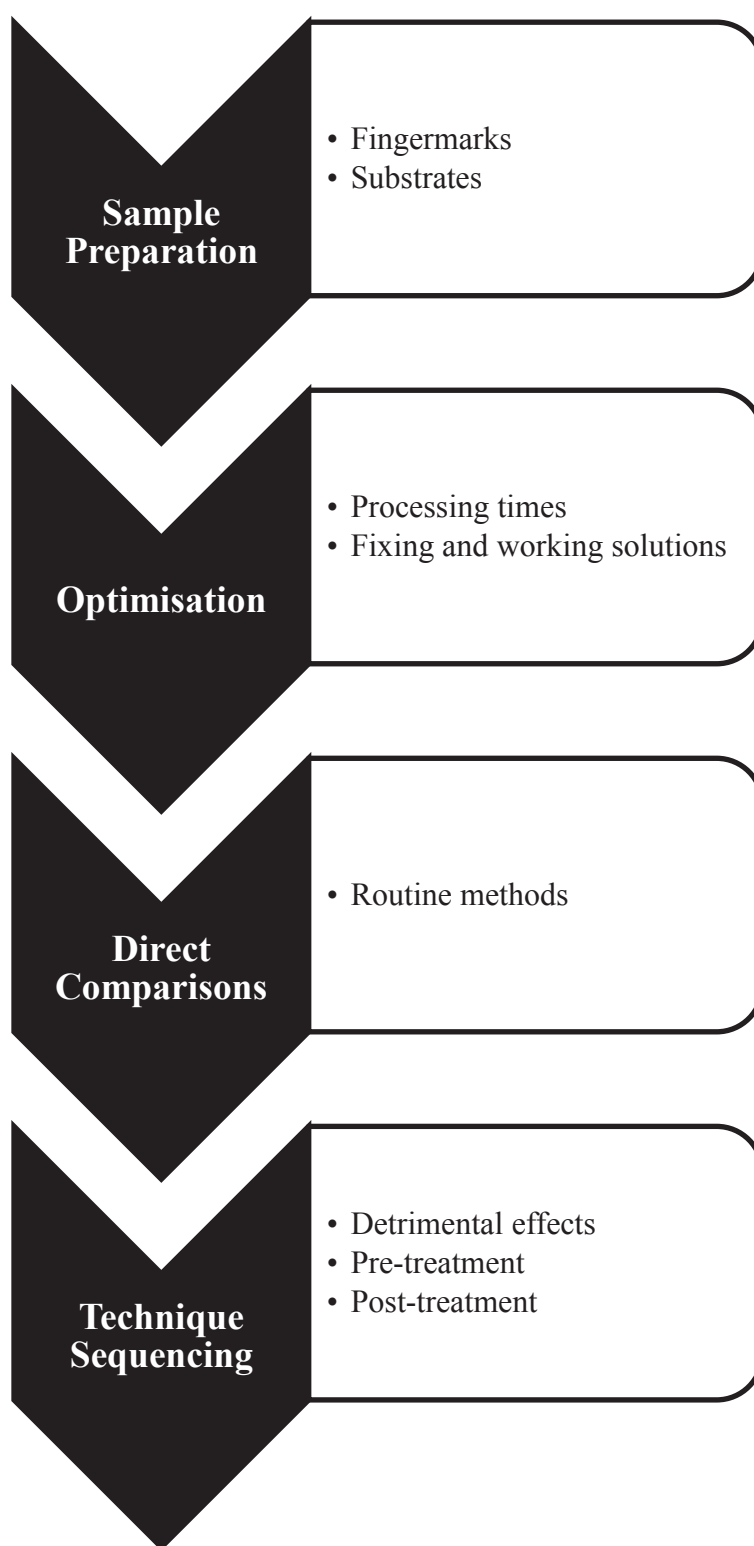


Figure 2-6 Approach overview for optimisation and validation of multi-target biomolecular recognition fingerprint enhancement reagents.

2.4.1 Materials and Instrumentation

Antibodies

All antibodies purchased were raised in rabbit, with the exceptions of anti-pan cytokeratin, anti-A HE195 clone, anti-B-HEB29 clone and anti-histatin 3 (clone 4G9) (raised in mouse), and anti-cAMP (raised in sheep).

Polyclonal anti-cAMP, anti-D-glucosamine, anti-human red blood cell (RBC), anti-semenogelin I, and anti-ODF3 antibodies were supplied by Abcam®.

Polyclonal anti-carnosine and monoclonal anti-histatin 3 (clone 4G9) antibodies were supplied by Abnova.

Polyclonal anti-acid phosphatase antibody was supplied by Aviva Systems Biology.

Monoclonal anti-pan cytokeratin [AE1/AE3], anti-A HE195 clone and anti-B-HEB29 clone, polyclonal anti- α -amylase, anti-mucin 5B, and anti-SPA17 [N1C3] antibodies were supplied by GeneTex Inc.

Polyclonal anti-SPAG11A (aa17-46) antibody was supplied by LifeSpan BioSciences Inc.

Polyclonal anti-cathepsin D antibody was supplied by Molecular Innovations.

Anti-L-amino acid and anti-L- α -hydroxy acid antibodies (raised in rabbit) were produced at Northern Illinois University by A/Prof. Oliver Hofstetter, as described in published methods [92, 102], and dialysed prior to use.

Polyclonal (whole antiserum) anti-cortisol, anti-serotonin, anti-haemoglobin, and anti-spectrin antibodies were supplied by Sigma-Aldrich.

Lectins

Helix Aspersa and Ulex Europaeus (lyophilised powders) lectins were supplied by Sigma-Aldrich.

Aptamers

Aptamers selected against catalase [103], “cathepsin D” (DGI, GEL, KAI tripeptide sequences) [104], cortisol [105], sperm [106], vitamin B12 [107], and vitamin D [108] were prepared and HPLC purified to order by Sigma-Aldrich’s Castle Hill oligonucleotide laboratory.

Luminescent Dyes

Fluorescent Orange 550 reactive and Fluorescent Red 630 reactive were supplied by Fluka.

AttoTec Atto 550 N-hydroxysuccinimide (NHS) ester, Atto 590 NHS ester, Atto 610 NHS ester, and Atto 647N NHS ester were supplied by Sigma-Aldrich and prepared according to manufacturer’s instructions.

Isoindole 1 was synthesised at the University of Technology Sydney (UTS) by Dr Xanthe Spindler (refer to ESI of [100]).

Multi-Target Biomolecular Reagents

Sodium citrate (ACS grade, Sigma-Aldrich), tetrachloroauric acid (Proscitech, Australia), sodium borohydride (99.99%, Sigma-Aldrich), O-(2-carboxyethyl)-O’-(2-mercaptoethyl) heptaethylene glycol (7EG, $\geq 95\%$, Sigma-Aldrich), NHS and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) ($\geq 97\%$, Fluka) were used as supplied for preparation of antibody-functionalised AuNPs.

2-(N-morpholino)ethanesulfonic acid (MES) was supplied by Merck.

Phosphate buffered saline (PBS) with 5% non-fat milk powder (dry milled, pH 7.3), 1% w/v bovine serum albumin (BSA, $\geq 98\%$), and ethylene glycol (EG, spectroscopic grade) were supplied by Sigma-Aldrich.

Current Fingerprint Detection Techniques

Indanedione (SHIRAN, Israel), ethyl acetate (99.8%, Sigma-Aldrich), glacial acetic acid (RCI Labscan Ltd.), zinc chloride (reagent grade; Scharlau), ethanol (EtOH, 100%, Chem-Supply) and HFE7100 (3M Novec) were used as supplied in the preparation of IND-Zn reagent.

Maleic acid (99%, Sigma-Aldrich), n-dodecylamine acetate (Optimum Technologies), Tween® 20 (Sigma-Aldrich), silver nitrate (99.5%, Chem-Supply), ferric nitrate (98%, Chem-Supply) and ammonium ferrous sulphate (AR grade, Chem-Supply) were used as supplied in the preparation of PD.

Cyanobloom low-density cyanoacrylate (CA, Foster + Freeman), basic yellow 40 (BY40, Lightning Powder Co.), rhodamine 6G (R6G, Sigma-Aldrich), isopropanol (99.9%, VWR) and methyl ethyl ketone (99.5%, Chem-Supply) were used for CA development and luminescent post-staining of latent fingerprints.

5-SSA (99.5%, BDH Chemicals Ltd.), EtOH (95%, Chem-Supply), glacial acetic acid (RCI Labscan Ltd.), AY7 (Optimum Technologies), AB (Hopkin & Williams) and MeOH (AR grade; Chem-Supply) were used as supplied in the preparation of blood reagents AY7 and AB.

Substrates

Beverage cans (primarily Coca-Cola Company), PET plastic water bottles (Cool Ridge), PE plastic ziplock bags (Coles Supermarkets Australia Pty Ltd, Woolworths Limited), polyvinylidene fluoride (PVDF, Amersham™ Hybond™ 0.45 µm, GE Healthcare), LDPE and microwave-safe PE cling film (Glad®, Woolworths Limited, respectively), and black PE garbage bags and light grey HDPE plastic shopping bags (Woolworths Limited) were used as non-porous substrates.

Glossy magazines obtained from UTS (UTS Playground publications) and Aldi (weekly catalogues), and glossy cardboard (Nabisco) were used as semi-porous substrates.

Additional Reagents and Materials

Semen from pooled human donors was supplied by Lee Biosolutions.

Amicon Ultra (Merck Millipore) 3 kDa spin columns were used to remove excess luminescent dye from incubation step with aptamers.

Instrumentation

Eppendorf miniSpinPlus was used for spin column purification of antibodies and aptamers incubated with luminescent dyes.

MVCTM 1000/D fuming cabinet (Foster + Freeman) was used to treat samples with CA.

Rofin Poliview IV [with either a PL500 or PL550XL forensic light source (FLS)] and V++ Precision Digital Imaging System software (version 4.0 or 5.0) were used to visualise and image processed fingerprints.

Magic Steam Press 7 (Singer) was used to apply heat to samples treated with IND-Zn.

2.4.2 Methods

2.4.2.1 Reagent Preparation

The antibody-AuNPs were synthesised using a similar method to Spindler et al. [70]. The multi-target biomolecular reagents, or multiplex solutions, were prepared as follows:

Gold-Citrate Nanoparticle Synthesis

To synthesise ~10 nm particles, 3.66 mL of freshly prepared 0.5% w/v sodium citrate solution was added to 18.5 mL milliQ (18.2 Ω) water in a two-neck round bottom flask, and then heated to reflux with constant stirring. Once condensation began to build up, 640 μ L of 0.011 M chloroauric acid was added rapidly and allowed to further reflux for 15 minutes, or until the solution had turned a merlot red colour. The

observed colour changes during the reaction were: pale yellow → colourless → grey → purple → burgundy → merlot red. The solution was then cooled to room temperature and stored in a clean bottle until use.

Thiol Linker Attachment

In a plastic vial tube, 115 μL of 21 mM 7EG linker was added to 5 mL of gold-citrate nanoparticles, mixed, and allowed to incubate at room temperature for four to six hours, or overnight in the refrigerator. The solution was then aliquoted into five 1.5 mL microcentrifuge tubes, which were centrifuged using the Eppendorf miniSpin Plus at 12,000 rpm for 16 minutes. The supernatant was carefully removed with a pipette and then 400 μL of MES buffer was added to reconstitute. If necessary, the aliquots were sonicated to break up and resuspend any clusters.

Antibody and Aptamer Labelling

Antibodies and aptamers were labelled with luminescent dyes prepared according to manufacturers' instructions. Quantities between 1.5 μL and 5 μL of different luminescent dyes were previously determined by Spindler [109], added to a variety of antibody and aptamer aliquots (10 μL to 100 μL), and mixed (Table 2-1). These were then left to incubate at room temperature for one or two hours, or longer if refrigerated. Labelled aptamers were purified using spin columns by centrifuging at 14,500 rpm for ten minutes. The unbound luminescent dye would be visible at the bottom of the centrifuge tubes. The bound materials were recovered by inverting the filter units in new centrifuge tubes and spinning at 6,000 rpm for two minutes.

Table 2-1 List of antibodies, aptamers, and luminescent dyes included in the multiplex solutions. All aptamers were DNA aptamers except vitamin B12. All luminescent dyes were commercially bought except isoindole-1, which was previously synthesised by Spindler (see ESI of [100]).

Type of Fingerprint	Antibodies	Aptamers	Luminescent Dyes
Latent	Anti-amino acid	Cathepsin DGI	Atto 550
	Anti-cAMP	Cathepsin GEL	Atto 590
	Anti-carnosine	Cathepsin KAI	Atto 610
	Anti-cathepsin	Cortisol	Atto 647N
	Anti-cortisol	Vitamin B12	Fluorescent Orange 550
	Anti-glucosamine	Vitamin D	Fluorescent Red 630
	Anti-L- α -hydroxy acid		Isoindole-1
	Anti-keratin		
	Anti-serotonin		
Blood-contaminated	Anti-A HE195 clone	Catalase	Atto 550
	Anti-A SAF clone		Atto 590
	Anti-B-HEB29 clone		Atto 610
	Anti-haemoglobin		Atto 647N
	Anti-spectrin		Isoindole-1
	Anti-RBC		
	Helix Aspersa lectin Ulex Europaeus lectin		
Saliva-contaminated	Anti- α -amylase	N/A	Atto 550
	Anti-histatin 3		Atto 590
	Anti-mucin 5B		Atto 610
			Isoindole-1
Semen-contaminated	Anti-semenogelin	Sperm	Atto 550
	Anti-acid phosphatase		Atto 590
	Anti-ODF3		Atto 610
	Anti-SPA17		Fluorescent Red 630
	Anti-SPAG11A		Isoindole-1

Tagged Antibody Attachment

The luminescently tagged antibodies were conjugated to the AuNPs without intermediate purification. For each microcentrifuge tube, 2 μ L of NHS/EDC solution was added, mixed, and allowed to sit at room temperature for two to five minutes.

Excess dye and unbound tagged antibodies were separated from the conjugates by centrifugation at 12,000 rpm for 16 minutes, the supernatant was removed, and the clusters were resuspended in 400 μ L MES buffer. The tagged antibodies were added, mixed, and left to incubate at room temperature for two hours, or longer if refrigerated.

Working Solutions

For aqueous (aq) solutions, aliquots of tagged antibody-AuNPs conjugates or labelled aptamers were placed in a new 15 mL Eppendorf tube and reconstituted to 5 mL, either with equal parts PBS with non-fat milk solution (one packet dissolved in 200 mL milliQ water, prepared at least 24 hours in advance):milliQ water, or with 1% BSA in PBS solution (prepared in-house using the protocol in [110]).

For glycolic (EG) solutions, the required amounts of tagged antibody-AuNPs conjugates or labelled aptamers were placed in a new microcentrifuge tube, which was then centrifuged at 12,000 rpm for eight minutes. The supernatant was removed and the clusters were resuspended in 5 mL ethylene glycol.

2.4.2.2 Sample Preparation

Non-porous and semi-porous substrates were chosen because they are often found at crime scenes or submitted as exhibits; they were deemed to be representative of casework materials. All of the substrates were pristine and used as is, except for the empty beverage cans and bottles. These were washed with warm soapy water, rinsed, dried, and then an acetone wipe was used to remove any residual traces from the outer surface prior to fingerprint deposition. Samples were prepared by drawing grid lines on the substrate surfaces to ensure that one fingerprint was present per cell and was divided evenly – in quarters for optimisation experiments and in half for comparison experiments – so equivalent areas of fingerprint residue were developed and assessed for each technique. The number of columns and rows varied depending on the number of donors and fingerprints per donor were required, respectively.

Male and female donors, ranging from poor to good fingerprint donors, were asked to deposit both natural latent and blood-contaminated fingerprints. Donors were asked

not to wash their hands for at least an hour prior to collecting natural fingermarks. Blood-contaminated fingermarks were collected by pricking the finger with a single-use blood sampling lancet and rubbing the blood over the finger pad prior to deposition. If there was an excess of blood, donors were asked to deposit one or two fingermarks on a blank piece of paper prior to depositing on the prepared substrates. The reason for this was to ensure that the fingermarks were faint and not readily visible. Saliva-contaminated fingermarks were collected from the same four donors by having them swab their inner cheeks with a cotton-tipped swab and then rubbing it onto their finger pad prior to deposition. Mixed donor semen was applied onto a fingermark rubber stamp with a cotton-tipped swab. All fingermarks were deposited in depletion series and then stored in the dark under ambient laboratory conditions for up to 4.5 months.

For the optimisation experiments, all four types of fingermarks (latent, blood-, saliva-, and semen-contaminated) were collected and processed. However, for the direct comparison and technique sequence experiments, only latent and blood-contaminated fingermarks were collected and processed. The reason for this being is that there are no routine detection methods for saliva and semen as they relate to fingermarks.

2.4.2.3 *Sample Processing*

The aged fingermarks were cut in half or in quarters depending on the experiment, with each portion processed with a different technique or set of conditions for direct comparison purposes (Figure 2-7). Fingermarks were air dried and then the corresponding halves or quarters were reconstructed, visualised under appropriate viewing conditions (Table 2-2), and imaged using the Rofin Poliview IV and V++ Precision Digital Imaging System software.

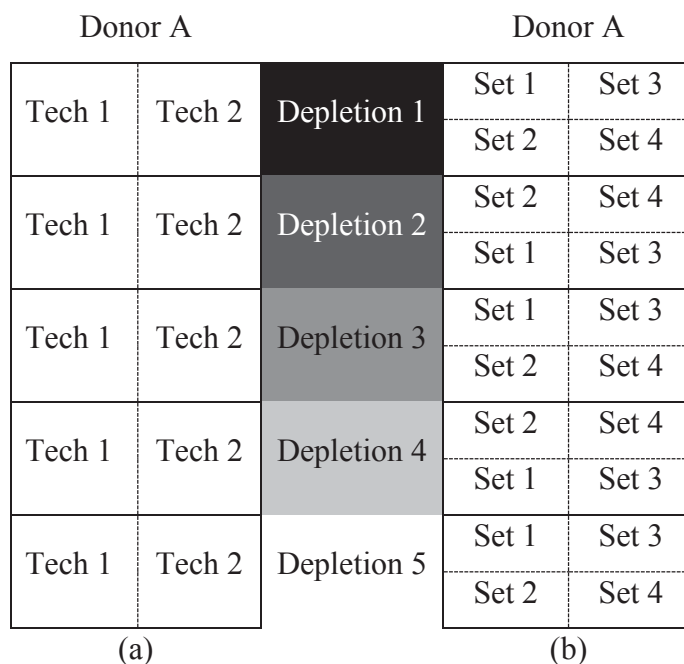


Figure 2-7 Schematic diagrams of how depleting fingerprints were processed when: (a) halved and (b) quartered. The gradient in colour between the two diagrams illustrates the decrease in fingerprint residues within a depletion series.

Table 2-2 Visualisation conditions utilised.

Technique	Excitation (nm)	Bandpass Filter (nm)
CA	White light	N/A
R6G	530	610
IND-Zn	530	610
PD	White light	N/A
AY7	450	530
AB	White light	N/A
Multiplex solution	555-620	650-750

2.4.2.4 Fingerprint Evaluation

Two different evaluation scales were used to assess the performance of the various techniques (Tables 2-3 and 2-4). The Centre for Applied Science & Technology

(CAST) scale [111] was used to assess the overall performance of the technique based on the area of developed ridge detail. The University of Canberra (UC) scale [112] was used to directly compare the corresponding fingerprint halves by assigning a score for the assessed technique (i.e., multiplex solution) based on the quality of development relative to the control method (i.e., routine technique). Corresponding fingerprint halves given CAST scores of 0 were removed from the UC scale analysis, as there was no evidence of development to assess or compare. This allowed for UC scores of 0 to solely denote no difference in enhancement between the two techniques being compared (rather than the absence of development for both halves).

Table 2-3 The CAST scale utilised to evaluate the halved fingerprints individually. Representative image examples of fingerprint halves are included.

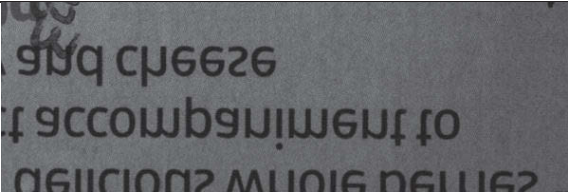
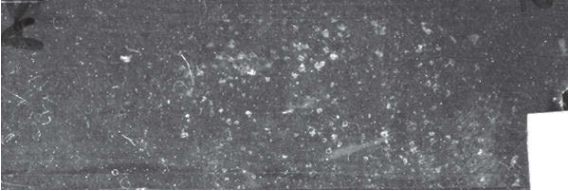
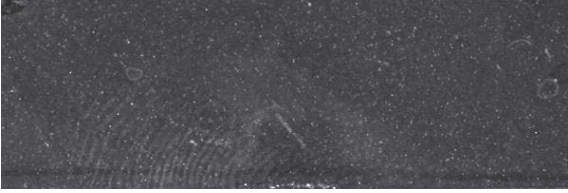





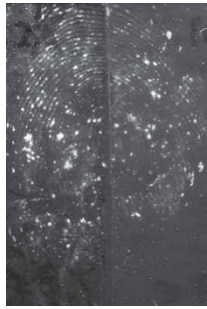

Score	Description	Representative Image
0	No development; no evidence of fingerprint	
1	Weak development; evidence of contact, but no ridge detail	
2	Limited development; about 1/3 of ridge details are present	
3	Strong development; between 1/3 and 2/3 of ridge details are present	
4	Very strong development; full ridge details present	

Table 2-4 The UC scale utilised to evaluate corresponding halved fingermarks. Representative image examples illustrate comparisons between the assessed technique (left) and the other technique (right).

Score	Description	Representative Image
-2	Significant decrease in enhancement of the assessed technique when compared to the other technique	
-1	Slight decrease in enhancement of the assessed technique when compared to the other technique	
0	No enhancement of the assessed technique when compared to the other technique	
+1	Slight increase in enhancement of the assessed technique when compared to the other technique	
+2	Significant increase in enhancement of the assessed technique when compared to the other technique	

2.4.2.5 Optimisation of Multiplex Solutions

Determination of Optimal Processing Time

Previous research by Spindler et al. had stated that fingermarks could be developed after ten minutes or up to two hours [70]. The aim of this experiment was to determine if there was an optimal length of time that the substrate should be in contact with the multiplex solution for successful development. It was hypothesised that fingermarks deposited by poor donors would require more time to develop. A total of twelve donors (four male, eight female) deposited two sets of three depleting natural fingermarks onto two substrates (total = 144 collected). The substrates chosen were an ideal experimental substrate, PVDF, and a commonly encountered in casework non-porous substrate, ziplock bag. The fingermarks were aged around 10 or 50 days before being quartered; each segment processed in a different time interval, ranging from 15 minutes to two hours, in 15-minute intervals (Figure 2-8). Various working solutions were used: aqueous (with and without a BSA blocking buffer) and glycolic solutions, all containing eight to ten components (i.e., antibodies and aptamers).

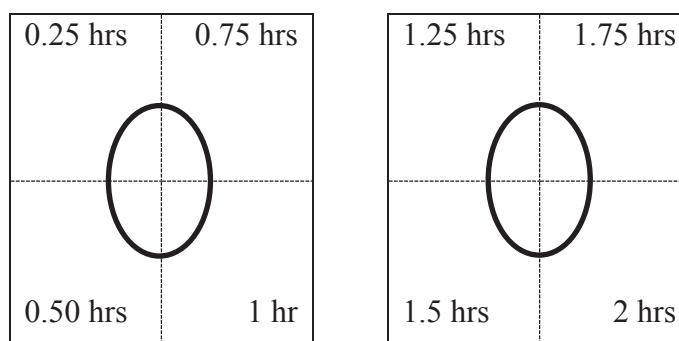


Figure 2-8 Schematic diagrams depicting manner in which quartered fingermarks were processed to compare the eight different time intervals.

Investigation of Optimal Multiplex Conditions

Once the optimal processing time was identified, other relevant parameters were investigated. This experiment aimed to determine whether the number of components within the multiplex working solution affected its performance. With an increased number of substrates compared to the proof-of-concept study [70], the relative performance of aqueous and glycolic working solutions was assessed. Also, if applicable, the choice of fixing solution (5-SSA or ice-cold MeOH) was also studied.

It was hypothesised that a higher number of components would result in better development.

This experiment was divided into four sub-experiments based on the type of fingerprints used: latent, blood-, saliva-, and semen-contaminated (Table 2-5). Six non-porous and two semi-porous substrates were used for all sub-experiments, with the exception of saliva-contaminated fingerprints which did not include cling wrap. The same fingerprint donors were used to deposit the latent (total collected = 320, aged 10 to 70 days), blood-contaminated (total collected = 480, aged 10 to 70 days), and saliva-contaminated (total collected = 140, aged 30 and 40 days) fingerprints. A rubber fingerprint stamp was used to deposit the depleting semen-contaminated marks (total collected = 195, aged 10 and 20 days). Only blood-contaminated fingerprints required a fixing solution prior to the application of the multiplex solution. A number of working solutions were used to assess the optimal number of components within the multiplex working solutions and whether aqueous or glycolic solutions were better (Figure 2-9).

Table 2-5 Summary of variables for optimisation study.

Sub-Experiments	Latent	Blood-Contaminated	Saliva-Contaminated	Semen-Contaminated
Substrates	Ziplock bag, shopping bag, garbage bag, cling wrap, can, plastic bottle, glossy magazine, glossy cardboard	Ziplock bag, shopping bag, garbage bag, cling wrap, can, plastic bottle, glossy magazine, glossy cardboard	Ziplock bag, shopping bag, garbage bag, can, plastic bottle, glossy magazine, glossy cardboard	Ziplock bag, shopping bag, garbage bag, cling wrap, can, plastic bottle, glossy magazine, glossy cardboard
Donors	2 males, 2 females	2 males, 2 females	2 males, 2 females	Rubber stamp
Fingermarks	2 sets of 5 depleting natural latent fingermarks (quartered)	3 sets of 5 depleting blood- contaminated fingermarks (quartered)	Set of 5 depleting saliva- contaminated fingermarks (halved)	Sets of 5 depleting semen- contaminated marks (halved)
Fixing solutions	N/A	5-sulfosalicylic acid for 5 minutes, ice cold methanol for 15 minutes	N/A	N/A
Working solutions	Aqueous (with and without blocking buffer) and glycolic containing 4, 6, 8, 9, and 10 components	Aqueous and glycolic containing 4, 6, and 8 components	Aqueous and glycolic containing 3 components	Aqueous and glycolic containing 5 components
Processing times	45 minutes for non-porous, 15 minutes for semi-porous	45 minutes for non-porous, 15 minutes for semi-porous	45 minutes for non-porous, 15 minutes for semi-porous	45 minutes for non-porous, 15 minutes for semi-porous

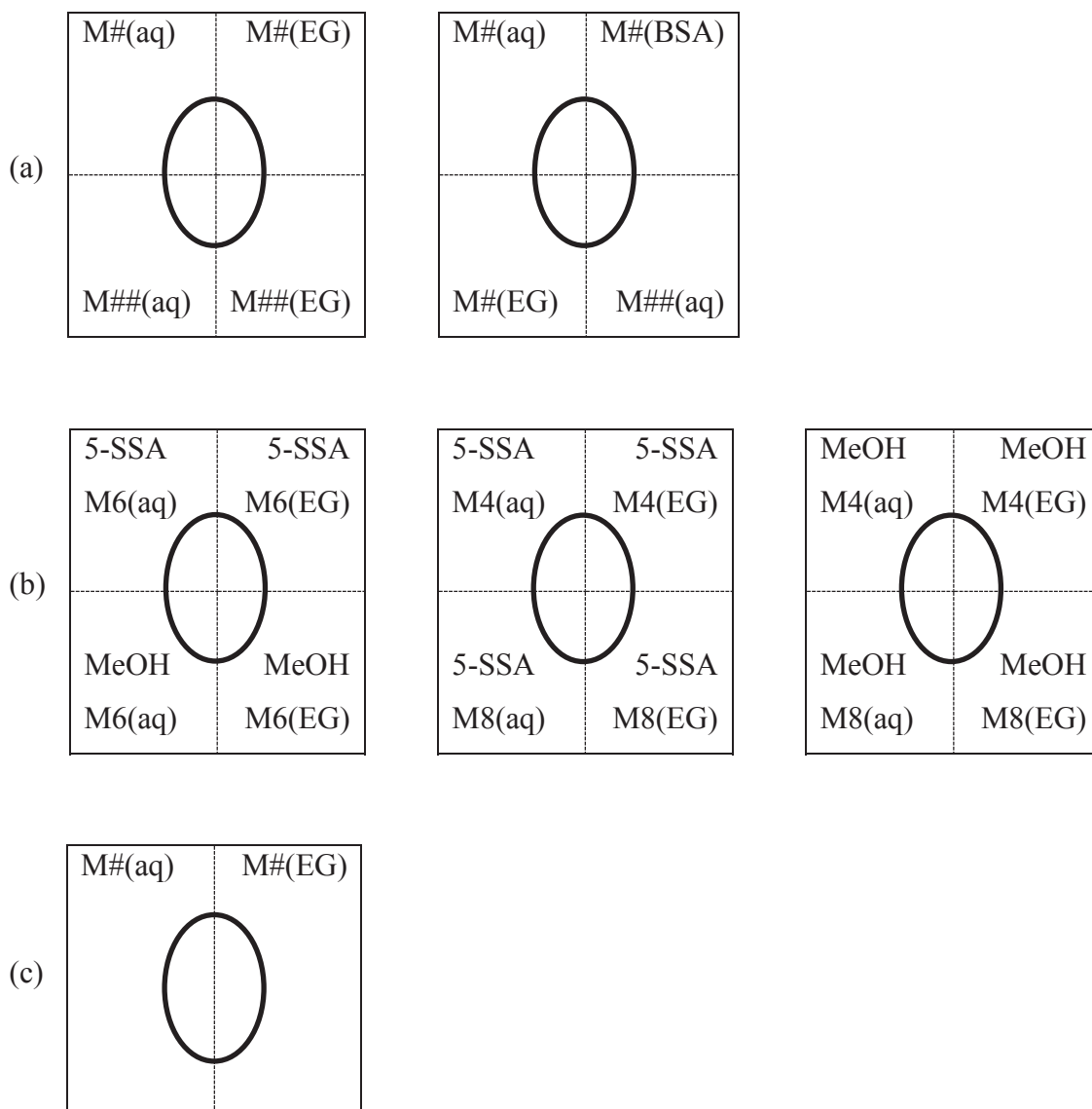


Figure 2-9 Schematic diagrams depicting the different processing configurations for: (a) latent fingerprints; (b) blood-contaminated fingerprints; and (c) saliva- and semen-contaminated fingerprints. (aq = aqueous working solution; BSA = aqueous working solution containing bovine serum albumin; EG = glycolic working solution; M# = multiplex solution containing # antibodies/aptamers; MeOH = ice-cold methanol fixing solution used; 5-SSA = 5-sulfosalicylic acid fixing solution used)

2.4.2.6 Direct Comparison to Routine Fingerprint Enhancement Techniques

As previously mentioned, there are no conventional detection methods for saliva- and semen-contaminated fingerprints. Therefore, this experiment only assessed the

relative performance of the multiplex solution with those of routine techniques. It was hypothesised that the routine techniques would perform better on fresh fingerprints, while the multiplex solution would perform better on aged fingerprints.

The same donors (two male, two female) and realistic substrates (six non-porous, two semi-porous) used in the optimisation study were used for consistency. Sets of five depleting natural and blood-contaminated fingerprints were deposited (total collected = 160 each) and aged up to 4.5 months. Aqueous working solutions containing eight components, with a solution height of approximately 0.75 cm in the processing tray, were applied to non-porous substrates for 45 minutes, while only for 15 minutes for the semi-porous substrates, with occasional gentle shaking.

The routine reagents used for comparison purposes – CA fuming; R6G luminescent post-staining; IND-Zn; PD; AY7; and AB – were used in sequence as listed in Table 2-6. The chemical formulations and applications were those reported by Stoilovic and Lennard [113] (Appendix I). The PD methodology was modified for local conditions and substrates as recommended by de la Hunty [114] (Appendix I). For latent fingerprints on semi-porous substrates, NIN was not included in the routine sequence, as it does not luminesce. However, PD was still included to see what effect the use of AuNPs would have on the technique in later experiments. As blood-contaminated fingerprints could still contain some latent fingerprint components, they were first treated with CA prior to the application of the blood reagent. Since AY7 is not suitable for semi-porous substrates, AB was used in these cases. A 2% w/v 5-SSA aqueous solution was used to fix the blood-contaminated fingerprints prior to processing with the detection methods.

Table 2-6 Routine fingerprint detection sequences selected for non-porous and semi-porous substrates. Only latent fingerprints deposited on semi-porous substrates were sequentially processed with three techniques.

Type of Fingerprint	Substrate Type	Routine Sequence		
		Technique 1	Technique 2	Technique 3
Latent	Non-porous		R6G	
	Semi-porous		IND-Zn	PD
		CA	→	→
Blood-contaminated	Non-porous		AY7	
	Semi-porous		AB	

The aged fingerprints were split in half; one half was processed with the multiplex solution and the other half was processed with the first technique of the routine sequence. Fingerprints were air dried, visualised under appropriate viewing conditions, and imaged. The routine fingerprint halves were further processed with the second technique in their sequences, dried, and visualised. If required, the routine fingerprint halves were processed with a third technique and photographed with their corresponding multiplex solution fingerprint halves.

2.4.2.7 Compatibility with Routine Technique Sequences

Assessment of Routine Technique Effects on Multiplex Solution Performance

As outlined in the IFRG guidelines, consideration into the value of a novel method placed in a detection sequence should be taken [96]. When a new technique is proposed for fingerprint detection, it should be assessed in conjunction with routine techniques. The new method may have a detrimental effect on the routine technique performance, or vice versa. Tests were conducted to determine if routine techniques had a detrimental effect on the biomolecular reagent prior to assessing its performance within routine technique sequences.

In order to assess the effect routine techniques may have on the multiplex working solutions, aged fingermarks were first pre-treated with routine techniques and then further processed with the multiplex solution. For latent fingermarks, sets of three natural depleting fingermarks were deposited by three female donors on shopping bag, garbage bag, can, and glossy magazine and aged up to 12 days (total collected = 99). For blood-contaminated fingermarks, sets of three depleting fingermarks were deposited by one of the three female donors on ziplock bag and aged for 37 days (total collected = 18). The split fingermarks were first treated by the same routine sequence as listed in Table 2-7. Blood-contaminated fingermarks were fixed with a 2% w/v 5-SSA for five minutes prior to the application of AY7 or AB. Once dried and photographed, one fingermark half was processed with fresh multiplex working solution and its corresponding half was processed with used multiplex working solution (i.e., exposed to other samples treated with routine methods), both containing eight components, 45 minutes for non-porous substrates and 15 minutes for semi-porous substrates (Figure 2-10).

Table 2-7 Routine sequences used to assess the effects of routine techniques on the multiplex solutions.

Fingermark Type	Routine Sequences
Latent	CA
	CA → BY40
	CA → R6G
	CA → IND-Zn
	CA → IND-Zn → PD
Blood-contaminated	CA → AY7
	CA → AB

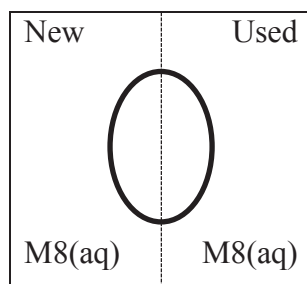


Figure 2-10 Schematic diagram showing how a split fingerprint was processed. Used multiplex solution was created by exposing it to additional fingerprints pre-treated with the various routine technique sequences. For blood-contaminated fingerprints, 5-SSA fixing solution was used prior to the multiplex solution application. (aq = aqueous working solution; M# = multiplex solution containing # antibodies/aptamers)

Impact of Routine Dyestain on Multiplex Solution Luminescence

Fingermarks treated with luminescent dyestains, BY40 and R6G, were directly compared to each other to determine if either would interfere with the luminescence of the multiplex solution. Although R6G is more commonly used in casework, it was hypothesised that it may interfere since they are viewed around the same wavelength range.

Four donors (two male, two female) deposited sets of five depleting natural fingerprints (total collected = 90) onto three non-porous substrates chosen due to their varying appearance: transparent ziplock bag, opaque black garbage bag, and multi-coloured can. A multiplex solution of eight components was used to process the aged (up to 3.5 months) samples for 45 minutes. The multiplex solution was placed before and after a common two-step routine sequence (i.e., multiplex solution → CA → dyestain, CA → dyestain → multiplex solution), as well as between the two techniques (i.e., CA → multiplex solution → dyestain). The only difference between the corresponding fingerprint halves was the dyestain; half was treated with BY40, while the other half was treated with R6G.

Effect of Multiplex Solutions on Routine Technique Sequences

Typically, technique sequencing would only be completed if the novel method had comparable or superior performance to routine techniques. However, since some of the multiplex solution targets are found in eccrine secretions, which are not typically

the intended fingerprint residue components on non-porous substrates, there may be an added benefit to its inclusion in a detection sequence. It is possible that there could be extra ridge development on non-porous substrates, or it could replace two techniques required for semi-porous substrates. The aim of this experiment was to determine if there was any benefit to including the multiplex solution in routine technique sequences, and if so, where it should be placed in the sequences. Two main questions were investigated:

- What effect does the multiplex solution have when used before a particular routine technique?
- Can the multiplex solution be used as a post-treatment after a particular routine technique?

The fingerprints collected for the direct comparison study were a portion of the total number of fingerprints collected for the technique sequence study; they were all deposited and aged simultaneously. As with the direct comparison study, only latent and blood-contaminated fingerprints were used in this technique sequencing study. The same eight substrates were prepared and the same four donors deposited sets of five depleting fingerprints. A total of 600 natural and 480 blood-contaminated fingerprints were collected and aged up to 4.5 months. The latter fingerprints were fixed with 5-SSA for five minutes prior to application of any routine method. The non-porous substrates were subjected to the aqueous multiplex working solution containing eight components for 45 minutes, while the semi-porous substrates only 15 minutes. The various sequences compared are listed in Table 2-8, which generalises the sequences found in Table 2-6; however, this time with the incorporation of the multiplex solution at different step positions. All possible combinations of direct comparisons between the sequences were performed (i.e., A vs B, B vs C, C vs A, etc.).

Table 2-8 Incorporation of the multiplex solution at different positions within the routine sequences. Only samples of latent fingerprints on semi-porous substrates included the steps in grey.

		Sequence			
		A	B	C	D
Step	1	Multiplex	Technique 1	Technique 1	Technique 1
	2	Technique 1	Multiplex	Technique 2	Technique 2
	3	Technique 2	Technique 2	Multiplex	Technique 3
	4	Technique 3	Technique 3	Technique 3	Multiplex

Since fingerprint halves were sequentially processed, not only could the same step in two sequences be compared (e.g., multiplex solution compared to CA), but subsequent steps as well. For example, for latent fingerprints deposited on non-porous substrates, a comparison between A1 and C2 in Table 2-8 would represent a comparison between the multiplex solution and the routine sequence of CA → R6G.

2.5 Results and Discussion

2.5.1 Optimisation of Multiplex Solution

2.5.1.1 Determination of Optimal Processing Time

The work presented in this subsection investigated how the processing time affected results. Several working solutions were prepared and compared over the eight 15-minute time intervals. As two fingerprints were required to compare the eight time intervals, it is possible that there was residue variability between the two fingerprints. Each time interval was not directly compared to each other; the four shorter intervals were compared to one another with one fingerprint and the four longer intervals were compared to each other with the other fingerprint. The fingerprints were quartered due to the large number of variables; as a result of the small development area to evaluate, the comparative scale was not used. All the time intervals seemed to exhibit

the same level of luminescence and the clarity of ridge detail, when present, also appeared comparable Figure 2-11.



Figure 2-11 Natural fingerprint deposited on PVDF and processed for 15 minutes (top right), 30 minutes (bottom right), 45 minutes (top left), and one hour (bottom left), visualised at 590 nm with a 650 nm bandpass filter.

The average CAST scores were calculated for each substrate and time interval. Since PVDF would be considered an ideal substrate, whereas the ziplock bag is a more realistic substrate, it was expected that PVDF would have much higher average CAST scores than ziplock bag. This demonstrated the importance of conducting experiments on surfaces more relevant to the desired application, in this case for fingerprint detection in police investigations. Development on the white portions of the ziplock bag, such as its logo or areas designated for writing, was better than on the clear portions. This indicated that the multiplex solution may interact with the substrate differently depending on its surface composition (Figure 2-12). Although poor results were observed on the ziplock bag, they may not have been indicative of the multiplex solution's performance, as other parameters had not yet been optimised. There are multiple reasons as to why the biomolecular reagent was not performing well. Some factors that affect the antibody-antigen interactions include the antibody concentration,

the number of antigen sites, the reaction time, as well as the pH and ionic strength of the surrounding medium.

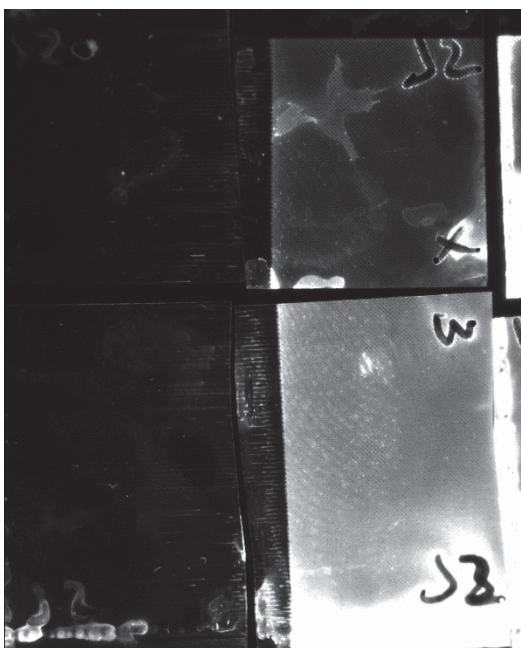


Figure 2-12 Natural fingerprint deposited on ziplock bag and processed for 1.25 hours (top left), 1.5 hours (bottom left), 1.75 hours (top right), and two hours (bottom right), visualised at 590 nm with a 650 nm bandpass filter.

The results indicate that the length of time in which the substrate was in contact with the multiplex working solution did not have a significant effect (Figure 2-13). Since there were no large differences between the intervals, one might be inclined to use a processing time of 15 minutes. However, it was observed that fingerprints deposited by poor donors did not normally develop enough usable ridge detail until after 45 minutes. One desirable of this research is to find a “universal” reagent. Therefore, fingerprints that may be deposited by poor secretors should not be overlooked. However, since the desired output is to implement the use of the biomolecular reagent by law enforcement personnel, processing an item for more than one hour is not favourable. If a large volume of exhibits required processing, leaving an exhibit in the solution for two hours is impractical. Therefore, the data for the four shorter time intervals, from 15 minutes to one hour, were examined more closely. The 45-minute interval seemed to have a slightly better distribution of scores with more fingerprints given a score of 4 and/or less fingerprints given a score of 0 (Figure 2-14).

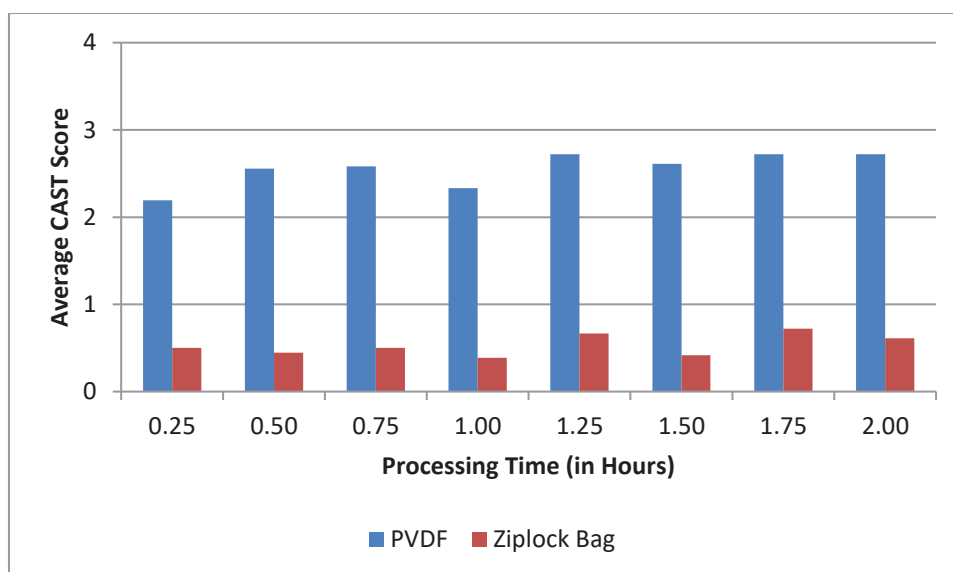


Figure 2-13 Average CAST scores calculated for latent fingerprints developed on PVDF and ziplock bag to compare eight different processing times.

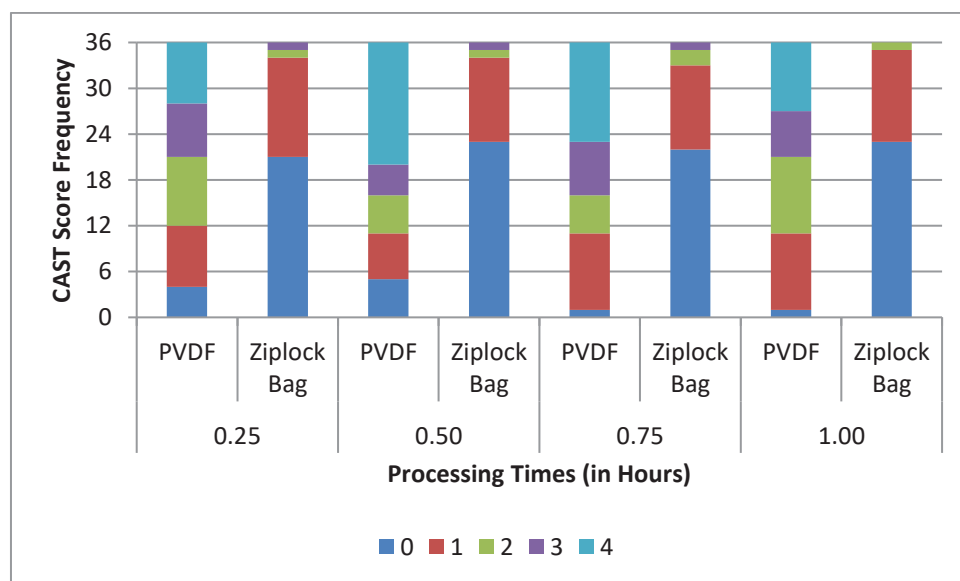


Figure 2-14 Frequency of CAST scores for latent fingerprints developed on PVDF and ziplock bag to assess four shorter processing times.

Processing a sample for 45 minutes may be considered too long or impractical from an operational perspective for routine use. It could be possible to process a sample for 15 minutes and if development is not visible, the sample could be re-processed again in 15-minute intervals. This could decrease the time in practice, however, is not

recommended because as the number of processing and rinsing steps is increased, the chance of recovering fingerprint evidence decreases, unless a subsequent technique targets different components of the fingerprints [21]. Examples of routine methods that are time-consuming are PD – due to all the different wash and processing trays – and VMD – processing time is very quick, but reaching ideal pressure takes time. Although time-consuming, these methods are still used as they have niche applications; wet porous substrates for PD and polymer banknotes or cold cases for VMD. If a niche application can be found for this biomolecular reagent, such disadvantages may be overlooked.

2.5.1.2 Investigation of Optimal Multiplex Conditions

Latent Fingerprints

The working solutions chosen for each substrate depended on the availability of antibodies and aptamers at the time of the sub-experiment. To maintain some consistency between the working solutions, the same components from the smaller multiplex solution (i.e., those with four or six components) were included in the larger multiplex solution (i.e., those with eight to ten components). With the donors being the same for all of the samples, this was intended to assist in determining the optimal number of components needed in the multiplex solution. Also, due to the poor results for the initial samples processed with aqueous solution, a 1% BSA blocking buffer was added to some of the comparisons. Therefore, the substrates that were processed with the same set of working solutions were grouped together in separate graphs (Figure 2-15). This was the best way to illustrate the results, as combining them in a single graph with all the substrates together would lead to the data being skewed. For example, the multiplex solution of ten components would appear superior; however, it was only used with one substrate.

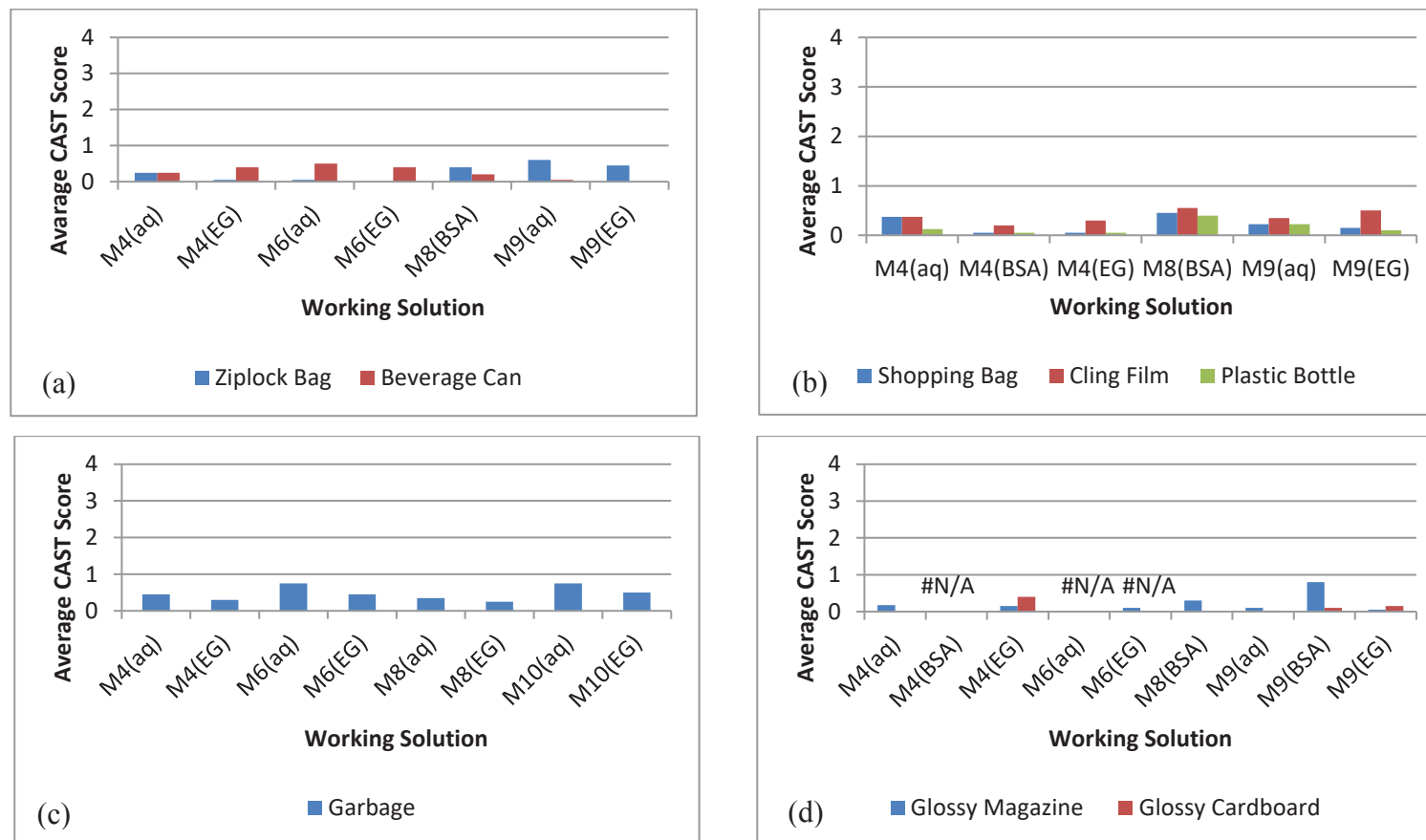


Figure 2-15 Results for latent fingerprints on non-porous substrates (a-c) and semi-porous substrates (d) organised by average CAST scores and working solutions. In Figure 2-15(d), glossy magazine was not processed with M4(BSA), while glossy cardboard was not processed with either M6 working solution. (aq = aqueous working solution; BSA = aqueous working solution containing bovine serum albumin; EG = glycolic working solution; M# = multiplex solution containing # antibodies/aptamers)

Contrary to expectations, the number of components did not seem to affect the multiplex solution's performance. Better development was anticipated since it was thought that there would be more selectivity and sensitivity towards more targets. A possible explanation could be that once some of the antibodies and aptamers interacted with their cognate targets in the fingerprint residues, they inhibit additional interactions between other multiplex solution components and their associated targets. Another explanation could also be that the fingerprints did not have an abundance of a particular target. A multiplex solution of eight components was chosen for subsequent experiments in an attempt to increase the chances of development since fingerprint composition is inherently variable between donors.

The performance of the working solutions was variable from substrate to substrate; background noise caused by the biomolecular recognition probes binding to the organic moieties of the substrates rather than the fingerprint constituents may be the reason. In general, there was better development on non-porous substrates than on semi-porous substrates. The processing time for semi-porous substrates was decreased from 45 minutes to 15 minutes after initial samples showed the diffusion of the fingerprint. It was difficult to visualise the development on multi-coloured or patterned backgrounds, such as cans, glossy magazines, and glossy cardboard. The range of luminescent dyes was useful to tailor and try to minimise distracting patterns under luminescence conditions. However, using too many luminescent dyes in the same working solution could affect the resulting luminescence. If various components are luminescing at different wavelengths and require different viewing conditions, not all the ridge detail development would be visible at the same time with conventional equipment; using hyperspectral imaging could address this issue. Another issue associated with the use of multiple fluorophores in the same solution could be the quenching effect one fluorophore may have on another, resulting in a decrease in luminescence intensity. It should be noted that the manner in which the multiplex solution was applied may not be well-suited for operational use. Curved exhibits like bottles and cans would require a large volume of solution in order for the whole surface to be in contact with the solution (i.e., immersed). For this research, bottle and flattened can strips were utilised. However, cutting an exhibit is not recommended, as fingerprint evidence may be damaged in the process.

Typically, the aqueous solution was better for the non-porous substrates, while glycolic was better for semi-porous. Unfortunately, these working solutions may be impractical for the intended end-users, especially for the semi-porous substrates. Semi-porous substrates processed with glycolic solutions took several days to dry, even though the sample pieces had dimensions of 2.5 cm by 3 cm. This could equate to weeks to dry larger exhibits like an entire glossy cardboard box. The inclusion of 1% BSA in the aqueous solution had noticeable effects on the semi-porous substrates as well. It increased the luminescence of the creases/wrinkles created by the aqueous solution, and as a result, making it harder to visualise the ridge details.

Blood-Contaminated Fingermarks

Blood-contaminated fingerprint development was greater than that obtained for latent fingerprints, although still variable between substrates (Figure 2-16). Weaker luminescence was observed with more heavily deposited blood-contaminated fingerprints due to the quenching effect of haemoglobin [8].

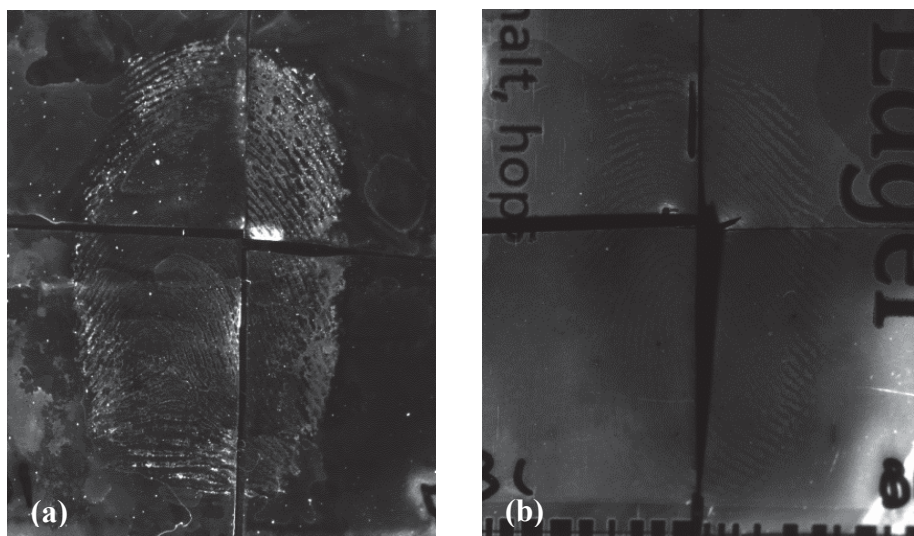


Figure 2-16 Blood-contaminated fingerprints on (a) garbage bag and (b) beverage can, both fixed with 5-SSA solution and processed with glycolic working solution with four components (top left), glycolic working solution with eight components (bottom left), aqueous working solution with four components (top right), and aqueous working solution with eight components (bottom right). Both developed fingerprints were visualised at 590 nm with a 650 nm bandpass filter.

In general, it was found that a 2% w/v 5-SSA fixing solution was less detrimental to blood-contaminated fingerprints than the use of an ice-cold MeOH fixing solution (Figure 2-17), once processed with a multiplex working solution (Figures 2-18 and 2-19). It could also be seen that the aqueous working solution typically had a higher average CAST score than the glycolic solution. When fingerprints were processed with multiplex solutions differing by four components, the resulting development was comparable (Figure 2-19). Similar to the results found with the latent fingerprints, the number of components did not seem to affect the biomolecular reagent's overall performance. The fixing-working solution combination which had the worse overall performance was the MeOH fixing solution with a glycolic working solution. Methanol has been known to be detrimental with some substrates, but it also caused inks on the substrates to bleed even prior to placing them into either working solution. This is not a desired effect especially if the exhibit is a document that also requires other forensic procedures, such as handwriting or thin layer chromatography analysis.

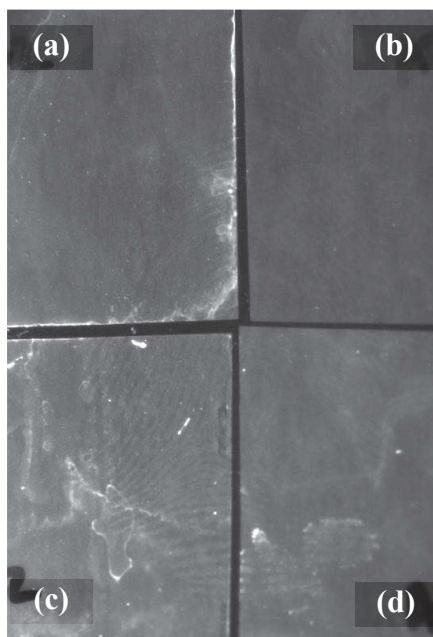


Figure 2-17 Quartered blood-contaminated fingerprint developed with a multiplex solution of six components on a light grey shopping bag with: (a) MeOH fixing/aqueous working solutions; (b) MeOH fixing/glycolic working solutions; (c) 5-SSA fixing/aqueous working solutions; and (d) 5-SSA fixing/glycolic working solutions. All quarter marks were visualised under an excitation of 590 nm with a 650 nm bandpass filter.

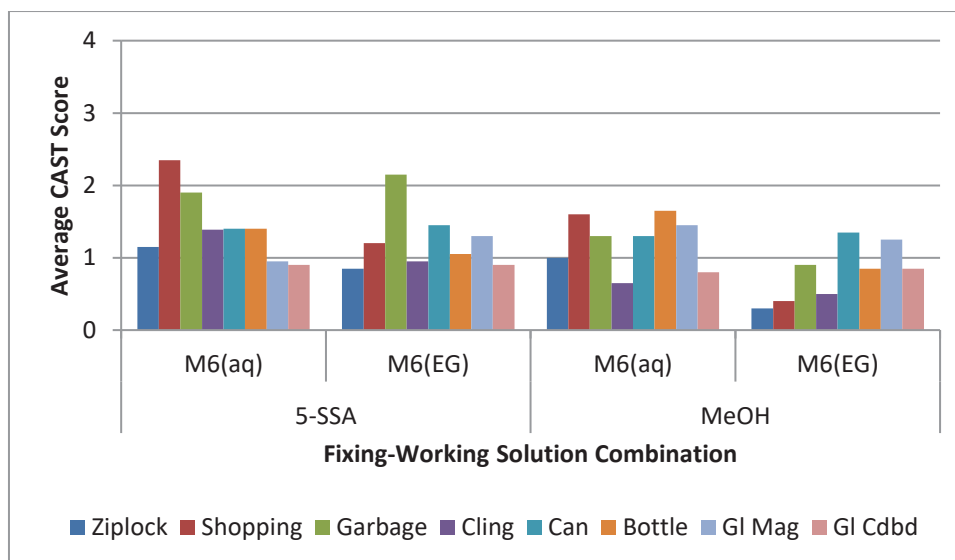


Figure 2-18 Average CAST score results comparing blood-contaminated fingerprints fixed with two different fixing solutions, but all processed with a multiplex solution of six components. (aq = aqueous working solution; EG = glycolic working solution; M# = multiplex solution containing # antibodies/aptamers; MeOH = ice-cold methanol fixing solution used; 5-SSA = 5-sulfosalicylic acid fixing solution used)

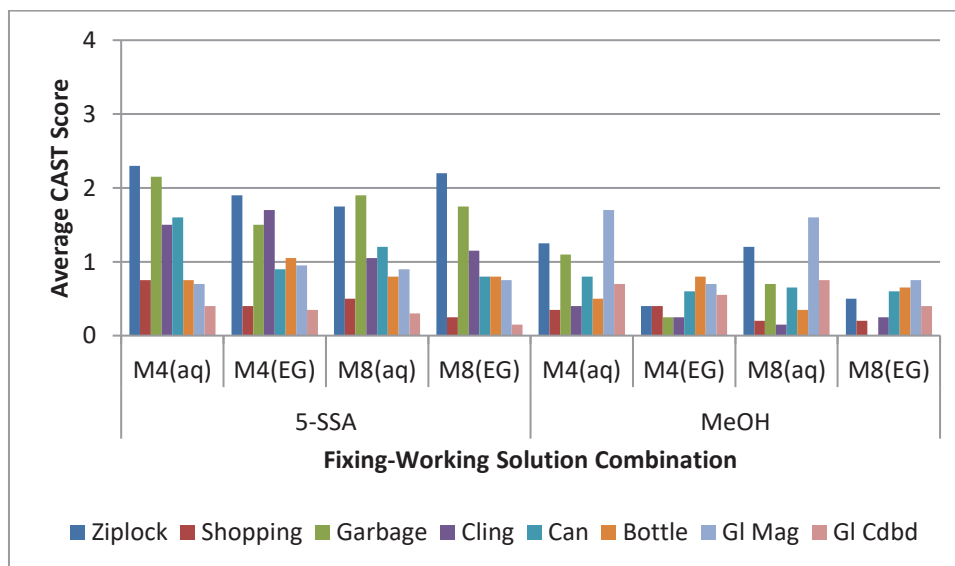


Figure 2-19 Average CAST score results comparing blood-contaminated fingerprints fixed with two different fixing solutions and multiplex working solutions containing different number of components. (aq = aqueous working solution; EG = glycolic working solution; M# = multiplex solution containing # antibodies/aptamers; MeOH = ice-cold methanol fixing solution used; 5-SSA = 5-sulfosalicylic acid fixing solution used)

Other Body Fluid-Contaminated Fingermarks

In forensic casework, blood may not be the only body fluid present; saliva and semen can also be used for DNA analysis. There are several conventional presumptive tests for body fluid detection, such as Hemastix® and Phadebas® [115]. An alternative detection method for blood and saliva, which involves the use of antibodies specific to blood and saliva components conjugated to iron oxide nanoparticles was proposed by Frascione et al. [116]. Not only could their sensitive method detect these two body fluids, but it could also differentiate between the two. It could be performed *in situ*; was effective on dark substrates; and did not interfere with subsequent DNA profiling. These are also desirables for a novel body fluid-contaminated fingerprint detection method. Currently, there are no routine techniques for the detection of saliva- or semen-contaminated fingerprints. The use of a biomolecular reagent specific for these body fluids would allow for simultaneous ridge detection and non-destructive presumptive testing. Any ridge detail that was developed with the biomolecular reagent could be additional information which may be useful for practitioners. Preliminary experiments by Spindler suggested that multi-target reagents for mixed body fluid-latent fingerprints may also be a promising avenue for detecting the entire mark, as well as gathering reconstructive information about the deposit [99]. Simultaneous detection of the fluid-latent components (e.g., semen-latent) is not currently utilised, as complete detection of the fingerprint ridges is reliant on technique sequencing.

All three and five biomolecular recognition probes readily available for saliva and semen, respectively, were used (Table 2-1). Therefore, comparisons were primarily designed to determine whether there was a difference in performance of the aqueous and glycolic working solutions. Development was variable between substrates (Figure 2-20), with evidence of a fingerprint best seen on garbage bag for saliva and beverage can for semen. The reagents performed the worst on semi-porous substrates; no development was observed at all on glossy magazine for saliva-contaminated fingerprints.

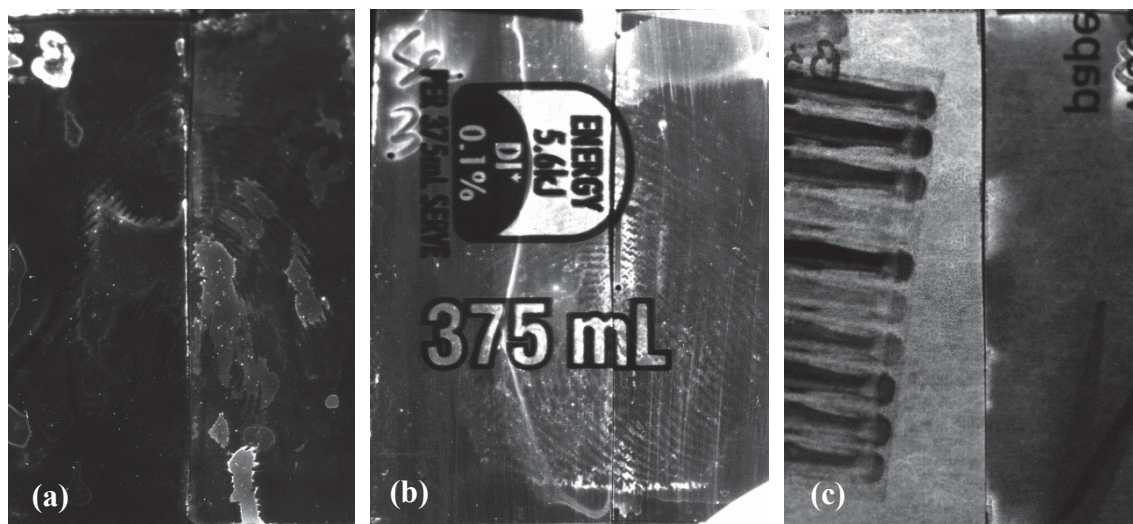


Figure 2-20 Comparison of aqueous (left halves) and glycolic (right halves) working solutions for fingermarks contaminated with (a) saliva on garbage bag; (b) semen on beverage can; and (c) saliva on glossy magazine. Saliva-contaminated fingermarks were visualised at 590 nm with a 650 nm bandpass filter, while the semen-contaminated fingermark was visualised at 530 nm with a 610 nm bandpass filter.

The frequency distribution of CAST scores was almost identical between the two working solutions for semen-contaminated fingermarks. When corresponding fingermark halves were evaluated using the UC scale, the glycolic solution had slightly better enhancement than the aqueous solution or was comparable (Figure 2-21). The apparent enhancement may be insignificant, as 73 saliva- and 64 semen-contaminated fingermarks were excluded due to CAST scores of 0 for both corresponding halves, the most from semi-porous substrates.

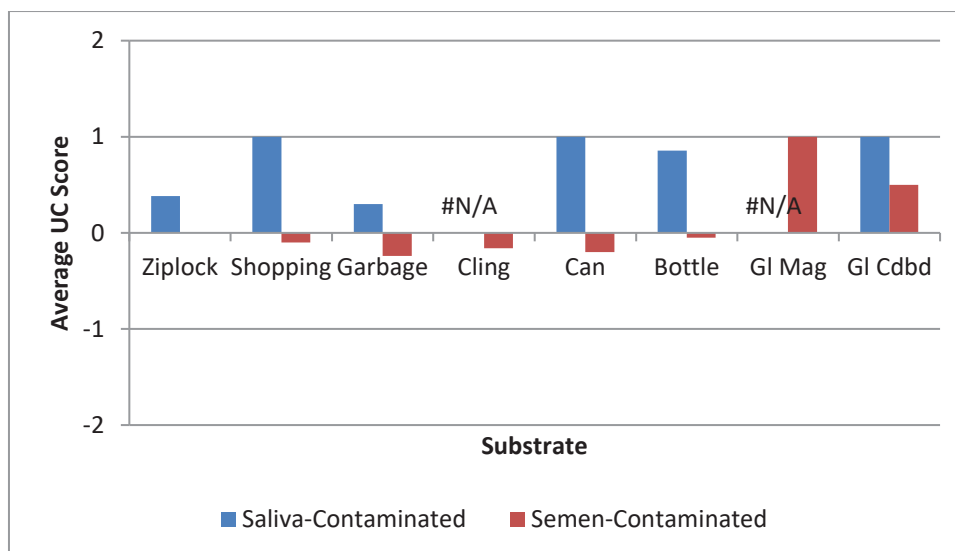


Figure 2-21 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the comparison between aqueous and glycolic working solutions for saliva- and semen-contaminated fingerprints (blue and red columns, respectively). Cling film was not used for saliva-contaminated fingerprints. All fingerprints with no development were removed from this analysis, resulting in no usable data for saliva-contaminated fingerprints on glossy magazine. A positive value favours the glycolic working solution.

It was difficult to get consistent deposition with the fingerprint rubber stamp and semen solution, which can be resolved in the future by determining the ideal amount of pressure required to apply the stamp marks onto the substrates. Also, a better stamp could be used; Clemons et al. created a silicone cast of a hand [117] and the National Institute of Standards and Technology in the United States has created ballistics gelatin fingers with 3-dimensional printed bones [118]. Evidence of contact was often visible; however, clarity of ridge detail was lacking. This occurred primarily along the edges of the fingerprints.

2.5.2 Direct Comparison to Routine Fingerprint Enhancement Techniques

Latent Fingerprints

As the multiplex reagent was a modified version of the single-target formulation used by Spindler et al. [70], which was shown to work well with aged fingerprints

compared to routine methods, it was anticipated that the multiplex reagent would perform better than the routine techniques as well since the fingerprints were aged for 4 to 4.5 months. However, as seen in Figure 2-22, the multiplex solution had the lowest average CAST scores when directly compared to the routine methods on all non-porous substrates except garbage bag, where it was only slightly better than CA. Such a wide range in fingerprint development on semi-porous substrates was unexpected, but possible explanations could be: the donors' natural fingerprints may not have sufficient sebaceous contaminants to be detected by CA; the glossy surfaces of the semi-porous substrates prevented the residues from being absorbed and later being detected by IND-Zn or PD; or the multiplex solution did not contain antibodies or aptamers for the remaining fingerprint residues. It is also important to note that the two female donors were considered poor natural fingerprint donors, meaning that they were at the limit of visualisation for the routine techniques.

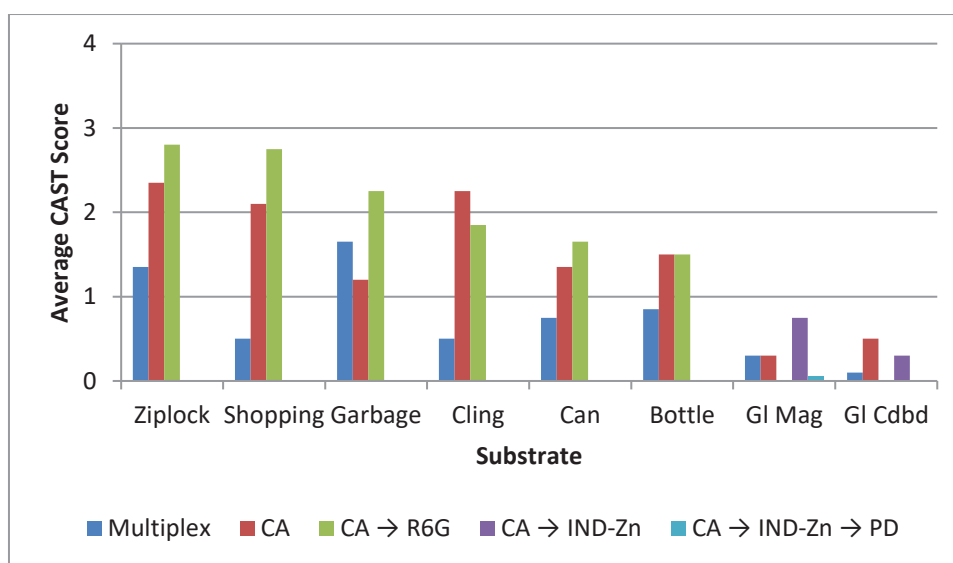


Figure 2-22 Comparison of average CAST scores for corresponding latent fingerprint halves. Non-porous substrates were only processed with the multiplex, CA, and CA → R6G, while semi-porous substrates were processed with the multiplex, CA, CA → IND-Zn, and CA → IND-Zn → PD.

When evaluated using the UC scale, the routine technique or sequence was better than the multiplex solution for all non-porous substrates, with the exception of garbage bag (Figure 2-23). For this substrate, the multiplex solution and CA had comparable

development, but the use of a luminescent dyestain increased enhancement slightly. The results for semi-porous substrates may not be representative, as a total of 48 fingermarks deposited on the glossy substrates was excluded due to double CAST scores of 0; this is equivalent to 60% of the results having no development at all for the multiplex solution and routine techniques. However, observations related to the backgrounds could be made. There were stronger luminescent creases or wrinkles on the glossy cardboard than on the glossy magazine when processed with the multiplex solution. This made the multiplex solution less preferred when compared to CA. There was poor contrast between CA development and the glossy magazine, as the majority of the substrate was white. The worst background development was seen with PD. Although the initial water bath steps in the procedure should soften creases, silver precipitated and resulted in a crackled appearance for glossy cardboard. These creases were imperfections in the substrate and became preferential sites for silver deposition [21] and accumulation of the antibody-AuNP conjugates, resulting in obliteration of both fingermark halves. Another observation for the substrate samples exposed to PD was a peeling or blistering appearance. A possible explanation for this was that the maleic acid wash had a detrimental effect [21]. There were also issues visualising PD development since there were dark substrates and PD development is dark grey or black.

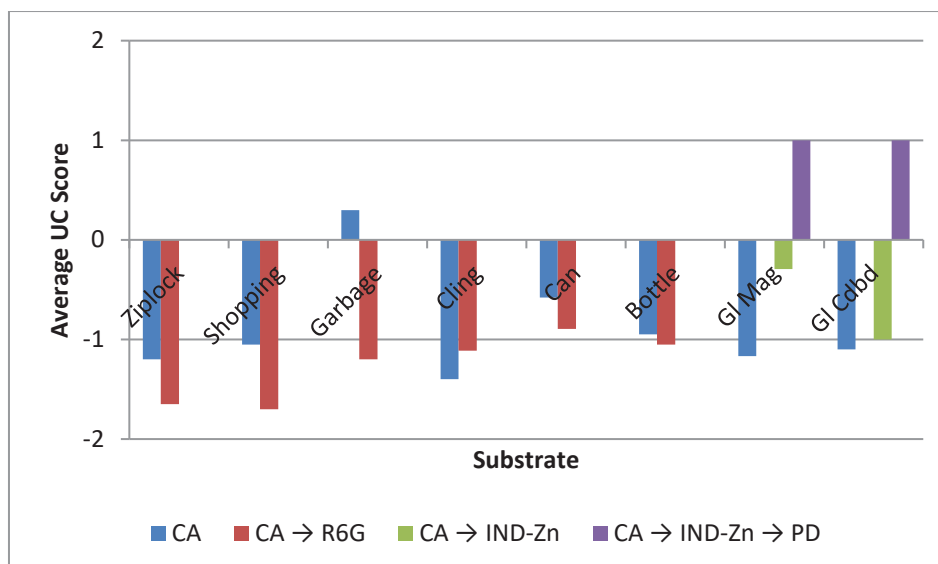


Figure 2-23 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the comparison between the multiplex solution with routine sequences for latent fingerprints. Assessments were performed under each technique's optimal viewing conditions (Table 2-2). Negative values correspond to a decrease in enhancement of the multiplex solution when compared to the routine technique sequence. The multiplex was compared to CA and CA → R6G on non-porous substrates, and CA, CA → IND-Zn, and CA → IND-Zn → PD on semi-porous substrates.

Since the multiplex solution and routine techniques target different secretions, eccrine and sebaceous, respectively, this may not be a fair assessment of the multiplex reagent's ability to detect fingerprint residues. Based on these preliminary results, it is unlikely that the multiplex reagent will replace existing techniques for these types of substrates, but there is a possibility it could be used as a complementary technique. If it targets different residue components, perhaps more ridge detail could be developed when used in sequence. It should also be noted that even though the multiplex reagent did not perform as well as routine techniques, the fact that there was still development being observed is still important because eccrine secretions are normally ignored on non-porous substrates. This actually supports the complementary (as opposed to the alternative) use of the multiplex reagent.

Blood-Contaminated Fingerprints

Similar trends were observed with the blood-contaminated fingerprints as with the latent ones. Prior to any development, some of the blood-contaminated fingerprints

were visible to the naked eye; however, ridge detail of untreated fingermarks was not assessed. Although CA is not a blood enhancement reagent, it was included in the technique sequences to first treat any latent fingermark components present; the CA results seemed to indicate that there were some present. Once again, the poorest performance of the multiplex solution was on the semi-porous substrates (Figure 2-24). It was not surprising to see that the multiplex solution and routine sequence were comparable for garbage bag, as this substrate yielded some of the better results during the optimisation experiment for blood-contaminated fingermarks (see Subsection 2.5.1.2). Amido black could have been used on the non-porous substrates as well, but since the multiplex fluoresces, it was preferred to compare it to a luminescent routine technique (i.e., AY7) as much as possible.

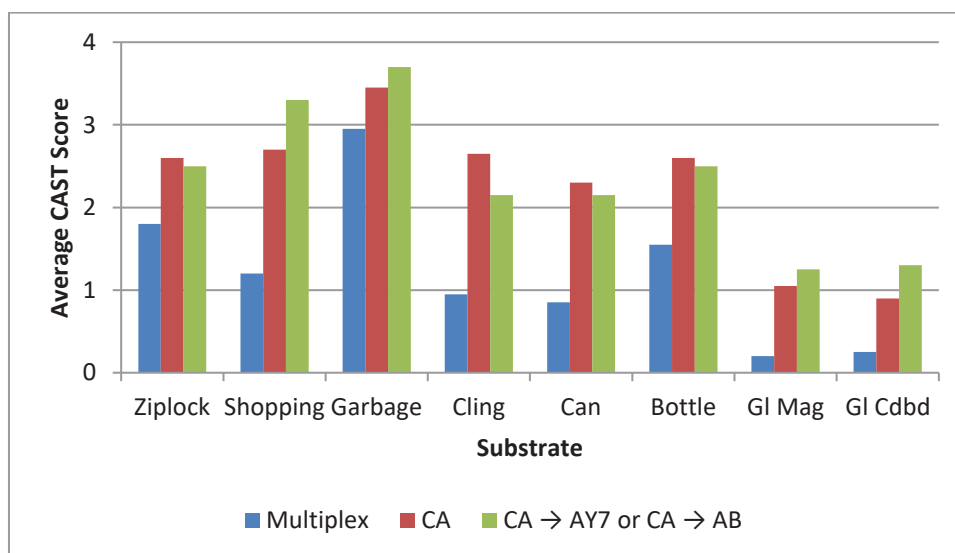


Figure 2-24 Comparison of average CAST scores for blood-contaminated fingermark halves processed with the multiplex solution and routine technique sequences.

Overall, the multiplex solution did not perform as well as the routine sequences (Figure 2-25). This does not necessarily indicate that the multiplex solutions were ineffective; some blood-contaminated fingermark halves were assigned CAST scores of 4. However, when compared to their corresponding halves, the routine methods were preferred. Multiplex-treated fingermark halves with CAST scores of 3 or 4 were rare (14%), while the majority showed spotted or discontinuous ridges (58% CAST score 1 or 2). On the other hand, the routine sequences had a more balanced

distribution: 44% gave CAST scores of 1 or 2; and 48% gave CAST scores of 3 or 4. These results support the argument that the multiplex solutions evaluated are not a replacement for existing techniques. However, this experiment alone cannot rule out the use of a multiplex reagent in sequence with existing techniques.

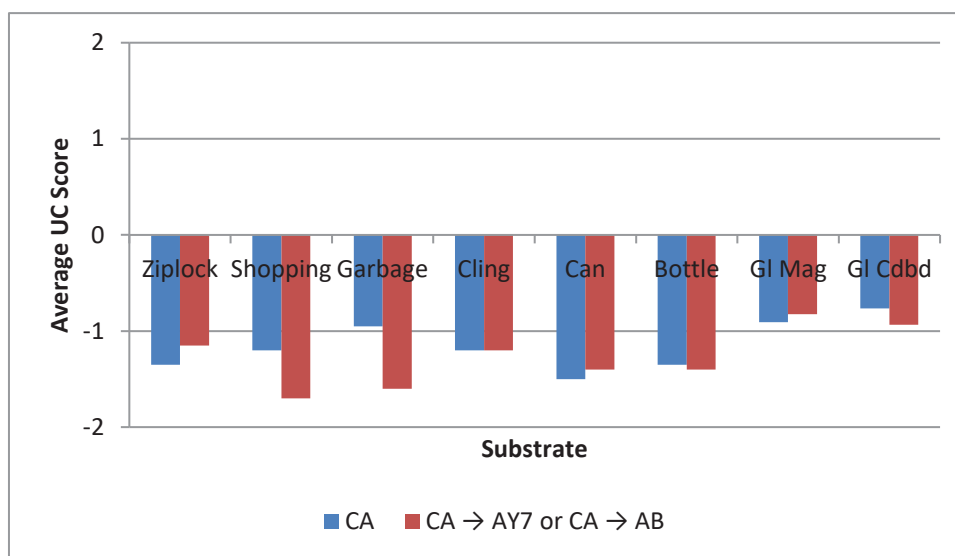


Figure 2-25 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the comparison between the multiplex solution with blood reagent sequences. Assessments were performed under each technique's optimal viewing conditions (Table 2-2). Negative values correspond to a decrease in enhancement of the multiplex solution when compared to the routine technique sequence.

2.5.3 Compatibility with Routine Technique Sequences

2.5.3.1 Assessment of Routine Technique Effects on Multiplex Solution Performance

Prior to assessing the effects of the multiplex solutions on routine techniques, tests were conducted to ensure that the routine techniques did not have any detrimental effects on the multiplex solutions, which could negatively affect the results. This was something that Bécue [55] had expressed interest in after reviewing studies by van Dam et al. [79, 90, 91]. Routine techniques did not appear to have a negative impact on the effectiveness of the multiplex working solutions for both latent and blood-contaminated fingerprints. This means that, should this be implemented into

operational use, practitioners would be able to process a batch of exhibits with the same multiplex working solution even if it had been previously treated. For example, if multiple plastic ziplock bags from a drug file are processed with CA, the practitioner would not have to process one CA-treated bag and then change the multiplex working solution before processing the next CA-treated bag. That being said, it is uncertain if greater exposure to the routine methods would make a difference to the performance of the multiplex solution. The effectiveness would likely be influenced more by the depletion of biomolecular recognition probes within the multiplex solution.

2.5.3.2 Impact of Routine Dyestain on Multiplex Solution Luminescence

Using the data collected, an attempt to observe whether there was a change in the multiplex solution's luminescence was performed; the sequences seemed to be relatively comparable based on their average CAST and UC scores (the latter is depicted in Figure 2-26 (blue columns)). This, however, was not a true representation since the only variable that was constant between the two sequences was the donor. Once the fingerprints were analysed, it was found that the manner in which the fingerprints were processed may not have been the best to study the effect of the dyestain on the multiplex solution's luminescence. Split fingerprints should have been processed as follows: one half with CA → multiplex and its corresponding half with CA → dyestain → multiplex, rather than directly comparing the same sequence with the different dyestains.

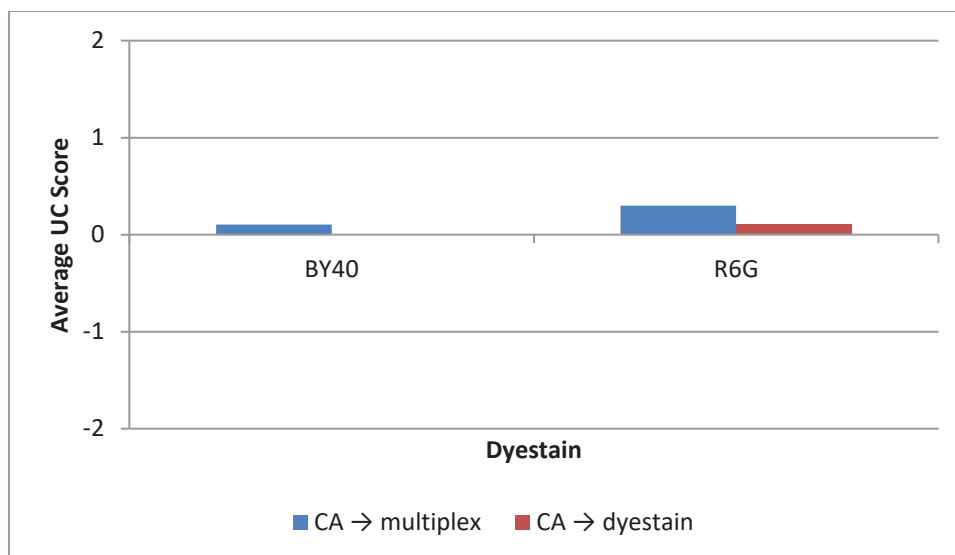


Figure 2-26 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the comparison of $CA \rightarrow dyestain \rightarrow multiplex$ with $CA \rightarrow multiplex$ (blue) and $CA \rightarrow dyestain$ (red). Negative values favour $CA \rightarrow dyestain \rightarrow multiplex$.

To reinforce the analysis, another way to assess how the choice of dyestain would influence the outcome was to evaluate the exact same fingerprint half with the addition of the multiplex solution to the routine sequence. Therefore, the following analysis was made: $CA \rightarrow dyestain$ compared to $CA \rightarrow dyestain \rightarrow multiplex$. As seen in Figure 2-26 (red columns), there was no significant difference. This can be interpreted as both dyestains having such strong luminescent properties that the addition of the multiplex solution had no effect. This suggests that the multiplex solution will not deliver any benefit as a post-dyestain treatment, which will be discussed next.

2.5.3.3 Effect of Multiplex Solutions on Routine Technique Sequences

Results from the direct comparison study indicated that the current multiplex solutions were not viable alternatives to the routine detection methods. Nonetheless, the compatibility of the biomolecular reagents with existing detection sequences was evaluated by varying their position within the sequences (e.g., $multiplex \rightarrow CA \rightarrow R6G$; $CA \rightarrow multiplex \rightarrow R6G$; $CA \rightarrow R6G \rightarrow multiplex$). More development was observed with blood-contaminated than latent fingerprints. The poorest results were

obtained on semi-porous substrates and, once again, a large number of latent fingermarks deposited on these substrates were removed from the UC scale analysis due to no development (i.e., both the sequence with and without the multiplex solution had CAST scores of 0). Similar explanations as mentioned with the direct comparisons (Subsection 2.5.2) could apply. Similar trends were observed with latent and blood-contaminated fingermarks when incorporating the multiplex solutions into the routine technique sequences.

Pre-Treatment

Firstly, the multiplex solution was placed in front of a routine technique or sequence to determine what impact it had as a pre-treatment (Figure 2-27). When the multiplex solution was the first technique of the entire sequence, it had a detrimental effect to subsequent CA development (Figure 2-28(a)). This was expected, as CA treatment is known to be ineffective on substrates that have been previously wetted [21]. As with subsequent CA processing, IND-Zn also had poor results due to the fact that the substrates had already been wetted. It was thought that the AuNPs would act as nucleation sites for the silver deposition once the substrate was further processed with PD. Unlike the iMMD [91], subsequent PD development was not good, which may be attributed to the differences in fingermark type and substrate composition used. When the multiplex solution was not the first technique in the sequence, development was comparable (Figure 2-28(b)).

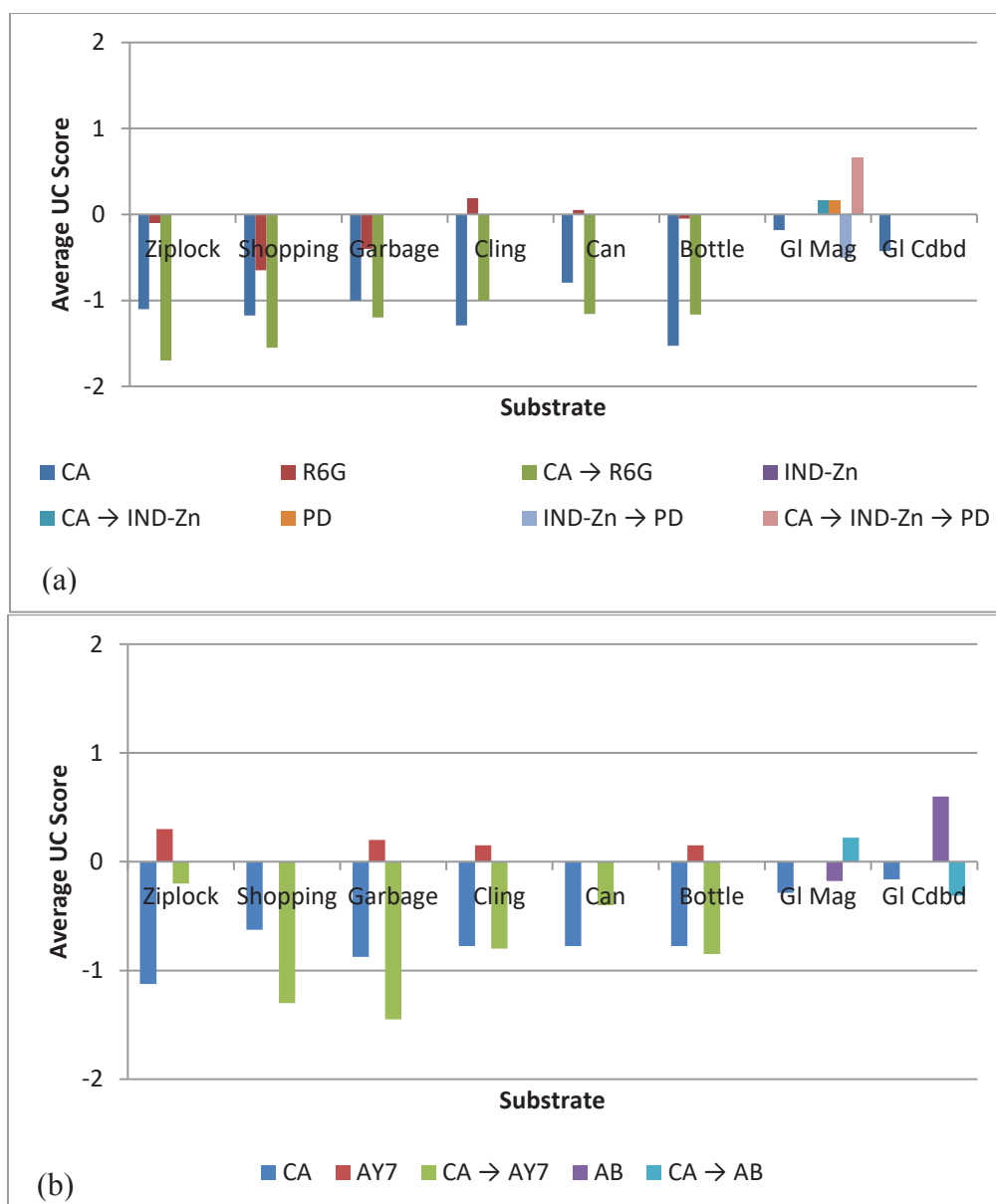


Figure 2-27 Average enhancement scores (for a comparative scale between -2 and +2) resulting from comparisons of routine (a) latent and (b) blood reagent sequences with and without the multiplex solution as a pre-treatment. Assessments were performed under each technique's optimal viewing conditions (Table 2-2). Negative values correspond to a decrease in enhancement of the sequences with the multiplex solutions when compared to the routine technique sequences alone.

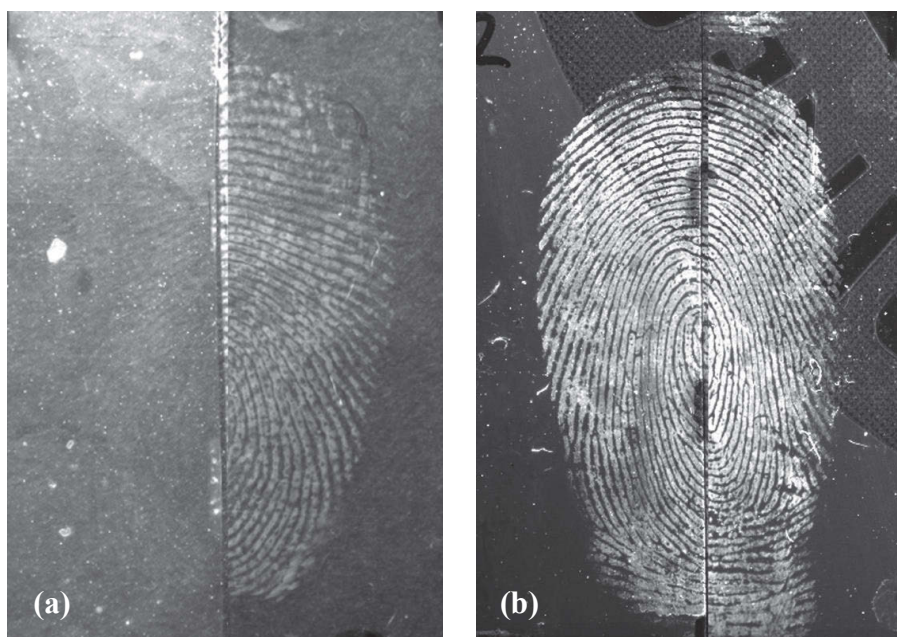


Figure 2-28 Representative images of split fingermarks visualised under their respective viewing conditions depicting results for the multiplex solution as a pre-treatment: (a) latent fingermark on a ziplock bag treated with multiplex → CA (left) and CA only (right); and (b) blood-contaminated fingermark on a beverage can treated with CA → multiplex → AY7 (left) and CA → AY7 (right). Each half illustrated was visualised and recorded under optimal viewing conditions for the last enhancement technique implemented (Table 2-2).

Post-Treatment

The multiplex solution was also placed at the end of the routine technique or sequence to determine whether there were any benefits to include it as a post-treatment (Figure 2-29). Enhancement increased when the multiplex solution was applied to CA-treated fingermarks. It acted as a luminescent stain and gave slightly sharper ridge detail (Figure 2-30(a)). If the routine sequence already had a luminescent technique (e.g., R6G for latent fingermarks and AY7 for blood-contaminated fingermarks), further processing with the multiplex solution had no significant effect. In other words, the existing luminescent technique was already strong and overpowered the multiplex solution's luminescent properties (Figure 2-30(b)). Although van Dam and co-workers reported successful results when sequencing their immunolabelling method after traditional fingermark detection techniques, their experiments were limited to fresh marks developed with one or two techniques per substrate [79, 90]. They also never directly compared two fluorescent techniques in sequence (i.e., used split

fingermarks); therefore, it is hard to get any real indication of whether using a fluorescently tagged antibody after a routine technique would have any impact.

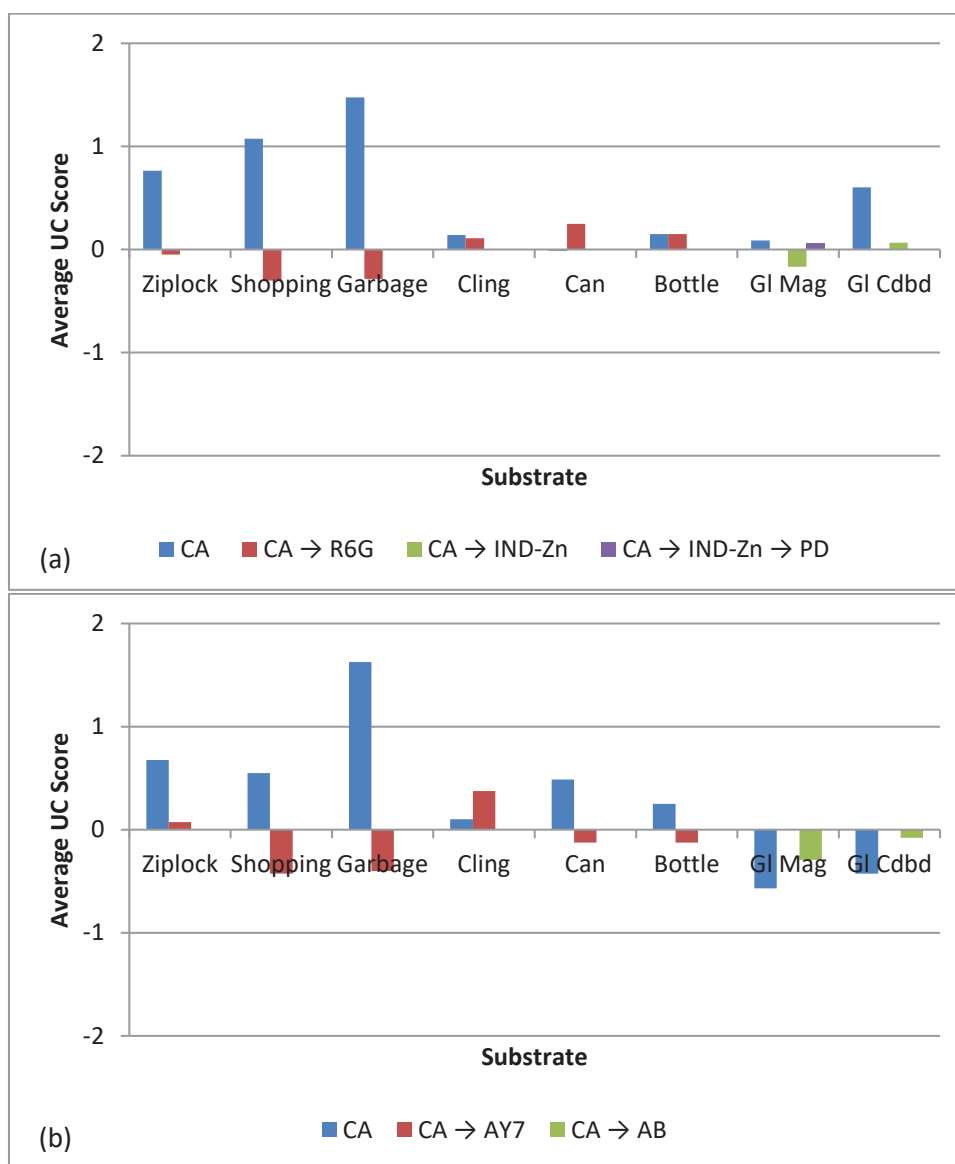


Figure 2-29 Average enhancement scores (for a comparative scale between -2 and +2) resulting from comparisons of routine (a) latent and (b) blood reagent sequences with and without the multiplex solution as a post-treatment. Assessments were performed under each technique's optimal viewing conditions (Table 2-2). Negative values correspond to a decrease in enhancement of the sequences with the multiplex solutions when compared to the routine technique sequences alone.

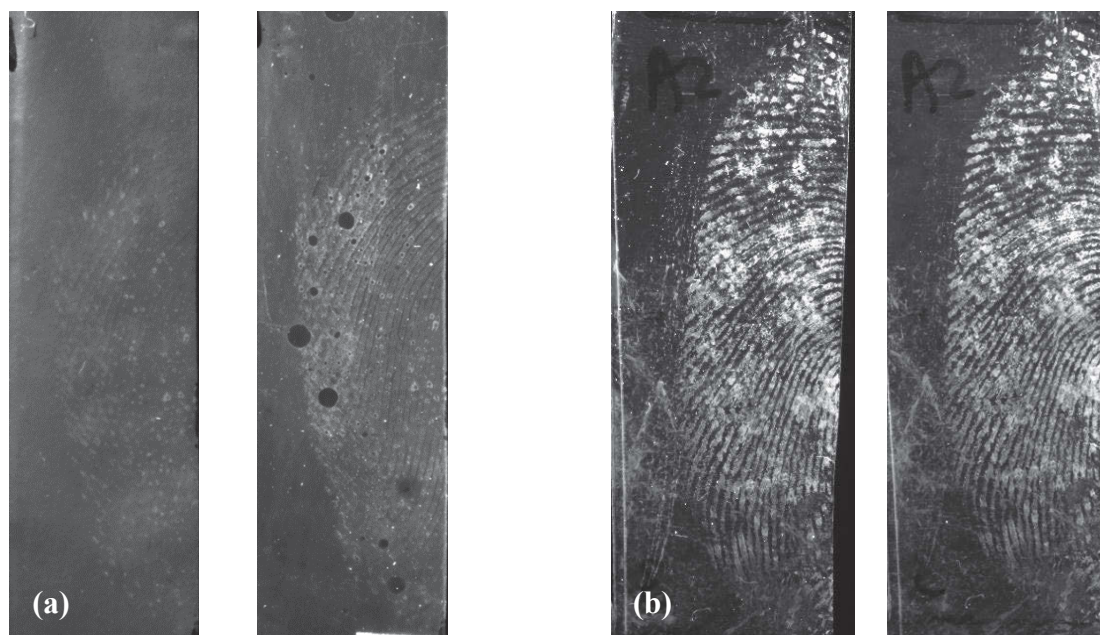


Figure 2-30 Representative images of fingerprint halves visualised under their respective viewing conditions depicting results for the multiplex solution as a post-treatment: (a) latent fingerprint half on a shopping bag treated with CA only (left) and then CA → multiplex (right); and (b) blood-contaminated fingerprint half on a plastic bottle treated with CA → AY7 (left) and then CA → AY7 → multiplex (right). Each half illustrated was visualised and recorded under optimal viewing conditions for the last enhancement technique implemented (Table 2-2).

2.5.4 Other Considerations

As mentioned in Chapter 1, there are several practical aspects to consider when developing or improving a fingerprint detection method, and several limitations to antibodies and published immunodetection methods have been reported [55]. While the biomolecular reagents are both selective and able to target low abundance analytes, their effectiveness may be limited by the inherent variability of fingerprint residues between donors. It is unknown what combination of antibodies or aptamers would be the most “universal”, especially as the fingerprints evolve or age.

The methodology of this multiplex reagent is relatively simpler than those of other published immunodetection methods; multiple incubation and wash steps are avoided. As previously stated, the more times the substrate is in contact with solutions, the

higher the chance that fingerprint residues will be washed away instead of detected. The synthesis of AuNPs could easily be increased in a similar manner as proposed by Moret and Bécue with their second generation single-metal deposition method [119].

Issues with the working solution include the limited volume of reagent, which could also possibly deplete over multiple uses. Most of the research presented in this chapter used working solution volumes of 40 mL, the lowest being 5 mL; however much larger volumes would be required for casework. Unlike routine amino acid-sensitive reagents that may exhibit cloudiness or layer separation in the working solutions, or PD with silver precipitation in the working tray, there is no visible indication of when the multiplex solution needs to be changed or replaced after multiple uses.

The cost associated with antibodies is high, but may be acceptable should a niche application be discovered. Costs could be reduced if upscaled as with many reagents, or if these *in vivo* molecular recognition probes could be translated into *in vitro* ones. Antibodies are fragile and can denature easily. Even if stored correctly, they still become ineffective over time. The incorporation of aptamers into the multiplex solutions, as well as shifting the direction of this research in the coming chapters, aims to address some of these concerns. Research into the biomolecular reagent's compatibility with other forensic analyses (e.g., DNA) also needs to be performed should such a method be used in an operational setting.

2.6 Conclusions

This chapter reported the optimisation of selected multi-target biomolecular reagents for the enhancement of latent and body fluid-contaminated fingerprints deposited on a wider range of substrates commonly encountered in casework, as well as their relative performances in comparison to those of established latent and blood detection methods. Investigations into the compatibility of the optimised multi-target biomolecular reagent with existing routine technique sequences were also performed. Experiments were executed to determine if it was a viable alternative to routine techniques or a complementary technique in routine sequences.

Parameters, such as processing time and formulation, were optimised. Although the 30- and 45-minute intervals were similar, the latter was selected for future experiments to ensure that fingerprints from all type of donors (i.e., including poor donors) had the best chance of being visualised. Although the working solutions were comparable for some fingerprint types, aqueous solutions were favoured due to practical reasons. The number of antibodies or aptamers within the multiplex working solution, and its condition, did not seem to affect its performance.

There was development variability among substrates, with semi-porous substrates having the poorest results. This may not be a true representation of the multiplex solution's potential as a fingerprint detection method. For latent fingerprints, the multiplex solution was targeting specific eccrine secretions that are not normally targeted on non-porous substrates by routine techniques, and still showed some development. As there are many factors that affect fingerprint composition and development, poor results could be due to the technique or the fingerprints themselves. The most significant issue was that the multi-target biomolecular reagents appeared to exhibit greater donor variability than routine techniques such as CA fuming and blood protein stains. Further developments in the formulation of multi-target biomolecular reagents – including application procedures or the selection of other more abundant or robust target residues – could considerably improve this problem.

Although initial research showed promising results [70], the multi-target biomolecular reagents investigated in this chapter do not currently provide a better alternative to existing methods. Progress in the field of fingerprint detection relies on the communication of both positive and negative results in order to develop techniques that could considerably increase the chances of identifiable fingerprint recovery in an operational setting [96]. Despite the theoretical benefits of using biomolecular fingerprint enhancement reagents, the current generation of reagents does not provide appreciable benefits over routine techniques or sequences for latent or blood-contaminated fingerprints. When the biomolecular reagents did show enhancement over the routine techniques, it was only marginal and not to the degree that would currently support the replacement of existing detection sequences, but may still possibly be considered should a niche application be identified.

***Chapter 3: Development of Novel
In Vitro Selection Variation
(Fingerprint-SELEX)***

Chapter 3 Development of Novel *In Vitro* Selection Variation (Fingerprint-SELEX)

3.1 Introduction

Until now, all published fingerprint immunodetection methods have incorporated antibodies and aptamers that each target a single fingerprint residue constituent. The majority of studies also focused on experimentally ideal substrates such as blotting membranes and low background surfaces. While these methods demonstrate the high selectivity of the biomolecular recognition probes, their main limitations are that some of their targets may not be endogenous; their targets have fast degradation rates; or they do not allow for continuous ridge detail, but rather spotty development, which in turn means that the choice in target may not be ideal. While some of these shortcomings could be due to the application mode – typically a working solution – a more fundamental issue resides in the nature of the single-target or multiple single-target approach. By only targeting a specific fraction of a fingerprint, one is limiting the amount of development possible. A pooled target approach may be a better avenue to pursue, where a biomolecular recognition probe can bind to multiple different targets simultaneously.

What we call single- and multi-target approaches can also be referred to as targeted approaches; there are specific targets of interest. On the other hand, there are untargeted approaches where the targets' identities are not necessarily known. An experiment is performed, but then further analyses need to be conducted in order to determine target identities. A pooled target approach is untargeted; there is not a single target of interest, but multiple ones that have not been identified yet. While it is possible for an antibody to be synthesised with this capability, aptamers were the route investigated.

3.2 Aptamers

Aptamers are chemically synthesised short single-stranded deoxyribonucleic acid (ssDNA) or ribonucleic acid (RNA) sequences, which bind to specific targets with high affinity. In the 1960s, Spiegelman and co-workers conducted the first evolutionary experiments with nucleic acids [120-122]. Shortly after the introduction of polymerase chain reaction (PCR) in 1986 by Mullis et al. [123, 124], which significantly influenced the field of molecular biology and evolutionary experiments [125], three research groups independently reported small nucleic acid structures with predefined functions as a result of the experiment process [126-128]. Ellington and Szostak termed the resulting motifs aptamers [126] (from the Latin word *aptus* meaning “fitting” and from the Greek word *meros* meaning “part”), while Tuerk and Gold termed the *in vitro* selection process Systematic Evolution of Ligands by EXponential enrichment (SELEX) [127].

3.2.1 Comparisons to Antibodies

Antibodies had long been thought to be the standard for biomolecular recognition. However, they are not without limitations, some of which are listed in Table 3-1. The specificity and affinity of man-made aptamers are determined by the folding geometry similarly to natural-made antibodies. Aptamers have been shown to have dissociation constants that are comparable or superior to those of antibodies [129].

Table 3-1 Summary of aptamer advantages over antibodies (Adapted from O'Sullivan et al. [130]; Jayasena [131]).

Antibodies	Aptamers
Requires the use of animals	Aptamers are identified through an <i>in vitro</i> process not requiring animals
Limitations against target representing constituents of the body and toxic substances	Toxins, as well as molecules that do not elicit good immune response, can be used to generate high affinity aptamers
Antibodies often suffer from batch-to-batch variation	Aptamers are produced by chemical synthesis resulting in little or no batch-to-batch variation
Kinetic parameters of antibody-antigen interactions cannot be changed on demand	Kinetic parameters such as on/off rates can be changed on demand
Antibodies have limited shelf life and are sensitive to temperature and may undergo irreversible denaturation	Denatured aptamers can be regenerated within minutes; aptamers are stable to long term storage and can be transported at ambient temperature
Identification of antibodies is restricted to <i>in vivo</i> parameters (i.e., target recognition in physiological conditions only)	Selection conditions can be manipulated to obtain aptamers with properties desirable for <i>in vitro</i> assay, e.g., non-physiological buffer
Labelling of antibodies can cause loss in affinity	Reporter molecules can be attached to aptamers at precise locations not involved in binding
Production is quite laborious and expensive	
Heterophilic antibodies could produce false positives	
Frozen stock should be stored at multiple sites to overcome accidental losses/death of cell lines	

3.3 In Vitro Selection

3.3.1 General SELEX Process

Although there are numerous variations of SELEX, they all have the same basic steps (Figure 3-1) [129, 132]. The starting point of a typical SELEX process is a large oligonucleotide library, or pool, of 10^{13} - 10^{16} random ssDNA or RNA sequences [133]. The pool is directly incubated with the target of interest. The bound complexes are then partitioned from the unbound or weakly bound sequences. The binding sequences are eluted and amplified by PCR for DNA, or reverse transcription-PCR (RT-PCR) for RNA. Before undergoing subsequent rounds of selection, a new enriched pool needs to be formed by separating the double-stranded DNA sequences and preparing the relevant ssDNA, or by *in vitro* transcription of RNA sequences. After the final amplification step, the enriched aptamer pool is cloned and the individual aptamers are characterised.

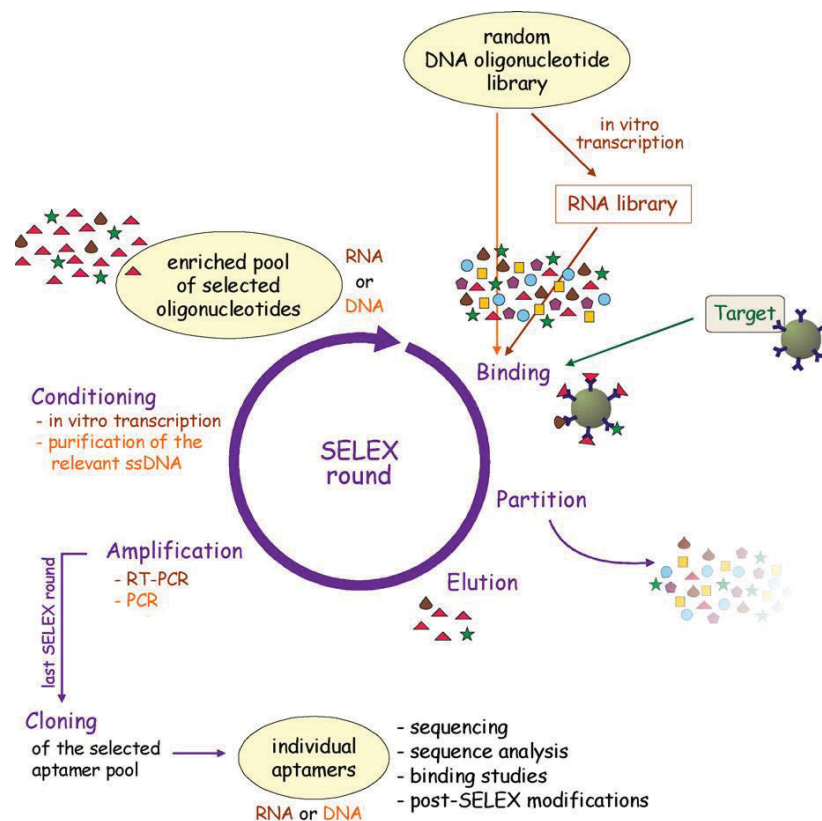


Figure 3-1 Schematic diagram of the SELEX process (Reproduced from Stoltenburg et al. [129]).

3.3.1.1 Library Design

As previously stated, the starting library can consist of either ssDNA or RNA. Earlier SELEX experiments were performed to develop RNA aptamers. It was then found that aptamers could also be developed from ssDNA, with tertiary structures comparable to those from RNA [134, 135]. While some researchers prefer RNA aptamers because they believe that RNA results in higher affinity binders, others believe DNA aptamers can have improved chemical and biological stability due to the lack of 2' hydroxyl of the DNA sugar. It turns out there is no significant correlation between the base content and aptamer affinity [136]. From a practical view, by starting with a random DNA library, *in vitro* transcription is not needed to convert to a random RNA library and neither is RT-PCR for amplification [133]. The choice of template length is also arbitrary, ranging from random regions of 22 to 200 nucleotides. Typically, a random region of 40 to 70 nucleotides is employed to ensure appropriate coverage of sequence space and to allow for greater structural complexity [129, 136]. After SELEX, aptamers can be further truncated to the shortest possible sequence that still has functionality [129].

3.3.1.2 Target Molecule

The majority of selections are performed against proteins due to their large, multifunctional surfaces [129]. Other target types include viruses, carbohydrates, nucleic acids, peptides, cells, and small molecules [136]. Theoretically, it seems as if SELEX is possible for virtually any target molecule. However, there are some criteria to make selection to the target molecule more likely to be successful. First of all, the target molecule should be in sufficient amount, preferably of high purity, to increase specificity and to decrease the enrichment of undesired binding [129]. Targets with positively charged groups, hydrogen bond donors and acceptors, or planarity facilitate aptamer selection more favourably than targets that are highly hydrophobic or negatively charged [129]. The reasons for these are intermolecular interactions in the aptamer-target complex. They bind together with hydrogen bonding, electrostatic interactions between charged groups, or because they are complementary in shape, and there can be stacking between aromatic compounds and the nucleobases [129, 137-139].

139]. Functional oligonucleotides typically have at least one simple secondary structural motifs such as stems, stable tetraloops, pseudoknots, and hairpins [140]. The higher the affinity the aptamer has when binding to a target, the more specific it is to that particular target [141]; dissociation constants can be in the nanomolar range. Flexible molecular conformation will result in a decrease in affinity [141]. According to Carothers et al. [142], the target molecular weight was proportional to the resulting aptamer affinity. This was supported by analysing data retrieved from the Aptamer Base [143], a free open-source database that included descriptions about almost 500 SELEX experiments that were published between 1990 and 2013 [136]. This could be a reason why small molecules are challenging targets for which to develop aptamers. Small molecule targets have less area for interactions compared to proteins, thereby reducing the number of functional groups available for binding interactions [141].

3.3.1.3 Selection Conditions

Careful consideration should be used when determining the selection conditions; they should be similar to those which will be used for the desired application. These include variables such as metal cation concentration, buffering agent of the selection buffer, pH, and temperature because it is possible for different aptamer sequences to be selected under different buffer conditions for the same target [144]. High levels of non-specific binding should be suppressed by the buffer conditions; this can be achieved by higher concentrations of monovalent cations; on the other hand higher concentrations of divalent cations like magnesium will promote nucleic acid structure formation and both specific and non-specific binding to a target [145].

3.3.2 SELEX Variations

Many SELEX variation research and review papers have been published [129, 133, 146]. The goal of changing the selection, partitioning, and recovery methods is to improve the partitioning coefficient or efficiency, to incorporate improved methods for monitoring enrichment, or to improve throughput [147, 148]. The following describes select SELEX variations.

Magnetic beads have been used in lieu of affinity chromatography as a separation method. These functionalised magnetic adsorbent particles can immobilise very small amounts of target and can be partitioned easily with a magnetic separation system [146, 147]. One downfall to bead-based selection is that there is less target or aptamer surface available for interaction and binding [133].

Capillary electrophoresis (CE)-SELEX uses the mobility shift from complex formation with the target [146]. High affinity aptamers can be obtained in very few rounds due to its improved selection and separation efficiency in free solution [146, 149]. However, CE-SELEX is only suitable for smaller targets [150, 151]. Another electrophoretic variation uses non-equilibrium CE of equilibrium mixtures and is a non-SELEX selection process, as only repeated partitioning steps are performed without amplification [152, 153].

Another SELEX variation is cell-SELEX. This is currently being used to develop aptamers for whole living cells like tumour cells for cancer research. One of the major advantages of this variation is that the identity of the target does not have to be known at the beginning of the selection process [133]. This SELEX variation can simultaneously identify target proteins and aptamers [154]. Cell-SELEX can generate a large number of aptamer sequences that target a variety of cells with unknown molecular markers [155]. Two steps that make cell-SELEX time-consuming are the required negative selections against non-target cells (e.g., non-cancerous healthy cells if the target cells are cancerous), and the identification of the target and subsequent specificity verification [133].

With such versatility, the area of fingerprint detection could benefit greatly from a process like SELEX. This was the justification to proceed with the following research objectives.

3.4 Objectives

One of the recommendations made by Wood [101] was to perform SELEX to develop aptamers specifically for fingerprint detection. He did not have the capability to do so

during his PhD research. The work presented in Chapter 2 showed that there was no significant benefit to using the multiplex solutions for latent and body fluid-contaminated fingerprints. Their performance could be greatly affected by the inherent inter-donor variability of fingerprint residues; it is unknown which eight components would be the most “universal”. Therefore, the issues of donor variability and target selection need to be addressed. Also, with such small quantities of luminescently labelled antibody-AuNPs and aptamers in the multiplex solutions, it is possible that the effectiveness of the working solutions decreased as the antibodies and aptamers depleted and were adhering to the previous fingerprint samples.

Rather than a targeted approach, be it single- or multi-target as described in Chapter 2, the goal was to design a new SELEX variation that involved an untargeted approach. In such an approach, *a priori* knowledge of the target is not needed, like with cell-SELEX. The overall end goal is to develop fingerprints; it does not necessarily matter what the reagent is detecting as long as ridge detail is visible. While not necessarily required, additional analyses were performed to determine potential targets of the resulting aptamer candidates. As this is a proof-of-concept, identifying limitations of this method will also be discussed.

3.5 Experimental Design

The work presented in this chapter was performed at the Laboratory for Aptamer Discovery and Development of Emerging Research (LADDER) at Carleton University in Ottawa, Canada. They specialise in developing new aptamers for neurotransmitters, viruses, disease biomarkers, and toxins; in developing biosensors and “smart” materials based on DNA aptamers; and in exploring new applications with their developed aptamers. Arrangements were made with the overseas facility for a four-month period, and during this time, important parameters in both areas of expertise – fingerprint detection and aptamer development – were considered and addressed. While a selection round was quite short (i.e., less than two hours), most of the time was spent preparing and conditioning the resultant DNA pool for the following selection round.

This study was performed under the assumption that it would be the only SELEX opportunity during this PhD project. Therefore, parameters were chosen to be as realistic as possible to increase the chances of incorporating the end product(s) into an effective fingerprint detection reagent. Aptamers selected for residues in charged fingerprints may not work for those in natural fingerprints, meaning some fingerprints would still remain undetected. Garbage bag plastic and copy paper were chosen, as they are often encountered in casework. Ten to sixteen donors deposited fingerprints to account for the inter-donor variability of fingerprint residues.

Figure 3-2 depicts a general overview of the experimental work presented in this chapter, starting with the novel SELEX variation, fingerprint-SELEX. First, a negative selection was performed to remove DNA sequences that had an affinity for the substrate, which may have organic moieties that act as interference or competing targets. Then, a piece of the stacked fingerprint sample was cut out and incubated with the remaining DNA library (positive selection). Unbound or weakly bound sequences were washed off before the strongly bound sequences were eluted and amplified. The resulting DNA was conditioned and used as the starting library for the next SELEX round. Portions of the collected fingerprint samples were analysed by gas chromatography-mass spectrometry (GC-MS) to aid in the identification of possible aptamer targets. This, in turn, assisted with the screening process of possible aptamer candidates after sequencing of select aptamer pools.

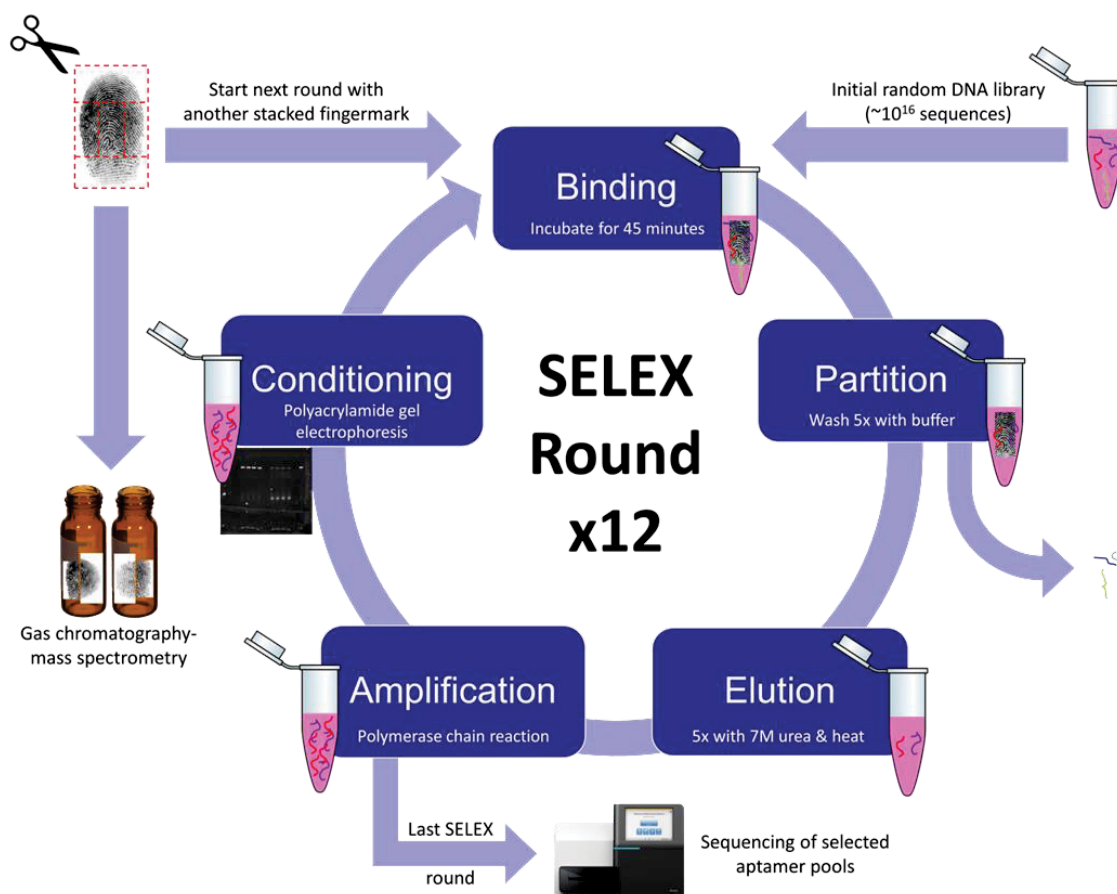


Figure 3-2 Overview of fingerprint-SELEX process, with the addition of GC-MS and sequencing analyses.

3.5.1 Materials and Instrumentation

In Vitro Selection

Ammonium hydroxide (28%, Anachemia) was used during the initial DNA library synthesis.

Disposable syringes without needles (Luer-Lok™, BD) and syringe filters (polyethersulfone (PES) membrane, 0.45 μm pore size) were used to filter out DNA in solution from gels.

Acetonitrile and standard support columns were purchased from BioAutomation.

Acrylamide stock (40%), ammonium persulfate (99%), boric acid (biotechnology grade), dNTP mix (10 mM), ethylenediaminetetraacetic acid (EDTA, disodium salt, dehydrate, biotechnology grade), formamide (99.5%), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 99.5%), magnesium chloride (MgCl_2 , 25mM), sodium chloride (NaCl , 99%), Taq DNA polymerase, tetramethylethylenediamine (TEMED, 99%), tris (BioUltraPure), and urea (99.5%) were supplied by BioShop® Canada, Inc.

Anhydrous and 95% ethanol were supplied by Commercial Alcohols.

CoStar® Spin-X® centrifuge tube filters (0.22 μm cellulose acetate in 2.0 mL polypropylene tubes, certified RNase/DNase free, sterile) and Corning® vacuum filter/storage bottle system (0.22 μm pore, 13.6 cm^2 , PES membrane, sterile) were supplied by Corning Incorporated.

Novagen® Pellet paint® co-precipitant (pellet paint co-precipitant, 3 M sodium acetate (NaOAc), pH 5.2) supplied by EMD Millipore Corp and stored at $-20\text{ }^{\circ}\text{C}$ until required.

Standard phosphoramidites (A, C, G, T) were purchased from Glen Research and prepared based on the Certificates of Analyses. Activator (0.25M 5-ethylthio-1H-tetrazole in acetonitrile), capping agents (tetrahydrofuran (THF)/2,6-lutidine/acetic anhydride and 16% 1-MeIm in THF), oxidising solution (0.02M iodine in THF/pyridine/water), and deblocking agent (3% dichloroacetic acid in dichloromethane (DCM)) for DNA synthesiser were also obtained from Glen Research.

Custom forward and reverse primers were prepared and purified to order by Integrated DNA Technologies (see Appendix III).

High Purity 5.0 argon gas was purchased from Praxair.

Amicon Ultra 0.5 mL Ultracel 3kDa tubes, calcium chloride (CaCl_2 , 93%), MgCl_2 (95%), potassium chloride (KCl , 99%), Triton X-100 were supplied by Sigma Aldrich.

Phenol-chloroform was used during the phenol-chloroform extraction and ethanol precipitation step.

Sequencing

Tris (99.9%) and sodium hydroxide (NaOH, 98 % purity, micro pearls) were supplied by BioShop® Canada, Inc.

MiSeq® reagent kit v2 (includes reagent cartridge, HT1 hybridisation buffer, PR2 bottle, MiSeq flow cell) and PhiX control kit v3 were supplied by Illumina, Inc.

GC-MS Analysis

Ethyl chloroformate (ECF, 99%) was supplied by Acros Organics.

Dichloromethane (99.8%), iso-octane (anhydrous), and N-Methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA, $\geq 98.5\%$) were supplied by Sigma Aldrich.

Substrates

Black PE garbage bag (Polykar Industries Inc.) and white eco-friendly copy paper (92 brightness, 20 lb, 75 g/m², Staples) were the non-porous and porous substrates, respectively, on which fingermarks were deposited.

Instrumentation

Cary 300 Bio UV-vis spectrophotometer (Agilent Technologies) with VARIAN Cary WinUV Scan Application version 3.00(182) and NanoDrop™ 1000 spectrophotometer (Thermo Scientific) with software version 3.7.1 were used to quantify DNA samples.

A 6890 Series GC system with 7683 Series injector (Agilent Technologies) was used with a DB-5HT column (15 m x 0.25 mm inner diameter x 0.10 μ m film, Agilent Technologies) and a DB-5MS-UI column (30 m x 0.25 mm inner diameter x 0.25 μ m film, Agilent Technologies) for GC-MS analyses. A 7820A GC system with 5977E MSD and 7697A headspace sampler was also used with a DB-WAXETR column (30 m x 0.25 mm inner diameter x 0.50 μ m film, Agilent Technologies).

AC600 PCR Workstation (AirClean® Systems) was used as the dedicated SELEX fumehood.

Multi-image light cabinet with camera and filters, AlphaEase®FC software version 6.0.0 (Alpha Innotech Corporation), and P93D printer (Mitsubishi) were used to image gels.

MerMade 6 DNA synthesiser (BioAutomation Corporation) with MerMade 12 version 2.3.7 and Bioautomation Inc LabVIEW 8.6 software were used synthesise the initial random DNA library.

Digital dry bath incubator (Boekel Scientific) was used for DNA preparation.

Thermocycler (Eppendorf) was used to amplify DNA.

Standard vortex mixer (Fisher Scientific) was used during *in vitro* selection process.

SE600 Series dual cooled vertical slab electrophoresis unit (Hoefer, Inc.) and FB1000 electrophoresis power supply (Fisher Scientific) were used for gel electrophoresis.

Various freezers were used to store elutions (Fisher Scientific™ Isotemp™ refrigerator/freezer), primers (Thermo Scientific -80 °C freezer), and PCR master mix reagents (VWR freezer).

Fluorolog (Horiba Jobin Yvon) with FluorEssence version 3.2.1.20 and Origin version 8.1090 software were used to monitor % DNA binding.

Magnetic stirrer and hot plate (RCT Basic, IKA Labortechnik) were used for polyacrylamide/gel solution preparation.

MiSeq sequencing system (Illumina, Inc.) was used to sequence select SELEX rounds.

FreeZone 4.5 Liter Benchtop Freeze Dry System (Labconco) was used to lyophilise the initial purified DNA library.

Innova®40 incubator/shaker (New Brunswick Scientific) with temperature and cycle controls was used to gently induce extraction of DNA from the gels.

Vortex-Genie 2 (Scientific Industries, Inc.) was used during SELEX column incubation.

Biosafety fumehood (1300 Series A2, Thermo Scientific) was used as the dedicated PCR fumehood.

Sorvall™ Legend™ Micro 21R centrifuge (Thermo Electron Corporation) with temperature control was used during the phenol-chloroform extraction and EtOH precipitation. Sorvall™ Legend™ Micro 21 centrifuge (Thermo Scientific) without temperature control was used during the desalting steps.

Savant Automatic Environmental SpeedVac® System AES2010, VaporNet® included (Thermo Scientific); was used to evaporate excess solvents with optional cryopump feature.

UVGL-25 compact UV lamp (UVP, LLC) was used to visualise gel bands.

3.5.2 Methods

3.5.2.1 Substrate Preparation and Fingerprint Collection

Strips of black garbage bag plastic and white copy paper were prepared for fingerprint deposition. Each substrate strip was comprised of three 3 cm x 2.5 cm boxes where fingerprint donors would deposit their fingerprints. Multiple donors (six males and ten females for garbage bag plastic, and three males and seven females of the same donors for copy paper) were instructed to not to wash their hands for at least an hour prior to fingerprint deposition. They were asked to deposit a pair of natural fingerprints, one on each side of the substrate boxes (e.g., left thumb and left index, left thumb and left middle, left thumb and left ring, right thumb and right index, right thumb and right middle, right thumb and right ring, left index and right index, left

middle and right middle, left ring and right ring). There was a waiting period of at least one minute before the next donor deposited their fingermarks onto the existing ones. The substrate strips were then hung to ensure that the fingermark residues would not get rubbed off of either side of the substrate if placed flat, and then stored under dark conditions until ready for the *in vitro* selection. For the garbage bag plastic samples, the fingermarks were aged between 10 to 106 days, while the fingermarks on the copy paper were aged for 12 to 25 days. The difference in fingermark age ranges was due to the setup of the selection process; fingermarks on the garbage bag plastic were used first and then those on the copy paper. Fingermarks on the porous substrate were only deposited once it was known that the results obtained from the garbage bag plastic were starting to show what was anticipated.

3.5.2.2 *In Vitro* Selection Process

A new random DNA library was synthesised for this study. The first round of SELEX took fifteen days to complete due to the inclusion of a phenol-chloroform extraction and EtOH precipitation step (Appendix IV), which resulted in a significant loss of DNA and multiple cycles of amplification by PCR, purification by polyacrylamide gel electrophoresis (PAGE), and quantification by ultraviolet-visible (UV-vis) spectrophotometry to be performed. This step was discontinued and the remaining rounds of SELEX only took four days each, as enough DNA was obtained for the following round.

Initial Random DNA Library Synthesis

Originally, a previously synthesised DNA library was going to be used for SELEX. However, after running some gels, it was found that the existing DNA library had degraded over the years. Therefore, a new random DNA library was synthesised using the MerMade 6 DNA synthesiser. The template DNA sequence used was:



The required phosphoramidites – monoamides of phosphite diesters and building blocks for oligonucleotide synthesis – and T 1000Å columns (since T is the first base

at the 3' end of the sequence) were placed in a desiccator to reach room temperature. The amidites were then prepared with acetonitrile according to the Glen Research Certificates of Analyses using syringes under argon gas, with care to avoid the introduction of excess air or moisture. The amidite bottles were then vortexed before being attached to the DNA synthesiser. For the N amidite, A, C, G, and T were mixed together and placed in the Amidite #5 position of the DNA synthesiser. The other reagents (e.g., acetonitrile, oxidiser, blocking agent, capping agent) were also attached to the DNA synthesiser. During calibration, it was found that Column #2 was blocked, resulting in only five columns being used to synthesise the starting random DNA library.

Once synthesis was complete, the beads in each of the columns were transferred into separate microcentrifuge tubes. Ammonium hydroxide (1 mL) was added to each tube, vortexed, and then placed on a heat block at 37 °C overnight. The tubes were allowed to reach room temperature before being vortexed and centrifuged. The column beads were rinsed with DI water, centrifuged, and the supernatant for each column tube was collected into new separate tubes. This rinsing and collecting process was repeated a few times. The collected supernatant (DNA) was then transferred to the speedvac with the cryopump option on and left overnight.

The DNA library was purified by PAGE (see Subsection “Amplification” on page 125), one gel for each of the five columns. The top visible band of each gel was cut out and placed into separate 50 mL tubes. The tubes were shaken to break up the gels. About 12.5 mL of DI water was added to each tube and shaken some more. All five tubes were placed in the shaker/warmer overnight.

Each of the 50 mL tubes were filtered into new tubes, placed in liquid nitrogen until frozen, and then lyophilised overnight. The tubes were removed from the lyophiliser and up to 1 mL of DI water was added to each tube. They were vortexed until the pellet inside was dissolved. The contents were desalted by adding 500 µL of the liquid into a desalting tube, centrifuging for 22 minutes at 13,000 rpm, collecting the liquid in new tubes, and repeating until all of the DNA was desalted. The DNA was then washed with DI water five times by centrifuge and collected. Once the DNA was

desalted, each column was quantified by UV-vis spectrophotometry (see Subsection “DNA Quantification” on page 128).

Buffer Solution Preparation

All glassware and equipment required to prepare the buffer solutions were bleached and rinsed with tap, distilled, then DI water. One litre of SELEX buffer (20 mM HEPES, 125 mM NaCl, 5 mM KCl, 2 mM MgCl₂, and 1 mM CaCl₂, pH 7.6) was prepared with DI water. To make 100 mL of PCR buffer, 100 mM KCl, 200 mM tris, and 2% Triton X-100 were combined with DI water and adjusted to pH 9. These two buffer solutions were filtered into sterile storage bottles.

SELEX Protocol

Everything required for this experiment (e.g., microcentrifuge tubes, pipettes and tips, KimWipes, paper towels, etc.) except the DNA was placed in the SELEX fumehood and exposed to UV radiation for 30 minutes. The desired amount of DNA per round was dissolved in 200 µL SELEX buffer. For the first round, 5 nmol was dissolved. For the second round, 1 nmol DNA was dissolved. For the third round and onwards, 200 pmol DNA was dissolved.

DNA Preparation

A fraction of the initial DNA pool (5 µL) was saved and placed in the freezer. The remaining DNA was heated at 90 °C for five minutes, placed in the fridge for ten minutes, and then returned to room temperature for 15 minutes.

Column Equilibration

For each round of SELEX, negative and positive columns (1.5 mL microcentrifuge tubes) were required. The negative column contained a piece of blank substrate, while the positive column contained a piece of the stacked fingerprint samples on the substrate. The columns were equilibrated by washing them with 200 µL SELEX buffer, vortexing (negative column) or inverting back and forth (positive column), and discarding the buffer. These steps were performed five times for both columns. The last buffer wash stayed in the column until the DNA pool was ready for incubation.

Binding

The SELEX buffer was removed from the negative column. The prepared DNA was added to the negative column with the blank piece of substrate and the column was then sealed with parafilm and vortexed for a 30-minute incubation period. Once done, the solution was transferred into a new microcentrifuge tube and 200 μ L SELEX buffer was placed in the negative column. A fraction of this pool (5 μ L) was saved and placed in the freezer, and then DNA preparation (i.e., heat, refrigerate, bring to room temperature) was repeated for the remaining DNA. The SELEX buffer was then removed from the positive column tube. The prepared DNA was added to the positive column with the piece of stacked fingerprint sample and the column was sealed with parafilm. This time, the positive column was vortexed for a 45-minute incubation period. Once done, the solution was transferred into a new microcentrifuge tube and 200 μ L SELEX buffer was placed in the positive column.

Partition

When the positive column was done its incubation period, this solution was transferred into a new microcentrifuge tube. This was considered the first wash of the positive column. SELEX buffer (200 μ L) was added to each column and vortexed. These washes were collected into separate 1.5 mL microcentrifuge tubes. This was repeated until five washes for each of the selection columns were collected. All ten washes were placed in the freezer.

Elution

A solution of 7 M urea was prepared and used to elute the DNA. Urea solution (200 μ L) was added to each column tube and vortexed. They were then placed on a heat block set at 90 °C for ten minutes with vortexing at least once while heating, typically at the five-minute mark. The columns were removed from the heat block and vortexed. The solutions were transferred to new separate 1.5 mL microcentrifuge tubes and this process was repeated until five elutions were collected for each column. All ten elutions were placed in the freezer.

Fluorescence Measurements

The % binding values of DNA to the substrate (negative selection) and to the fingerprints (positive selection) were measured using the fluorolog for all but the first

round of SELEX. An average urea baseline spectrum was obtained prior to the actual samples by running a 7 M urea solution sample in emission mode (excitation = 554 nm, emission range = 570 to 650 nm) in triplicate. Starting with the positive elutions, and working from the last one (i.e., fifth in the series of elutions), 60 μ L of each elution was run under the same parameters as the urea solution in triplicate, and recovered after measurement. The cuvette was then rinsed with DI water and dried with argon gas before repeating the process with the negative elutions. The two 5 μ L DNA pools that were saved were made into 40x dilutions by adding 195 μ L urea solution to each and vortexing them. Then, the DNA pool that was saved prior to the positive incubation (positive pool) was run through the fluorolog in triplicate, followed by the DNA pool that was saved prior to the negative incubation (negative pool) in triplicate.

All of the fluorescence measurements were recorded at 575 nm. The average urea baseline was subtracted from each average elution intensity to get the elution fluorescence (f_{eluted}). For the total fluorescence (f_{total}), the intensities of the DNA saved prior to the incubation steps were used. Therefore, the % DNA bound was calculated by dividing the f_{eluted} by f_{total} .

The positive elutions were placed in the speedvac overnight. Deionised water (100 μ L) was added to the first tube and vortexed until dissolved. The contents were heated around 90 °C for about ten seconds before being transferred into the second tube. The vortex, heat, transfer procedure was repeated until all the positive elutions were combined in the last tube. Half of the contents was transferred into a new microcentrifuge tube. To each of these two tubes, 2 μ L of pellet paint co-precipitant, 30 μ L NaOAc, and 600 μ L anhydrous EtOH were added and vortexed. There was a two-minute wait period before these two tubes were centrifuged at 14,800 rpm for five minutes. A small pink pellet (DNA) formed at the bottom of the tubes; the liquid was discarded and then the tubes were placed on the heat block with the caps open for about one minute to evaporate any residual EtOH. The DNA was then reconstituted in 100 μ L DI water, by vortexing and heating to break up the pellet. Once completely dissolved, the tubes were placed in the freezer until ready for amplification by PCR.

Amplification

The surface of the dedicated PCR fumehood was rinsed with 70% EtOH and then exposed to UV radiation for one hour prior to use to prevent contamination. Immediately prior to PCR, the required reagents, including the desalted DNA pool, were removed from their respective freezers to thaw for a few minutes. Enough PCR master mix was prepared (Table 3-2, primer sequences can be found in Appendix III) in a 2 mL microcentrifuge tube for up to 32 PCR reaction tubes. The tube was gently inverted back and forth to mix the contents to minimise solution bubble formation. Aliquots of PCR master mix (99 μL) were pipetted into PCR reaction tubes. Deionised water (1 μL) was added to one reaction tube as a negative control. The positive control included 1 μL of the initial random DNA library which was synthesised. Desalted DNA from the selection round (1 μL) was added to each of the remaining reaction tubes.

Table 3-2 Formulation for PCR master mix

Reagent
50 μL PCR buffer
8 μL 25mM MgCl_2
2 μL dNTP
0.5 μL forward primer
0.5 μL reverse primer
1 μL Taq polymerase
37 μL DI water

The PCR program used with the thermocycler can be found in Figure 3-3 and usually took around two hours. The reaction tubes were then transferred into microcentrifuge tubes: each control into their own, and then all of the sample reaction tubes together. The microcentrifuge tubes were then placed in the speedvac overnight.

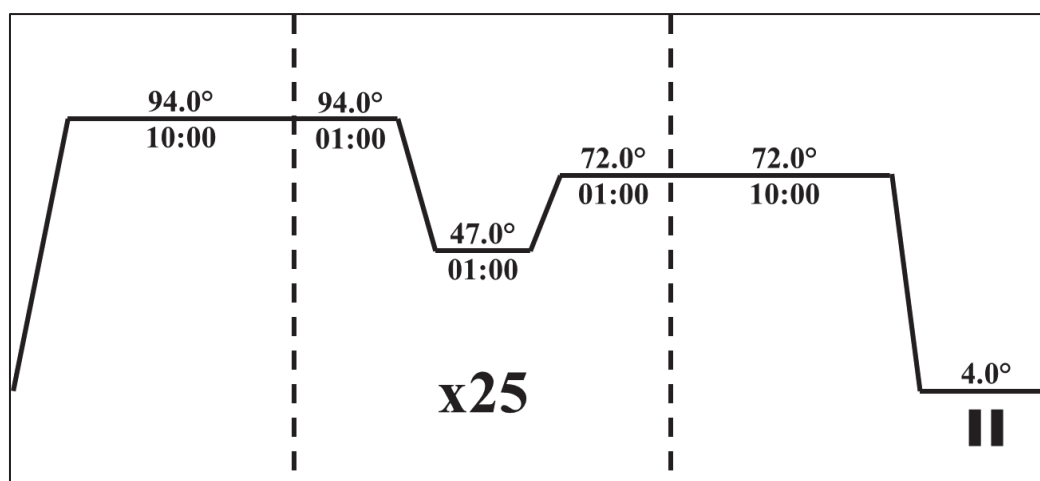


Figure 3-3 Polymerase chain reaction temperature program used.

Conditioning

Prior to preparing the polyacrylamide/urea gels for PAGE, all of the necessary glassware, etc. were bleached and rinsed with distilled water to prevent contamination. Since the length of the oligonucleotide was between 40-100 nucleotides, 12% polyacrylamide gels were prepared. The following solution was used to prepare two gels: 31.5 g urea, 23.5 mL acrylamide stock, 15 mL 5x TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA), and 14 mL DI water. The solution was placed on a hot plate/stirrer set at 37 °C and stir setting of 230 until dissolved. The solution was then filtered by gravity using Whatman No. 1 Filter paper and allowed to cool to room temperature. A 20% solution of ammonium persulfate was prepared with DI water in a 2 mL microcentrifuge tube and vortexing until dissolved. Once the acrylamide solution reached room temperature, 450 µL of the prepared 20% ammonium persulfate solution was added in a swirling motion, followed quickly by 35 µL TEMED, and then swirled to mix. This solution was then poured between the gel plates that were assembled according to protocol. Once the plates were filled with the gel, the appropriate comb was inserted in a manner to avoid air bubbles. More gel solution was added to top off the plates once the combs were in place. The acrylamide was left for 30 minutes to polymerise. Once polymerised, the combs were removed and the gels were rinsed with DI water five times. The rest of the PAGE system was put together (e.g., fill with 1x TBE buffer) and run for 15 minutes without the samples at a constant voltage of 250V.

During this time, the DNA was prepared by adding a 1:1 ratio of formamide and DI water to each tube and then vortexed for 30 seconds. The DNA was then denatured for five minutes at 90 °C, placed in the fridge for ten minutes, and then left out at room temperature for ten minutes. The gels were loaded with the prepared DNA samples and the PAGE units were run for approximately 2.5 hours, again at constant voltage of 250V. Once stopped, the gels were imaged using the Alpha Imager under UV and fluorescence. Typically, two bands would be visible under UV and only one band under fluorescence due to the Atto 550-tagged DNA in the bottom band (Figure 3-4). The bottom band was cut out, placed in a microcentrifuge tube, crushed, covered with DI water, and vortexed. The tube was then wrapped in aluminium foil and placed in a warmer/shaker set at 170 rpm and 37 °C overnight to induce the extraction of the DNA from the gel pieces.

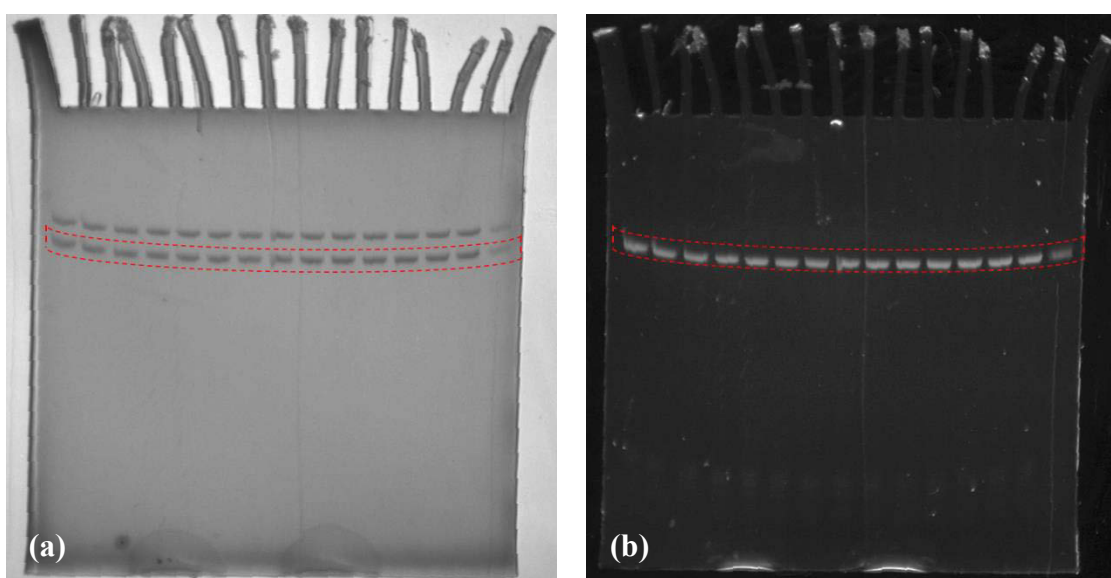


Figure 3-4 Example PAGE gels from Round 11 visualised under (a) UV and (b) fluorescence. The bottom band outlined in red was cut out for subsequent desalting and DNA quantification.

In the bleached SELEX fumehood, the contents of the microcentrifuge tube were filtered into a 15 mL Eppendorf tube. The DNA underwent a long desalting process. The centrifuge was bleached and two desalting tubes were filled with DI water and centrifuged for 22 minutes at 13,000 rpm; this water was then discarded. Into each desalting tube, 500 μ L of the filtered DNA was added. They were then centrifuged for 22 minutes at 13,000 rpm. The desalting liquid was collected into a new 15 mL

Eppendorf tube. This was repeated until all of the filtered DNA had been centrifuged. The desalting tubes – still containing the DNA in their filters – were washed with 500 μ L DI water and centrifuged for 22 minutes at 13,000 rpm. This liquid was again collected and this process was repeated for a total of four washes. The desalting tube filters were then flipped over into new desalting tubes and centrifuged for three minutes at 3,500 rpm. If there was more than one desalting tube used, the contents were combined prior to DNA quantification.

DNA Quantification

Deionised water (70 μ L) was placed in a 0.9 mL cuvette to determine a blank sample baseline before running samples through the UV-vis spectrophotometer. A 50x dilution of the DNA was prepared and a fraction of the diluted sample (70 μ L) was analysed for DNA quantification. The absorbance value at 260 nm was used to quantify the amount of DNA. If the absorbance reading, x , was not in the range of $0.1 < x < 1$ at 260 nm, further dilutions were required to be prepared (Figure 3-5). If the desired amount of DNA for the next SELEX round was not obtained, further PCR, PAGE, and desalting was performed.

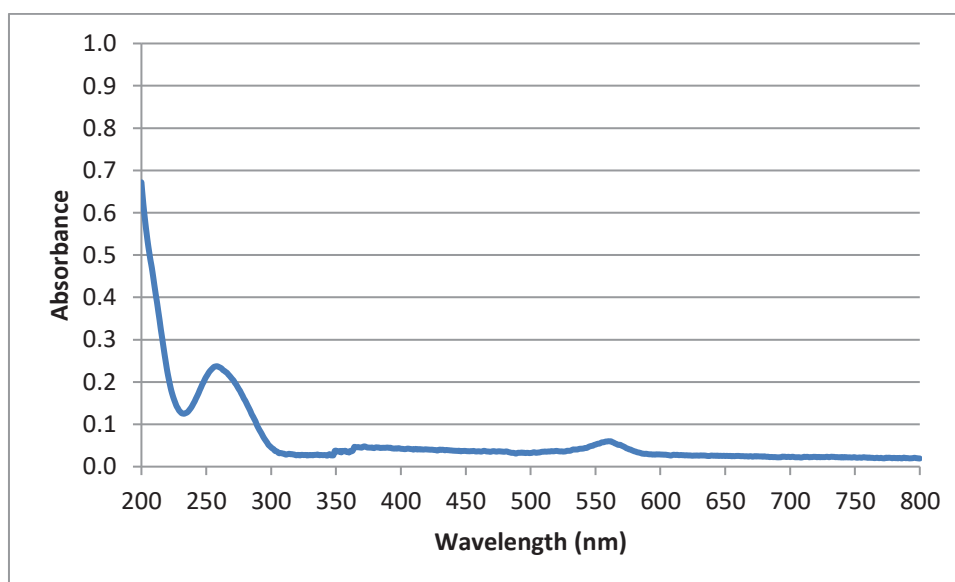


Figure 3-5 Example UV-vis absorbance spectrum from Round 9 with a 50x dilution of DNA sample.

The SELEX protocol was repeated for twelve rounds; nine rounds for stacked fingerprints deposited on black garbage bag plastic and three rounds for stacked fingerprints deposited on white copy paper. The DNA that was quantified from one SELEX round was used as the starting DNA pool for the next round. This also applied to the DNA that was quantified from the last round of garbage bag (Round 9) and subsequently used as the starting DNA pool for the first round of paper (Round 10).

3.5.2.3 Sequence Analysis for Aptamer Sequence Candidate Screening

Sample Preparation

In-house sequencing could be performed on eight DNA pools simultaneously. The SELEX rounds selected were: the initial DNA pool (Round 0); a good positive selection for garbage bag plastic (Round 6); both the negative and positive selections for the last round of garbage bag plastic (Round 9); the positive selection for the first paper round (Round 10); a good positive selection for paper (Round 11); and both the negative and positive selections for the last round of paper (Round 12). A good positive round was defined as a selection round where the % DNA binding to fingerprint residues was high in comparison to other selection rounds with the same substrate.

Each of the DNA pools was tagged and amplified using different pairs of forward and reverse primers (Table 3-3, primer sequences can be found in Appendix III). This was done so they could be differentiated when viewing and interpreting the resulting sequencing data.

Table 3-3 Primer pairing for selected DNA pools for sequencing. (# = selection round; P = positive selection round; N = negative selection round)

Round	Forward Primer	Reverse Primer
0	F1	R1
6P	F1	R2
9N	F2	R3
9P	F2	R4
10P	F3	R5
11P	F3	R6
12N	F4	R7
12P	F4	R8

The PCR biosafety fumehood was prepared as before for each round. The required forward and reverse primers for the round were each diluted in 20 μ L DI water and vortexed. While the PCR master mix was prepared for the round, the required DNA was removed from the freezer to thaw. If negative elutions were required (Rounds 9 and 12), the quick desalt procedure with pellet paint co-precipitant was performed. The annealing temperatures on the thermocycler were adjusted due to different primers being used compared to during the SELEX process. Once the DNA samples were amplified, they were purified using 8% gels (31.5 g urea, 16 mL acrylamide, 15 mL 5x TBE, 43 mL DI water). The gels were imaged and the appropriate bands were cut out, placed in the warmer/shaker overnight, and then placed in the freezer. They were replaced into the warmer/shaker the night before the long desalting procedure was performed.

DNA Quantification

Rather than quantifying the DNA using the Cary 300 Bio UV-vis spectrophotometer, the NanoDropTM was used. A blank of water was run. Proceeding in descending order of the selected rounds, 2 μ L of each desalted DNA pool (2.5 μ L for Round 12P) was placed on the NanodropTM lever and the concentrations calculated by the instrument at 260 nm were recorded.

MiSeq Sample Preparation

Two buffers (10 mM tris, pH 8.5 and 0.2 N NaOH) were prepared. The concentration for each DNA pool was converted from ng/ μ L to nM. Each DNA pool was then diluted to 4 nM with the 10 mM tris buffer and vortexed. In a 1.5 mL microcentrifuge tube, 5 μ L aliquots of each diluted DNA library were combined together to create a pooled library sample and vortexed. The preparation protocol [156] was followed and only modified in the last step.

To denature the DNA, 5 μ L of the 4 nM pooled library was combined with 5 μ L 0.2 N NaOH in a microcentrifuge tube and briefly vortexed. The sample solution was centrifuged at 2,000 rpm for one minute and then left to incubate at room temperature for five minutes to denature the DNA into single strands. Pre-chilled HT1 reagent (900 μ L) was added to the tube containing the denatured DNA, resulting in a 20 pM denatured library in 1 mM NaOH. This was placed on ice until ready for the final dilution. The final dilution consisted of diluting 120 μ L 20 pM denatured library with 480 μ L pre-chilled HT1 reagent. The tube was inverted several times to mix and the DNA solution was then pulse centrifuged and placed on ice. The PhiX control used in the research was already previously prepared as follows. The PhiX library was diluted to 4 nM by combining 2 μ L 10 nM PhiX library with 3 μ L 10 mM tris (pH 8.5). Then the diluted PhiX control was combined with 5 μ L 0.2 N NaOH and briefly vortexed, resulting in a 2 nM PhiX library solution. This was incubated at room temperature to denature the PhiX library into single strands. To make a 20 pM PhiX library, 10 μ L of the denatured PhiX library was combined with 990 μ L pre-chilled HT1 reagent.

The denatured 20 pM PhiX library was diluted to the same loading concentration as the DNA library (4 pM) by combining 120 μ L 20 pM denatured PhiX library with 480 μ L pre-chilled HT1 reagent. The tube was inverted several times to mix and then the solution was pulse centrifuged and placed on ice. The denatured and diluted PhiX library (480 μ L) was combined with the denatured and diluted DNA library (120 μ L). The tube was inverted to mix and put on ice. The tube contents were then incubated at 93 °C for two minutes. The tube was again inverted a couple of times and then placed on ice.

Loading the MiSeq Sequencing System

From the MiSeq® reagent kit v2, the flow cell was rinsed with EtOH, dried with a KimWipe, rinsed with EtOH and dried again with a KimWipe. The total contents of the tube (600 µL) were injected into the MiSeq sequencing sampler. The sample was left to run for approximately 24 hours, which was equivalent to 300 cycles in total. AptaGUI software, an open-source and platform-independent graphical user interface for dynamic visualisation of sequencing data [157], was used to analyse the resulting data.

3.5.2.4 GC-MS Analysis for Target Identification

All GC-MS analyses were performed in-house at the Carleton Mass Spectrometry Centre (CMSC). While target identification was not required for fingermark-SELEX, attempts to identify possible targets were made as supporting evidence of this novel variation and to possibly help narrow down the aptamer candidates for a new aptamer-based reagent. Three different GC-MS extraction and derivatisation methods previously published for fingermark composition studies were executed as closely as feasible (e.g., temperature run program) with available resources for the remainder of the stacked fingermark samples. These methods were initially performed on the same day as the SELEX selection, or as soon as possible based on the GC-MS technician's availability. If not on the same day as the selections, then the samples were extracted and derivatised on the same day that the samples could be analysed by GC-MS. Controls (i.e., blank substrate pieces without any fingermark residues present) were also prepared and analysed in the same manner to differentiate between compounds identified by GC-MS that were due to the fingermark residue composition rather than those due to the substrate background.

The method used by Girod et al. [158, 159] was chosen as it was able to elicit various fingermark residue compounds and it appeared manageable to perform and only one reagent needed to be ordered; in other words, experimental delays were minimised. Half of the remaining stacked fingermark sample was cut into approximately 5 mm x 5 mm pieces placed in a glass amber GC-MS vial. Twenty drops (approximately 300 µL) of DCM was added to the vial, which was then vortexed for one minute at a

moderate speed. The pieces of garbage bag plastic were removed from the vial and the liquid contents were then concentrated by drying down with argon gas and reconstituted with ten drops of DCM and vortexed. Samples prepared this way were analysed on either a 15 m or 30 m non-polar column.

The method used by Wehrli included MSTFA as a derivatising agent [160]. Briefly, 200 μ L DI water was combined with 50 μ L 5:1 chloroform:pyridine and 20 μ L MSTFA. The fingerprint sample pieces were vortexed for 30 seconds in the solution and then they were removed. The sample vial was then placed on a heat block for 80 °C for one hour. Samples prepared this way were analysed on either a 15 m or 30 m non-polar column.

Two separate research groups, Croxton et al. [161, 162] and Mink et al. [163], used an alcohol with alkyl chloroformate as the derivatising agent; in this case, ECF was used. Pieces of fingerprint samples were placed in a vial with 1 mL of 1% (w/v) NaOH, EtOH, and pyridine, 75:40:10 (v/v/v), which was then sonicated for 10 minutes. A 1:3 (v/v) ECF:iso-octane (200 μ L) was added to the sample and vortexed for 30 seconds, releasing the carbon dioxide (CO₂) produced every ten seconds. After one minute, 40 μ L pyridine and 50 μ L ECF were added and vortexed for an additional 30 seconds before left to stand for three minutes. The solvent was transferred to a second vial containing 1 mL chloroform with 1% (v/v) ECF, vortexed for 30 seconds with the CO₂ produced released every ten seconds, and then allowed to separate. The upper aqueous layer was discarded. Hydrochloric acid (1 M, 1 mL) was added to the vial and vortexed for 30 seconds with the CO₂ generated released every ten seconds. The lower organic layer was transferred to another vial and evaporated to dryness. On the day that the GC-MS technician was ready to run the sample, 50 μ L chloroform were added and the sample was sonicated for one minute and not injected into the GC-MS system until at least 30 minutes after the chloroform addition. Samples prepared this way were analysed on either a 30 m non-polar or polar column.

3.6 Results and Discussion

3.6.1 In Vitro Selection Process

One of the main disadvantages to the SELEX process is the uncertainty of successfully producing an aptamer to the target molecule [125]; the *in vitro* selection process is essentially performed blind. Ideally, the desired outcome would be to have the negative selection values (i.e., amount of DNA that binds to the substrate) decrease, while the positive selection values (i.e., amount of DNA that binds to the fingerprint residues) increase or plateau as more selection rounds are performed. These would indicate that there is an enrichment of aptamer sequences for the desired target. The % binding results for all selection rounds can be seen in Figure 3-6.

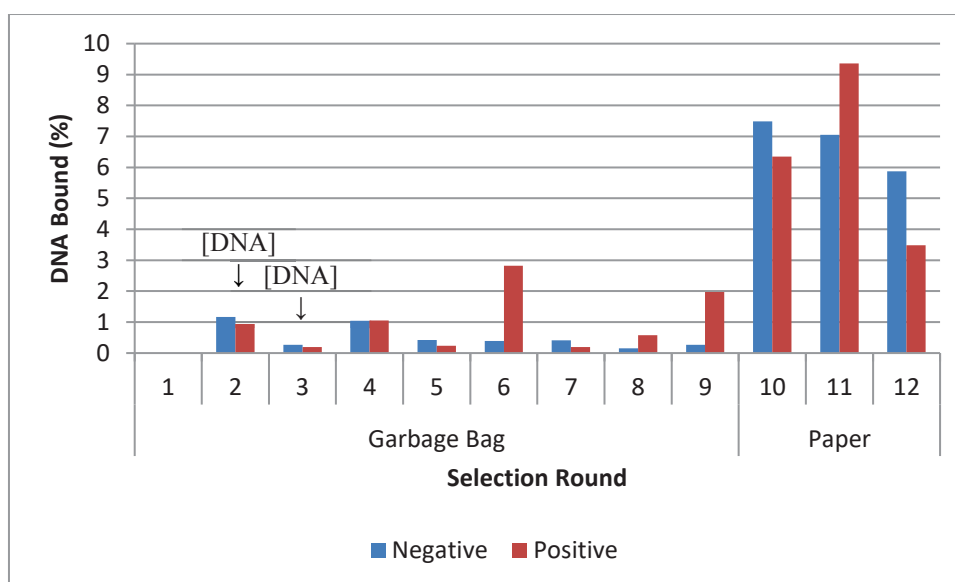


Figure 3-6 Graph showing percentage of DNA bound in each negative and positive selection round of fingermark-SELEX. Selection was monitored by the fluorophore attached to the DNA in all rounds except Round 1. The concentration of DNA was lowered in Rounds 2 and 3 to increase stringency.

The first nine rounds of SELEX were performed with fingerprints, aged 10 to 106 days, on garbage bag plastic. No % binding could be calculated for Round 1, as the Atto 550-labelled primer was not available at the time. Atto 550 was chosen due to its compatibility with forensic light sources available at UTS as previously assessed by

Spindler [164]. Although at first glance, the % binding values are very low, 2.8% (highest positive selection for this substrate) of 10^{16} sequences (the starting library) is still quite high. SELEX is known to be challenging; it is estimated that less than 30% of selections result in aptamers [165, 166] and especially for small molecule targets. Most SELEX experiments tend to target larger molecules, such as proteins [136]. The % binding values for the positive columns in both Rounds 6 and 9 were over seven times greater than those for the corresponding negative rounds. Since these are further along in the course of selection, it is reasonable to assume that the pool was composed of more high affinity binders to fingermark residues than non-specific or low affinity binders.

While published aptamer research has been analysed in bulk to identify trends in selection parameters [136], there are no set protocols to follow. The desired trends were not obtained; however, this could be due to several reasons. More selection rounds per substrate may have been required before the desired trends become apparent. Typically, approximately 5-15 rounds are required for SELEX [167]; it all depends on when the researchers deem that the selection has reached completion, typically by monitoring enrichment. It was unfortunate timing that the positive selection values were increasing from Rounds 7 to 9, but there were no more aged garbage bag plastic samples left to include in the selection process. Also, the decision was already made to switch to paper samples to increase stringency conditions.

The DNA pool becomes enriched with better binding sequences when stringency is increased. Other stringency conditions used in this SELEX experiment included decreasing the amount of DNA used, as well as using different aged fingermarks, for subsequent selection rounds. The latter variable may have made it even more difficult to interpret the effect of stringency. Typically, one or two known targets are used through the whole selection process. Fingermark residues are inherently variable within and between sources. Aged fingermarks add an extra dimension of complexity and variability. Components within the fingermark residues will begin to degrade over time and may not be present if used in a later selection round. Different aged fingermarks could be used in a future SELEX experiment, but it would probably be best to do multiple rounds with one fingermark age or age range to try and achieve

enrichment of binders before moving onto the next fingermark age or age range with subsequent rounds.

The last three rounds of SELEX were performed with fingermarks, aged 12 to 25 days, on copy paper. This was to increase stringency, as it was hypothesised that it would be harder for the DNA sequences to bind to fingermark residues that had been absorbed into the substrate as the samples aged. As seen in Figure 3-6, this did not appear to be the case. The % of DNA bound ranged between 3.5% and 9.4% compared to 0.2% to 2.8% for the non-porous substrate. For meaningful interpretation, more rounds needed to be performed. Unfortunately, while *in vitro* selections typically take a year or two to perform, this was not possible given the limited time for this offsite research placement. It was expected that the negative selection would have a high value in the first round since the substrate changed. Even though there were only three rounds with the paper substrate, the negative selection values were slowly decreasing. While it was thought that the % binding values on paper were going to be lower than those on garbage bag plastic, the positive selection values were unexpectedly high. This could be due to non-specific binding. On the other hand, it could also be due to the fact that the integrity of the paper was compromised. During the elution step, the layers of the paper substrate were coming apart and fibres became loose. This could have resulted in the fingermark residues being more accessible than initially thought. Another possibility could be that fresher fingermarks have components that are more favourable to DNA binding or at least to the sequences remaining in the elutions. Round 2 used 31-day-old fingermarks on garbage bag plastic, and the fingermark age for subsequent rounds on this substrate only increased, while Round 10 used 12-day-old fingermarks on paper, the freshest fingermarks used in the SELEX experiment.

The partition step of the SELEX process is not perfect. Some sequences that should be removed during the negative selection are not, while some sequences that should not be removed during the negative selection are. This is why the number of rounds varies from SELEX experiment to experiment. It is typically performed until those desired results are obtained.

As this was a proof-of-concept study in a short timeframe, optimisation could not be performed. If favourable results were not obtained after a particular round, there was

no time to go back to a previous round and redo or modify the parameters. It could be possible that the incubation time may need to be longer; this would increase the exposure of the sample to the DNA pool. However, typically for cell-SELEX (which fingermark-SELEX is based on), the binding reactions occur in incubation periods of less than an hour to address concerns of degradation and internalisation of the oligonucleotides [133]. Conversely, since the positive column was gently shaken during the incubation phase, a longer time could also increase the chances of the fingermark residues to disengage from the substrate and be free in solution. Therefore, there is the possibility that they could get removed from the positive column even before the washes and elutions. Five elutions were performed after each round. More elutions may have been necessary to remove more highly binding sequences from the substrate.

3.6.2 Sequence Analysis for Aptamer Sequence Candidate Screening

The decision to perform MiSeq sequencing was a happy medium between conventional low and high throughput sequencing (HTS). Low throughput sequencing involves bacterial cloning and Sanger sequencing, which typically results in fewer than 100 sequences being identified [168]. This may not be a true reflection of the aptamers that bind the best and it would not accurately illustrate the highly diverse nature of the resulting aptamer population [129, 169]. High throughput sequencing can provide significantly more information – complete pool diversity, enrichment trajectory of individual sequences as the selection process progresses, best-performing sequence predictions – that would allow for better analysis and interpretation of the enriched aptamer pools [168, 170]. It could also radically shorten the aptamer discovery process and reduce or eliminate truncation experiments to locate the core binding domain [171]. Unfortunately, HTS can be costly and take months to perform. Therefore, MiSeq sequencing was chosen, as the identity of a large number of sequences would still be obtained in significantly less time.

Preliminary analysis of the sequencing results showed that, after 300 cycles, about 1.2 million usable data were collected; on average 150,000 per sample pool (Figure 3-7). Using the AptaGUI software, this data could be arranged by count (frequency of the

sequence), fraction (percent of aptamer with respect to its pool size), and enrichment (fold change in fractions between consecutive cycles). While enriching highly bound sequences may be the end goal of SELEX, careful consideration needs to be taken if organising the sequencing data by enrichment, as the same amount of sequences per pool is not always present.

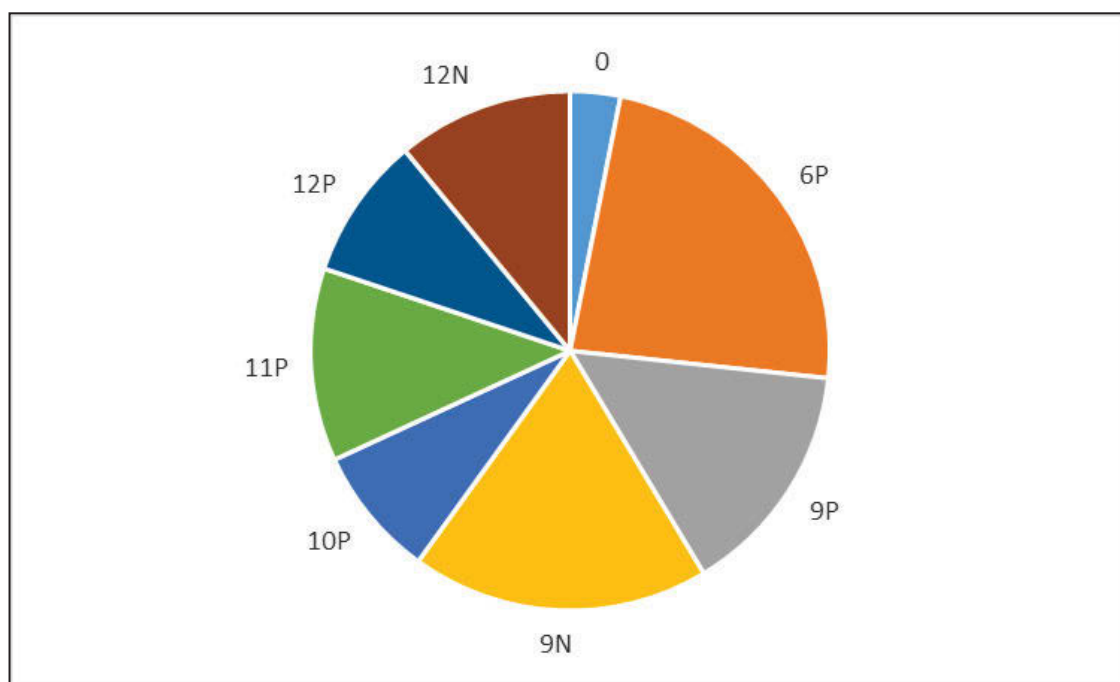


Figure 3-7 Sequencing data distribution for select SELEX rounds. (# = selection round; N = negative selection round; P = positive selection round)

It was determined that more usable data should have been acquired. Unfortunately, the ratio of the solutions during the last step of the sequencing preparation was reversed; 120 μ L denatured and diluted PhiX control should have been combined with 480 μ L denatured and diluted DNA library. The PhiX control was too high at around 85% [172]; Illumina recommends using $\geq 25\%$ PhiX control spike-in [156]. While enrichment may be deemed the main focus when analysing the data, other observations could not be disregarded. The drastic decrease of one DNA sequence in particular (1KjJqCJD; Table 4-1) may correspond to the presence/absence of squalene observed in the GC-MS results described below (Subsection 3.6.3). There were cases where DNA sequences would be counted in later rounds (e.g., GiAGIIGI; Table 4-1), but not in earlier rounds. The only way that they could be counted in later rounds was if they

were present in the earlier rounds, but just not loaded onto the sequencing cartridge to get detected due to only a fraction of the pooled library being used.

The sequencing data could also be viewed by cluster size by a particular round. If another round was grouped together with that particular round, then the others would fall into the same cluster. The AptaGUI software can also predict two-dimensional structures. Due to time and technical restrictions, only the first 100 sequences organised by count per round were able to be exported and viewed at UTS. While this was not ideal, this was one way to screen out sequences to incorporate into an aptamer-based fingerprint enhancement reagent.

3.6.3 GC-MS Analysis for Target Identification

The fingerprint-SELEX variation is similar to cell-SELEX, where *a priori* knowledge of the target is not necessary. The purpose of conducting GC-MS analyses was not to identify all compounds present in the fingerprint samples. Rather, it was used as an aid during the aptamer candidate screening process and provided direction as to what targets should be used for chemical spot tests (Subsection 4.4.2.1). Amongst the many techniques available to analyse the chemical composition of fingerprints [30, 173-175], GC-MS was used in this research project due to ease of accessibility at the overseas research facility. Basic information was also collected from the donors (Appendix II), which may provide explanations to some findings.

The DCM method was used for Rounds 1 to 9; the MSTFA method was used for Rounds 1 to 8; and the ECF method was used for Rounds 9 to 12. These decisions were made in attempt to elicit usable data with current instrumentation setups and no optimisation. Data was presented in total ion chromatograms and preliminary peak searches were performed. Some fingerprint compounds identified include: squalene, isopropyl palmitate, octadecanoic acid, and hexadecanoic acid. Other compounds were identified and linked to fragrances (e.g., nonanal, decanal) and the garbage bag plastic (e.g., 1,4-benzenedicarboxylic acid, phthalates, long alkyl chains). It is interesting to note that squalene was detected up to and including Round 6 (up to 66 days old), but no longer detected in subsequent rounds; Round 7 used 73-day-old

fingermarks (Figure 3-8). Isopropyl palmitate was first reported as an endogenous constituent of fingerprint residues by Girod et al. [158]. However, this was also listed as an ingredient in one of the donors' hand lotion; therefore its true source is unknown.

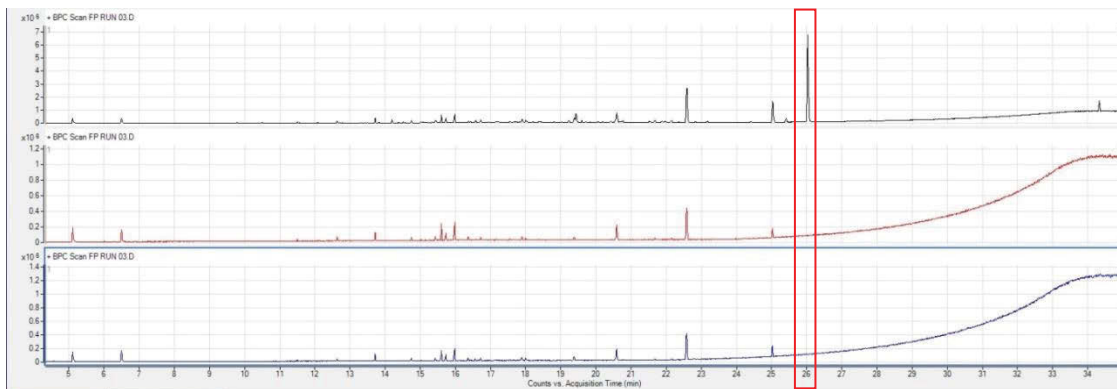


Figure 3-8 Squalene peak (highlighted in red box) was detected in Round 6 (top), but not in subsequent Rounds 7 (middle) and 8 (bottom).

Archer et al. reported that squalene degrades or transforms to other compounds very quickly; it was present up to 33 days in one donor's samples stored in the dark [176]. Squalene was detected up until 66 days in the fingerprint-SELEX experiment, with the samples stored in the dark until GC-MS sample preparation; however, this time difference is likely due to the fact that there was an abundance of fingerprint residues from 16 donors per garbage bag plastic sample. Archer et al. reported that short-chain fatty acids (e.g., hexanoic, octanoic, nonanoic acids) were detected more often in aged samples; longer chains did not appear to vary much as time increased; and saturated (e.g., tetradecanoic, palmitic, stearic acids) and unsaturated (e.g., palmitoleic, oleic acids) fatty acid levels increased in the early stages of ageing, but then decreased to around the original levels [176]. Fatty acids present in fingerprint residues are believed to be a result of the microbial degradation of glycerides; their presence in older fingerprints could be due to the breakdown of wax esters or triglycerides. Their disappearance could also be due to volatilisation or degradation.

Like Mong et al. [177], no quantification of the fingerprint residue components could be performed due to the fingerprint collection method. True variation trends could not be observed due to fingerprints from multiple donors being analysed simultaneously in

a single specimen. The amount of residues to analyse was inconsistent because the distribution of residues from the remaining stacked fingerprint sample may have been uneven, as well as the pressure applied. However, this is not critical for the current project as the focus is on qualitative analysis. Besides asking the fingerprint donors that they could not wash their hands for at least an hour before deposition, no other special instructions were needed. As such, the possibility of exogenous substances being present in the GC-MS data was not minimised to focus solely on the endogenous components of fingerprint residues; if donors happened to run their fingers through their hair, put on face cream, etc., this was allowed to mimic real-world scenarios.

As seen in Figures 3-9 (garbage bag plastic) and 3-10 (copy paper), the choice of extraction solvents and GC-MS column polarity will result in different chromatograms. The ECF extraction/derivatisation method was coupled with a mid-polar column in the cited papers. Unfortunately, CMSC only had the extremes; therefore, the ECF samples were run on both polar and non-polar columns to see which would provide better results. Although more peaks were obtained on the non-polar column, many of them were also present in the control samples (i.e., substrate without fingerprints extracted/derivatised in similar manner).

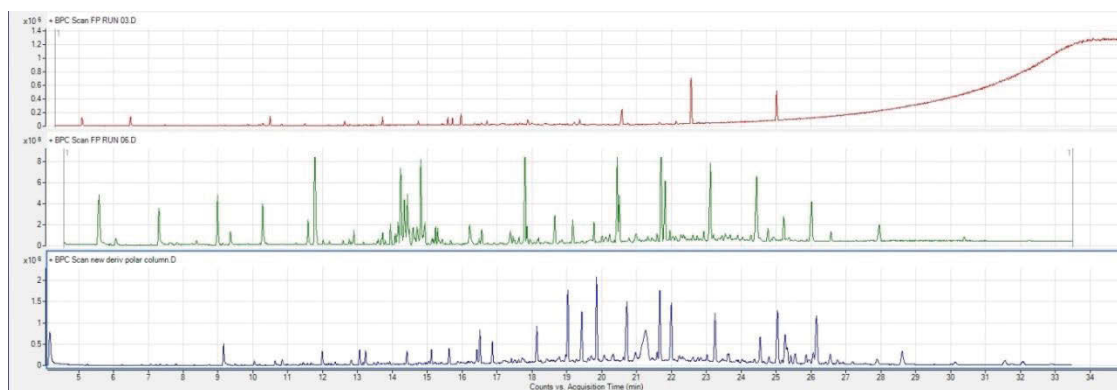


Figure 3-9 Round 9 samples (fingermarks on garbage bag plastic) using DCM on non-polar column (top), ECF on non-polar column (middle), and ECF on polar column (bottom).

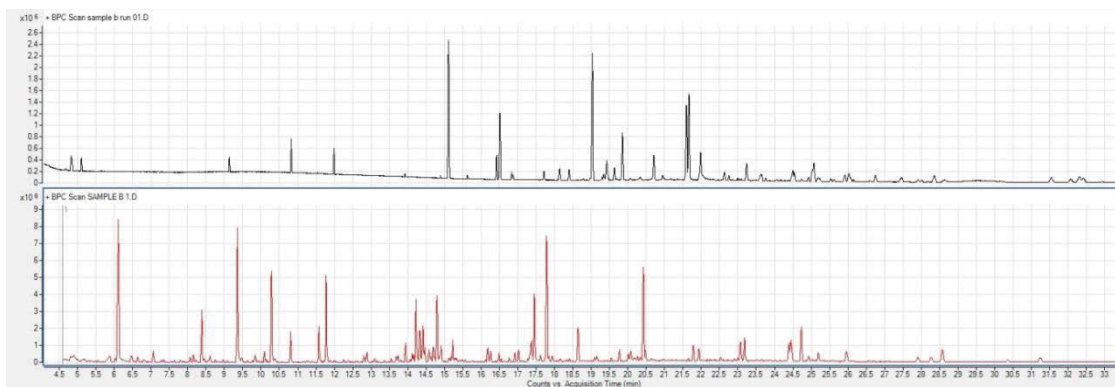


Figure 3-10 Round 10 samples (fingermarks on copy paper) using ECF and run on a polar column (top) and non-polar column (bottom).

If a more thorough analysis of GC-MS results was within the scope of this research project, the existing data could have been re-analysed and presented in extracted ion chromatograms. It is possible that even more compounds are present, but not easily observed since they are eluting at the same time as others.

3.6.4 Advantages and Limitations

With a process like (fingerprint-)SELEX, the possibilities are endless. There is a countless number of compounds that could be targets in this *in vitro* selection. Not only could an existing SELEX variation be used in a targeted approach developing aptamers for a specific target, but the research presented in this chapter demonstrates that an untargeted approach is also possible with fingerprint-SELEX. With an untargeted approach, aptamers could be developed for eccrine and sebaceous constituents simultaneously. This means that their aptamer-based reagent has the potential to replace a routine technique sequence for semi-porous substrates. The development of aptamers is not restricted by *in vivo* or physiological conditions as are antibodies. SELEX experiments could also be tailored to address difficult scenarios, for example an arson investigation by developing aptamers that target pyrolytic products, such as those identified by Chiavari and Galletti [178].

As this was a proof-of-concept study, there were bound to be limitations, the major ones being time and scale. It was not possible to significantly deviate from the initial plan (e.g., start over with different substrates, change the manner in which the fingermarks were deposited, etc.), as the research in this chapter had to be completed within the 4-month period. An initial plan was brainstormed and carried through no matter what issues presented themselves. The fact that anything was obtained was a feat considering the low success rate associated with SELEX experiments. Not only was time a factor in the *in vitro* selection process, but also for the GC-MS and sequencing analyses. There was insufficient time to optimise the GC-MS analysis protocol, an aspect that could have provided more information on possible aptamer targets present in fingermark residues than currently obtained. Thorough sequencing data analysis could not be performed at the offsite facility; better interpretation of the results could have been discussed.

The fingermarks were deposited in a stacked manner due to the study being performed on a microscale (e.g., selections were performed in only 200 μ L solutions), and only a portion of each stacked fingermark sample was used for the positive selection. Using a fraction of the stacked fingermark sample, as well as the possibility that a donor could have removed some fingermark residues already deposited by other donors, could have significantly decreased the amount of potential targets. If more fingermark sample was used, more DNA would have been required to proceed to the next round, which in turn would mean more PCR, PAGE, etc. would need to be done. Increasing the scale was not feasible in the allocated time period. Also, the amount of stacked fingermark sample remaining for GC-MS analysis was limited by the manner in which the samples had to be collected.

While there have been many technological advances to improve the generation of potential aptamer candidates, as seen with the abundance of SELEX variations, aptamer discovery tends to be stalled by the low-yielding and laborious characterisation and validation of individual aptamer sequences [179]. Unfortunately, due to limited time and resources, these areas could not be explored during this PhD project. However, as shown in Chapter 4, the omission of these steps did not hinder the progress of this research.

3.7 Conclusions

This chapter demonstrated, for the first time, a SELEX variation protocol designed specifically for fingerprint residues. Natural fingerprints from multiple donors were collected on commonly encountered substrates and subjected to the *in vitro* selection process. Changes in the % DNA binding data indicated that weakly bound or unbound DNA sequences were being removed from the selection process, while highly bound DNA sequences continued to be present in later rounds as stringency conditions were introduced. Sequencing data analysis revealed several potential aptamer candidates to be incorporated in a fingerprint detection reagent (Subsection 4.4.2.1). Fingerprint-SELEX did not require *a priori* knowledge of the targets; however, GC-MS analyses were attempted to identify some possible compounds.

While optimisation could not be performed during the short time period available at LADDER, a novel SELEX variation was still able to be designed and executed, resulting in the development of potential aptamer candidates against fingerprint residues. The experience was also a good example of the interdisciplinary nature of the forensic science field.

***Chapter 4: Investigation into
“Fingerprint” Aptamer Candidates
for Fingerprint Detection***

Chapter 4 Investigation into “Fingerprint” Aptamer Candidates for Fingerprint Detection

4.1 Introduction

Due to the versatility of aptamers, there are very few areas of research where they cannot be applied [180]. Aptamers are commonly found in therapeutic and biotechnological applications. They are useful as pharmacological tools for drug discovery target validation and as aptasensors [181, 182]. Aptasensors are biosensors that use aptamers as the biological recognition probe to recognise the analyte of interest [183, 184]. Since aptamers can bind to specific target molecules with a high affinity, they have also been incorporated in analytical chemistry applications, such as affinity CE and chromatography [185]. As with antibodies, aptamers have also been incorporated into lateral flow devices [186].

Aptamers are also starting to appear in the forensic science community. Forensic toxicology has started to explore this avenue for the detection of toxins and illicit drugs [187]. Some examples include the colorimetric detection of arsenic [188], an aptamer-based electrochemiluminescence biosensor for cocaine [189], and the electrochemical detection for cocaine [190, 191] and codeine [192] using aptamers.

SomaLogic, Inc. has been working in collaboration with the Denver Police Department Crime Laboratory to develop the first aptamer-based system for sperm binding, isolation and purification specifically for DNA analysis in sexual assault cases [106, 193]. This sperm cell separation method is more rapid than existing extraction methods, which in turn will reduce the backlog of forensic samples in this type of investigation.

4.2 Aptamers in Fingerprint Detection

In relation to the field of fingerprint detection, there has been little published about aptamer development to forensically relevant targets. Aptamers to some amino acids and proteins present in fingerprint residue have been published [129, 132, 194]; however, they are not ideal targets, as their abundance in fingerprint residue is low compared to other components. As amino acids are small molecules, aptamers do not possess high binding affinities towards these targets like they would for larger molecules. Wood attempted to develop an RNA aptamer-based fingerprint detection reagent for the L-histidine and L-isoleucine; however, results were unsuccessful [101].

The first known use of aptamers in fingerprint detection was published by Wood et al. [195]. Two previously published aptamer sequences for lysozyme, a polypeptide component identified in sweat [88], were synthesised with luminescent tags, and then incorporated into aptamer-based reagents. They were capable of developing charged fingerprints with minutiae clearly visible and no negative background interaction was observed on PVDF (Figure 4-1). One concern was that the aptamer-based reagent had long development times (i.e., one to four hours), which is not practical from an operational standpoint. Also, although logical for a proof-of-concept study, lysozyme is not an ideal target in a fingerprint detection context. This research showed great potential, especially since the use of aptamers does address some of the concerns with the use of antibodies. Wood et al. believed that aptamers were the next logical step to creating a biomolecular reagent that could be utilised by practitioners [43].

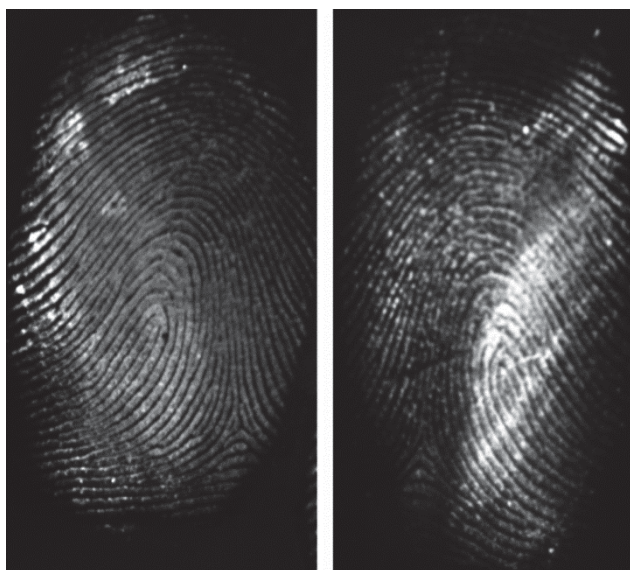


Figure 4-1 Charged fingerprints deposited on PVDF, aged for 24 hours, and then developed with aptamer-based reagent, each with a different lysozyme aptamer sequence (Reproduced from Wood et al. [195]).

Once again, a form of “information” gathering method was demonstrated with cocaine aptamers bound to AuNPs by Li et al [196]. When cocaine was present in the latent fingerprints, AuNPs would aggregate and induce a colour change from green to red (Figure 4-2). The aptamer-bound AuNPs acted both as an imaging probe and a molecular recognition probe. Often times, sophisticated and expensive instrumentation is required for these novel fingerprint imaging and detection methods, however, the authors claim that their dark-field microscopy method is a rapid, simple, and non-destructive method to visualising latent fingerprints.

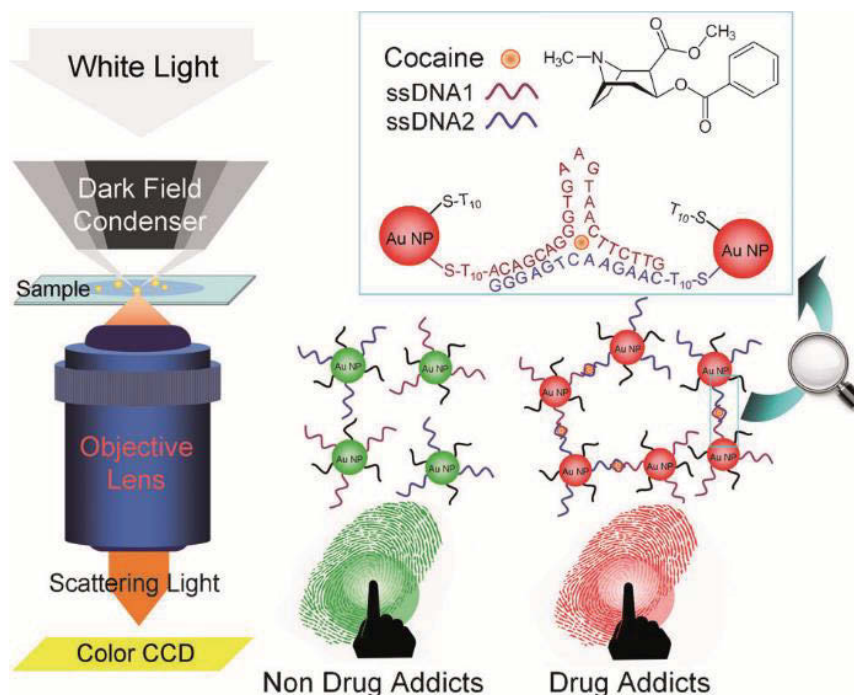


Figure 4-2 Principle of nanoplasmonic imaging of latent fingerprints with and without cocaine present (Reproduced from Li et al.[196]). With respects to detection, a more acceptable term for “addicts” would be “users”.

Even though lysozyme was deemed an unideal target for fingerprint detection due to its fast degradation rate [101], researchers continued to develop novel aptamer-based methods with it. Upconversion nanoparticles (UCNPs) were functionalised with lysozyme- and cocaine-binding aptamers [197]. Sebaceous and cocaine-contaminated fingerprints were only aged for 12 hours before processing. The use of UCNPs took advantage of near-infrared-light-mediated imaging to suppress background fluorescence as illustrated when compared to fluorescein-labelled aptamers and functionalised quantum dots (Figure 4-3).

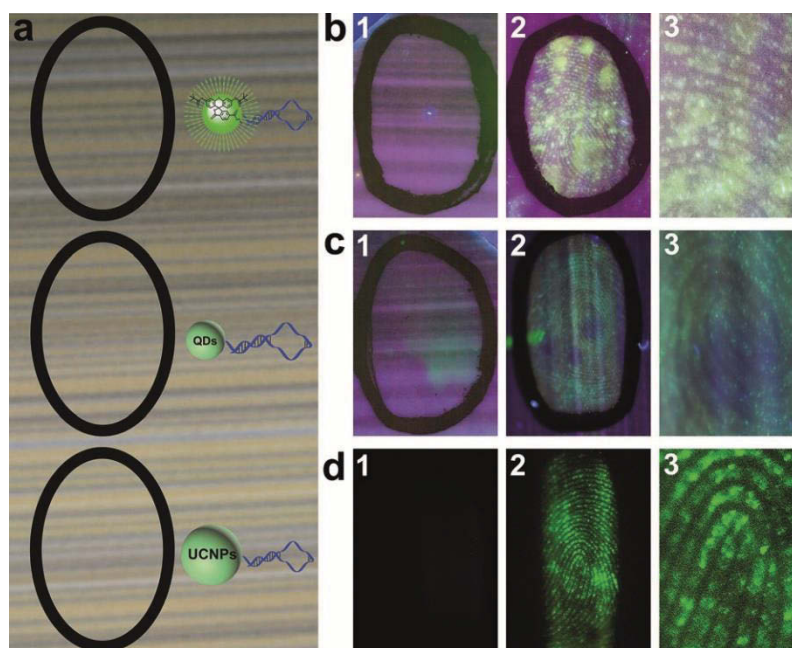


Figure 4-3 (a) Latent fingerprints deposited on marble and visualised with the aid of (b) FAM-labelled, (c) quantum dot-functionalised, and (d) UCNP-functionalised lysozyme-binding aptamer (Reproduced from Wang et al. [197]).

DNA-silver nanoclusters (AgNCs) show potential in chemical and biological detection as fluorescent reporters. Ran et al. decided to use aptamer-modified AgNCs [198] to detect endogenous (i.e., lysozyme) and exogenous (i.e., trinitrotoluene (TNT)) material of fingerprints deposited onto quartz chips. While the main goal was to develop fingerprints, the authors also noted that the fluorescence intensities were proportional to the TNT concentration, which may be useful to quantitatively identify different fingerprint residues or contaminants.

Zhao et al. combined sandwiched gold/*p*-nitrothiophenol/silicon dioxide (Au/pNTP/SiO₂) SERS nanoprobe with a lysozyme-binding aptamer to develop groomed fingerprints [199]. They were able to visualise 16-hour-old sebaceous fingerprints on glass, stainless steel, and PVDF, in addition to a fresh eccrine-rich fingerprint on glass and a sebaceous fingerprint aged over month on a plastic Petri dish. While the authors believe this is a facile and universal visualisation method, with no pre- or post-treatment of the substrate as was the case with Zhang et al. [95], there are a lot of time-consuming steps to prepare the Au/pNTP/SiO₂ nanoprobe and to

immobilise the aptamers to the nanoprobe. The authors also noted that their method required long imaging times.

As shown, most aptamer-based fingerprint detection methods have focused on lysozyme or exogenous substances and fresh fingerprints. A more reliable or “universal” fingerprint detection reagent is desired, which led to the following research objectives.

4.3 Objectives

The objectives of this chapter were not purely to design a fingerprint detection reagent using aptamers; that was previously achieved by Wood et al. [195]. Firstly, work was performed to determine if the “fingerprint” aptamer candidates developed during fingerprint-SELEX could be incorporated into working solutions to detect latent fingerprints, and if they varied in effectiveness from one another. This would support the idea of using the *in vitro* selection process to develop more aptamers against targets relevant to fingerprint detection.

Secondly, fingerprint samples were processed to directly compare the relative performance of the working solutions containing the “fingerprint” aptamer candidates to that of a routine technique sequence. This would provide a preliminary idea of whether they could be a viable alternative for routine use. Thirdly, the relative performance of working solutions containing biomolecular recognition probes developed through a pooled approach (i.e., fingerprint-SELEX) was compared to those of single- or multi-target approaches to determine which approach would be best to address donor variability and selectivity issues currently observed with routine methods.

4.4 Experimental Design

According to the IFRG guidelines, initial investigations of a novel fingerprint detection reagent would be considered a Phase 1 or proof-of-concept study [96]. To

ensure that the results accurately reflect the feasibility of the method, the manner in which fingerprints are collected and subsequently treated need to be considered carefully. Determination of the latent fingerprint components being targeted by the novel reagent could be achieved using chemical spot tests. Some initial tests of the performance of the novel reagent compared relatively to those of relevant routine detection methods should be conducted. As shown in Figure 4-4, these areas of interest have been covered. Typically, three donors deposited natural fingerprints that were aged more than 24 hours on three substrates as recommended in the IFRG guidelines [96]. While performing the proof-of-concept work, the following considerations were also taken into account to ensure that this reagent containing “fingerprint” aptamer candidates would be a feasible technique for operational use:

- Minimal time required for conjugation and reagent preparation steps;
- Reagent can be upscaled for realistic processing situations to avoid the use of hydrophobic pen barrier and microliter volumes; and
- Compatibility with existing imaging equipment typically found in a forensic identification laboratory.

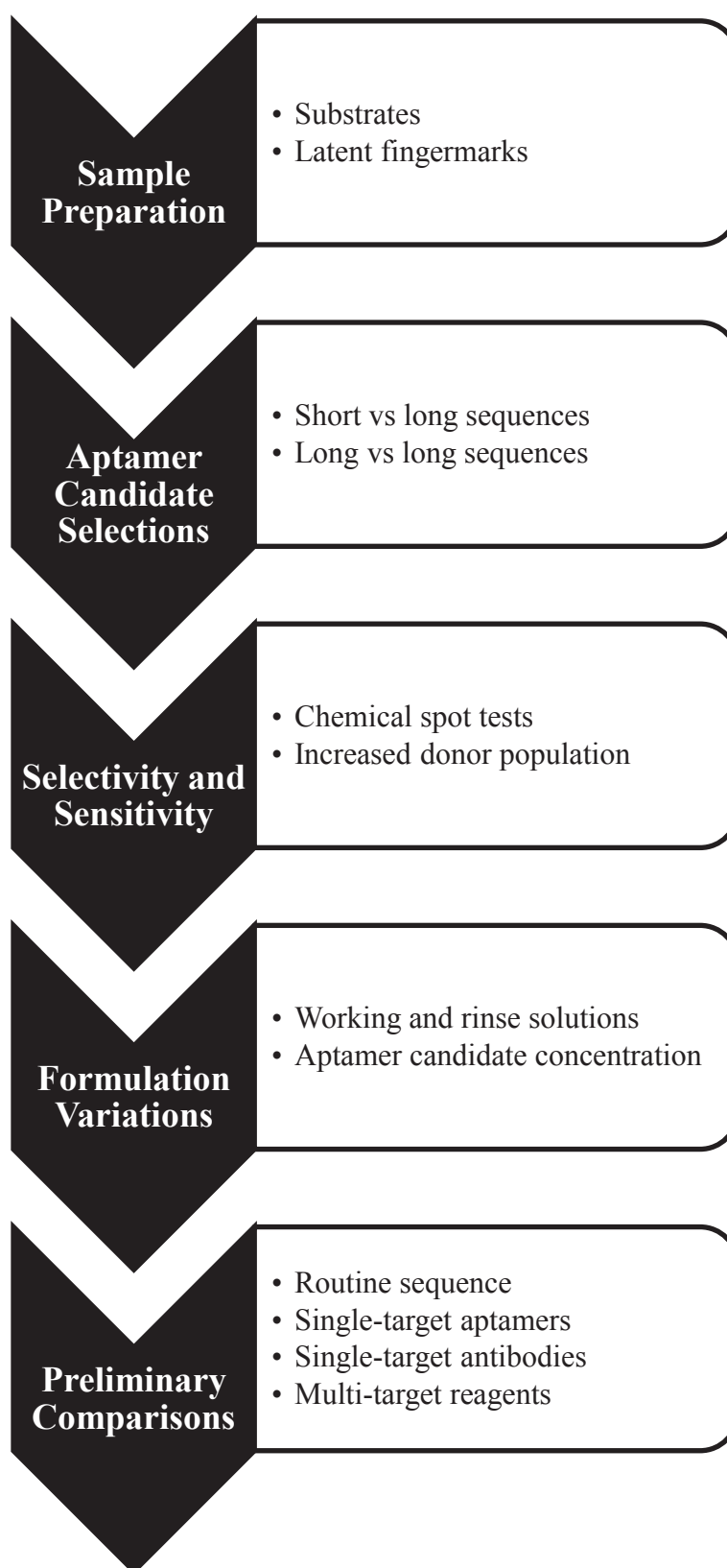


Figure 4-4 Overview of pilot study involving “fingerprint” aptamer candidates from fingerprint-SELEX process.

4.4.1 Materials and Instrumentation

Antibodies

All antibodies purchased were raised in rabbit, with the exception of anti-pan cytokeratin and anti-IgE (raised in mouse) and anti-human IgG (raised in goat).

Polyclonal anti-carnosine antibody was supplied by Abnova.

Monoclonal anti-IgE antibody was supplied by Aviva Systems Biology Corporation.

Monoclonal anti-pan cytokeratin [AE1/AE3] antibody was supplied by GeneTex Inc.

Polyclonal anti-cathepsin D antibody was supplied by Molecular Innovations.

Anti-l-amino acid antibody (raised in rabbit) was produced at Northern Illinois University by A/Prof. Oliver Hofstetter, as described in published methods [49, 53], and dialysed prior to use.

Polyclonal (whole antiserum) anti-cortisol, anti-serotonin, and anti-human IgG (whole molecule) antibodies were supplied by Sigma-Aldrich.

Aptamers

Two lysozyme aptamers [200, 201] were synthesised with fluorescent tag (CAL Fluor® Orange 560) modifications by Biosearch Technologies Inc., received lyophilised, and used as supplied.

Eight custom oligonucleotide sequences were synthesised and HPLC purified to order by Sigma-Aldrich’s Castle Hill and overseas oligonucleotide laboratories.

Aptamers selected against “cathepsin D” (DGI, GEL, KAI tripeptide sequences) [55], cortisol [56], IgE, [202], sperm [57], vitamin B12 [58], and vitamin D [59] were synthesised and HPLC purified to order by Sigma-Aldrich’s Castle Hill oligonucleotide laboratory.

Chemical Spot Tests

L-alanine ($\geq 98\%$ TLC), cholesterol (Sigma grade, 99+%), glycine (reagent grade, 98%), L-histidine (ReagentPlus®, $\geq 99\%$ TLC), L-isoleucine (min. 98% TLC), L-leucine (min. 98% TLC), L-lysine ($\geq 98\%$ TLC), L-ornithine monohydrochloride (approx. 99%), palmitic acid (SigmaUltra, approx. 99%), L-phenylalanine (reagent grade, $\geq 98\%$), L-serine (ReagentPlus™, $\geq 99\%$), squalene (min. 98%), L-threonine (reagent grade, $\geq 98\%$), and L-valine (min. 98% TLC) were supplied by Sigma-Aldrich.

Stearic acid powder (general purpose reagent, Hopkin & Williams Ltd), oleic acid (Laboratory Supply Pty Limited), and dichloromethane (analytical reagent, Rowe Scientific Pty Ltd) were also used for chemical spot tests.

Substrates

Black PE garbage bags (Glad® Wavetop Tie®; Kirkland Signature™; Polykar Industries Inc.), PVDF (Amersham™ Hybond™, 0.45 μm , GE Healthcare), light grey HDPE plastic grocery bag (Woolworths), PE ziplock bag (Hercules® Clip Zip®), aluminum foil (Cast Away®) were used as the non-porous substrates.

White eco-friendly copy paper (92 brightness, 20 lb, 75 g/m^2 , Staples) and carbon neutral 20% recycled copy paper (91.2% opacity, 80 g/m^2 , Staples) were used as the porous substrates.

Additional Reagents and Materials

AttoTec Atto 550 NHS ester (prepared according to manufacturer’s instructions) and PBS with 5% non-fat milk powder (dry milled, pH 7.3) were supplied by Sigma-Aldrich.

Amicon Ultra (Merck Millipore) 3 kDa spin columns were used to remove excess luminescent dye from incubation step with aptamers.

HEPES (buffer grade, AppliChem GmbH), NaCl (analytical reagent, Chem Supply), KCl (analytical reagent, Chem-Supply), magnesium chloride hexahydrate

($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, analysis grade, Merck Millipore), CaCl_2 (laboratory reagent, Unilab Chemicals Ltd.), and milliQ water were used to prepare the SELEX buffer.

Tris (UltraPure, ICN Biomedicals, Inc.), NaCl (analytical reagent, Chem Supply), and KCl (analytical reagent, Chem-Supply) were used to prepare the tris-buffered saline (TBS) buffer.

Tris base (UltraPure, ICN Biomedicals, Inc.), NaCl (analytical reagent, Chem Supply), and Tween® 20 (laboratory reagent, Chem-Supply Pty Ltd) were used to prepare the TBS with Tween® 20 solution.

Tris (UltraPure, ICN Biomedicals, Inc.), NaCl (analytical reagent, Chem Supply), and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (analysis grade, Merck Millipore) were used to reconstitute lysozyme aptamer as selected in [200].

Trizma® base (primary standard and buffer, $\geq 99.9\%$ titration), glycine (reagent grade, 98%), potassium phosphate dibasic (ACS reagent, $\geq 98\%$) were supplied by Sigma-Aldrich and used to reconstitute lysozyme aptamer as selected in [201].

Instrumentation

Rofin Poliview IV with a PL550XL FLS and V++ Precision Digital Imaging System software (version 5.0) were used to visualise and image processed fingermarks.

Eppendorf miniSpinPlus was used for spin column purification of antibodies and aptamers incubated with luminescent dyes.

JULABO SW22 shaking water bath (John Morris Scientific Pty Limited) was used to gently agitate samples while in working solutions.

VSC6000 video spectral comparator (Foster + Freeman) was used to image samples under white light.

4.4.2 Methods

Similar sample preparation was followed as in Chapter 2. Grids were drawn onto pristine substrates to ensure that fingerprints were deposited in known locations and could be subsequently cut in half for direct comparison experiments. Only natural latent fingerprints were collected unless otherwise stated; fingerprint donors were again asked not to wash their hands for at least one hour prior to deposition. The fingerprints were stored in the dark under ambient laboratory conditions until the desired age intervals were obtained. Processing times for the working solutions containing the biomolecular recognition probes were set at 45 minutes with constant gentle agitation, with the exception of porous substrates (no more than 15 minutes) and lysozyme aptamer-based reagent (two hours). Once the samples were dried, these fingerprints were imaged using the Poliview at 530 nm with a 610 nm bandpass filter tilted at 30° (equivalent to 590 nm) due to the incorporation of the Atto 550 dye into the reagent. Additional visualisation conditions are listed as needed in the following subsections. Fingerprints – whole and split – were assessed using the CAST and UC scales (Tables 2-3 and 2-4).

4.4.2.1 Proof-of-Concept

Selection of Aptamer Candidates

Eight DNA sequences were ordered: four were the random regions identified during the sequencing analysis (Subsection 3.6.2); the other four were the same random regions with the primer regions used during the SELEX process also included (Table 4-1). The sequences were chosen due to various reasons. Sequence 1BwJHFIHZ was chosen because, according to the sequencing data available, it was only counted in the positive SELEX rounds with the garbage bag substrate; therefore, it could be a substrate (or substrate type)-specific aptamer candidate. Sequence IHDeIHA was chosen because it was counted in the positive SELEX rounds for both garbage bag and paper substrates, meaning it could possibly be a “universal” aptamer candidate. While the purpose of SELEX is to promote enrichment of an aptamer candidate sequence, Sequence 1KjJqCJD was chosen because there was actually a reduction in its count that may have correlated with the loss of squalene during the

GC-MS analyses. Therefore, this sequence was tested due to the unexpected possibility that it could be squalene-specific. Finally, Sequence GiAGIIGI was chosen because of its presence after the final positive SELEX round. While it was not observed in the other rounds, the only way it could be present is if it was carried through the previous rounds. The longer sequences (N₄₅ region with primers) were tested in addition to the shorter sequences (N₄₅ region only) due to the predicted secondary structure strength. The secondary structure of Sequences 1KjJqCJD and GiAGIIGI drastically increased once the primer regions were included. With regards to the predicted secondary structures (Appendix V), at this point, it was not known if these sequences had high affinity to the targets, nor was it known which part of the sequences would bind. Aptamer characterisation and functional verification still need to be performed, but were not within the scope of this PhD project.

Single deposition natural and sebaceous fingerprints from three donors (one male, two female) were collected (total = 240 fingerprints) on an ideal experimental substrate (PVDF), a realistic non-porous substrate (two different black garbage bags) and a realistic porous substrate (two different white copy paper). One garbage bag and one copy paper substrates were the same used in the SELEX process (purchased in Ottawa, Canada), while the other realistic substrates were purchased locally in Sydney, Australia. The fingerprints were aged for three and twelve days prior to being cut in half and processed with working solutions containing one of the eight “fingerprint” aptamer candidates and rinsed with milliQ water. The working solution followed a similar formulation as in Chapter 2: 15 µL DNA sequence incubated with 5 µL dye and filtered by spin column, and then diluted into 5 mL 1:1 PBS with non-fat milk:milliQ water. The relative performance of each corresponding pair of short and long sequences was compared. The fingerprints developed on PVDF were also imaged using the video spectral comparator VSC6000 under direct white light.

Table 4-1 “Fingermark” aptamer candidate sequences tested. *Predicted secondary structure strength based on manufacturer’s website. Secondary structures predicted by RNAstructure software [203] can be found in Appendix V.

	Aptamer Candidate	Sequence	Predicted 2° Structure Strength*
N ₄₅ region only	#1 – short 1BwJHFIHZ	CAGCACGGCAACGGGCGCAGGGCAGGATGGGATACGGCTGGTATG	Strong
	#2 – short IHDeIHA	TAGCGCGGCAACGGGCGCAGGGCAGGATGGGATACGGCTGGTATG	Very strong
	#3 – short 1KjJqCJD	TCGCAGCAGAGAAGGAGTGGGTACGTGGTGATGGTGGATGGGGTA	Very weak
	#4 – short GiAGIIGI	CAAATCGACCACGCAATCCCTTTAATGTGAATATACAAGCATAGC	Weak
N ₄₅ region with primers	#5 – long 1BwJHFIHZ	ATCCAGAGTGACGCAGCACAGCACGGCAACGGGCGCAGGGCAGGATG GGATACGGCTGGTATGTGGACACGGTGGCTTAGT	Strong
	#6 – long IHDeIHA	ATCCAGAGTGACGCAGCATAGCGCGGCAACGGGCGCAGGGCAGGATG GGATACGGCTGGTATGTGGACACGGTGGCTTAGT	Very strong
	#7 – long 1KjJqCJD	ATCCAGAGTGACGCAGCATCGCAGCAGAGAAGGAGTGGGTACGTGGT GATGGTGGATGGGGTATGGACACGGTGGCTTAGT	Strong
	#8 – long GiAGIIGI	ATCCAGAGTGACGCAGCACAAATCGACCACGCAATCCCTTTAATGTGA ATATACAAGCATAGCTGGACACGGTGGCTTAGT	Strong

The goal of SELEX is to obtain more than one sequence to be a viable aptamer for a particular target [204]. Therefore, while it would have been less time-consuming to focus solely on a working solution containing one of the aptamer candidates, all four long sequences were continued to be tested. Working solutions containing the longer DNA sequences were then directly compared to each other using split natural fingerprints (total = 288 fingerprints collected), which were aged for one and three weeks. The same three donors deposited two depleting fingerprints on four non-porous substrates ranging in opacity and reflectiveness (same two garbage bags, ziplock bag, aluminium foil).

Selectivity and Sensitivity of Novel Reagent

Chemical spot tests were conducted on PVDF to identify possible fingerprint residue components to which the “fingerprint” aptamer candidates were binding. Various eccrine and sebaceous components (Table 4-2) were dissolved with water and DCM, respectively, to concentrations of 0.1 M or 0.05 M, 0.01 M, and 0.001 M by serial dilution. These concentrations were chosen to demonstrate reactivity rather than sensitivity at this stage. The chemical spots, as well as water and DCM controls and blank substrate control, were allowed to dry overnight before processing, air drying, and imaging with both the VSC6000 and Poliview. This was performed in duplicate, once with used working solutions and once with fresh working solutions.

*Table 4-2 Fingerprint constituents used for chemical spot tests. (*0.5 M instead of 0.1 M [36])*

Eccrine Components		Sebaceous Components
L-serine	L-valine	Stearic acid
Glycine	L-leucine	Oleic acid
L-ornithine	L-isoleucine	Squalene
L-alanine	L-lysine	Palmitic acid*
L-threonine	L-phenylalanine	Cholesterol*
L-histidine		

The donor population was also increased to investigate the sensitivity of the aptamer candidates. Sixteen donors (six male, ten female) were instructed to deposit natural

single deposition fingerprints on three non-porous substrates: Canadian garbage bag, grocery bag (some preliminary tests seemed to indicate that it was a good substrate to use as seen in Appendix VII), and aluminium foil. The whole fingerprints (total = 384 fingerprints collected) were aged for three days or two weeks prior to processing and imaging with the Poliview system.

Issues of reverse development and background staining were investigated by comparing four different working and rinsing solution combinations as listed in Table 4-3. These methods covered working solutions with and without a surfactant present and rinse solutions with and without a surfactant, as well as the absence of a rinse treatment altogether.

Table 4-3 Various working solution and rinsing solution combinations compared.

Method	Working Solution	Rinse Solution
1	PBS with milk + water	Water
2	PBS with milk + water + surfactant	Water
3	PBS with milk + water	Tris + surfactant
4	Tris	N/A

Method 1 had a similar formulation to that used in Chapter 2; 30 μL of 100 μM aptamer was incubated with 10 μL Atto 550 dye. Once purified by filter column, it was added to a 10 mL 1:1 solution of PBS with non-fat milk and milliQ water. MilliQ water was also used as the rinse solution. Method 2 had the same working solution as Method 1, but with 0.5% Tween® 20 incorporated, and water was once again used as the rinse solution. Method 3 had the same working solution as Method 1; however, the rinse solution was different. Rather than milliQ water, the TTBS rinse solution reported by Reinholz [78] was prepared: 0.1 M tris base, 0.05 M NaCl, 0.5% Tween® 20, pH 7.5. The processed samples were rinsed in the TTBS solution three times, five minutes each. Lastly, the working solution for Method 4 was adapted from Wood [101]: 67 μL of 10 μM aptamer candidate was incubated with 22 μL Atto 550 dye. Once purified by filter column, it was added to 10 mL tris solution (25 mM tris,

150 mM NaCl, 2 mM KCl, pH 8). No rinse solution was applied to the processed samples.

A total of 72 natural single deposition fingerprints were collected from three fingerprint donors (one male, two female) on three substrates that exhibited a lot of background staining and/or reverse development (Australian garbage bag, grocery bag, ziplock bag) and aluminium foil which did not. The two female donors were chosen due to them having high frequencies of reverse development and also development localised at pore sites during the larger donor population study. The three donors were instructed to deposit two sets of fingerprints: one without rubbing their hands together and one with rubbing to homogenise whatever residues and contaminants were present on their fingers prior to deposition. All fingerprints were aged for three days before being split in half and processed in order to directly compare the relative performance of Method 1 to the other three (Methods 2 to 4). Only Sequence #6 (long IHDeIHA) was used in this experiment.

Another experiment attempted to minimise reverse development was performed by varying the aptamer concentration of the working solutions for Methods 1 and 4. Two fingerprint donors (one male – good, one female – poor) deposited two depleting natural fingerprints on three substrates (aluminium foil, Australian garbage bag, grocery bag) and single deposition on PVDF. All fingerprints (total = 56 collected) were aged for three days prior to processing with a working solution containing Sequence #6 (long IHDeIHA). The aptamer candidate concentration in Method 1 working solution (100 μ M) was compared to 10 μ M (that of Wood [101]) and 50 μ M. The aptamer concentration in Method 4 working solution (10 μ M) was compared to 50 μ M and 100 μ M (that of Lam et al. [100]). The working solutions with the best results for Methods 1 and 4 were then supposed to be compared to each other.

4.4.2.2 Comparisons to Routine Fingerprint Enhancement Reagents

The relative performance of the working solution formulation containing 50 μ M “fingerprint” aptamer candidate (67 μ L incubated with 22 μ L dye and purified by filter column) in tris buffer (10 mL) was compared to that of the common routine technique

sequence for non-porous substrates, CA → R6G. Three donors (two male, one female) each deposited two depleting natural fingerprints onto four non-porous substrates (Australian and Canadian garbage bags, grocery bag, and aluminium foil). The fingerprints (total = 96 collected) were left to age for a week before being split in half. The fingerprint halves were photographed after CA treatment with the VSC6000 (white light), and the Poliview was used to image the fingerprint halves treated with the “fingerprint” aptamer candidate-containing working solutions and after R6G (530 nm with 610 nm filter, tilted at 30° and untilted, respectively).

4.4.2.3 Comparisons to Single- and Multi-Target Biomolecular Recognition Approaches

Further investigation went into comparing the relative performance of a reagent containing the longer “fingerprint” (pooled target) aptamer candidates compared to those of fingerprint detection reagents containing single-target aptamers, single-target antibodies, and multiple single-target antibodies. A summary of the variables can be found in Table 4-4.

Table 4-4 Summary of variables used to compare working solutions containing “fingerprint” aptamer candidates to previous UTS biomolecular recognition methods.

Variable	Single-Target Aptamers	Single-Target Antibodies	Multi-Target Antibodies
Donors	2 males, 1 female	2 males, 1 female	2 females, 1 male
Fingerprints	Natural Single deposition Split marks	Natural Single deposition Split marks	Natural Set of 2 depletions Split marks
Ages	2 days (for lysozyme only), 1 week	1 week	1 week
Substrates	Two garbage bags, plastic grocery bag, aluminium foil	Two garbage bags, plastic grocery bag, aluminium foil	Two garbage bags, plastic grocery bag, aluminium foil
Targets	Cathepsin, IgE, lysozyme	L-amino acid, cathepsin D, serotonin	For M4: L-amino acid, cathepsin D, serotonin, keratin For “M8”: L-amino acid, cathepsin D, serotonin, cortisol, carnosine, IgE, IgG

The same fingerprint donors were used for the comparisons to single-target aptamers and antibodies; while only two of those donors were available at the time to deposit for the multi-target antibody samples. They all deposited natural fingerprints on four non-porous substrates, which were the same across the three experiments. Split fingerprints were used to directly compare the working solutions containing a “fingerprint” aptamer candidate (formulation as described in Subsection 4.4.2.2) to those containing the other biomolecular recognition probes. All of the targets were

chosen due to previous work performed at UTS and the availability of the antibodies or aptamers during the experimental period.

Single-Target Aptamer-Based Reagents

One immunoglobulin (IgE) and two proteins (cathepsin D and lysozyme) were chosen as the aptamer targets. IgE is one of five major immunoglobulin classes. While it has not been specifically characterised in latent fingerprint secretions, medical research suggests that perspired IgE is involved in skin contact allergic reactions [205]. Cathepsin D was selected since it is one of the most abundant proteins involved in the desquamation phase of skin regeneration process [71]. While lysozyme is not a forensically relevant target for fingerprint detection, it was still selected due to previous experiments [101], resulting in a strong response with fresh fingerprints; these could be used as an aptamer-based reagent performance benchmark. Preliminary tests were conducted to ensure that the lysozyme aptamers used by Wood [101] and stored in the fridge were still effective (Appendix X). As development was comparable, only one lysozyme aptamer (from [200]) was chosen to be incorporated in a working solution for the direct comparisons to working solutions containing the “fingerprint” aptamer candidates. One set of fingerprints were left to age for two days for comparisons against the lysozyme aptamer only, due to its fast degradation rate [101] (number of fingerprints collected = 48), while additional sets of fingerprints for all three aptamers were aged for one week (number of fingerprints collected = 144). The working solutions containing either the cathepsin or IgE aptamer were prepared following Method 1 (see page 161). The working solution containing the lysozyme aptamer was prepared as outlined in Appendix X.

Single-Target Antibody-Based Reagents

As mentioned, the three selected antibodies – anti-L-amino acid, anti-cathepsin D, and anti-serotonin – were previously used by UTS [206]. L-amino acid was selected as a target due to the abundance of amino acids in eccrine secretions. It was also selected since fingerprint detection with this particular antibody had already been published [70]. Serotonin was selected as a target since it is less water-soluble than amino acids. A total of 144 natural fingerprints were collected. The single-target antibody working solutions were prepared following Method 1 (see page 161). These antibodies were not conjugated to AuNPs as was the case in Chapter 2. Based on

previously unpublished work by Spindler, development was comparable between the reformulated aqueous working solutions containing antibody AuNPs and those just containing free antibodies [207].

Multi-Target Antibody-Based Reagents

As the “fingerprint” aptamer candidates were hypothesised to bind to various components in pooled fingerprint residues, it was better to compare it to a multiplex solution – multiple single-target antibodies and/or aptamers – as investigated in Chapter 2. Two different multiplex solutions were prepared, one containing four antibodies and the other containing seven antibodies (Table 4-4). The working solution containing seven antibodies was prepared as if it contained eight due to unforeseen circumstances with the eighth antibody stock. As with the previous comparisons with single-target antibodies, these antibodies were also not conjugated to AuNPs. The fingerprints (total = 192 collected) were aged for one week before being split in half for processing with their respective working solutions.

4.5 Results and Discussion

4.5.1 Proof-of-Concept

Selection of Aptamer Candidates

All eight working solutions were able to develop both natural and sebaceous fingerprints with varying success on the different substrates. For non-porous substrates, 93% of fingerprints were detected, while only 30% of fingerprints were detected on porous substrates. This was not unexpected as previous research performed by Spindler and colleagues [97, 98, 101] indicated that porous substrates were problematic for these biomolecular recognition fingerprint enhancement reagents. First and second level details were observed on PVDF and bag plastics, with quality and clarity of ridge detail better on the ideal experimental substrate. This showed the potential of conducting future SELEX experiments without a major concern about the resulting aptamer candidates being donor-specific – this would help with the development of a “universal” reagent – as only one fingerprint donor participated in both the fingerprint-SELEX process and this proof-of-concept study.

A total of 77 fingerprints (32% of fingerprints collected) were not detected at all under luminescence conditions, which can be attributed to the problematic porous substrates and the poor fingerprint donor. This donor was the sole constant between the fingerprint-SELEX process and the research presented in this chapter. One might expect this donor’s fingerprints to develop due to this fact. However, during fingerprint collection for the fingerprint-SELEX experiment, this participant was always the last or second last to deposit fingerprints. This meant that their fingerprint residues were on the outermost layers on the substrates and would be exposed to the selection buffer first, increasing their chances of being washed or removed rather than bound and eluted.

Sebaceous fingerprints were developed more frequently than natural fingerprints. The manner in which the fingerprints were collected during the SELEX experiment (stacked natural fingerprints) could be viewed as similar to the collection of sebaceous fingerprints; there was the possibility of excess sebaceous material compared to a single natural fingerprint. Sebaceous material may have also been less affected during the fingerprint-SELEX process (i.e., less likely to be washed away from the substrate), increasing the chances that the DNA sequences were targeting sebaceous over eccrine constituents. With this type of fingerprints, the difference between the enhancement of working solutions containing the shorter sequences and corresponding longer sequences was more evident (Figure 4-5).

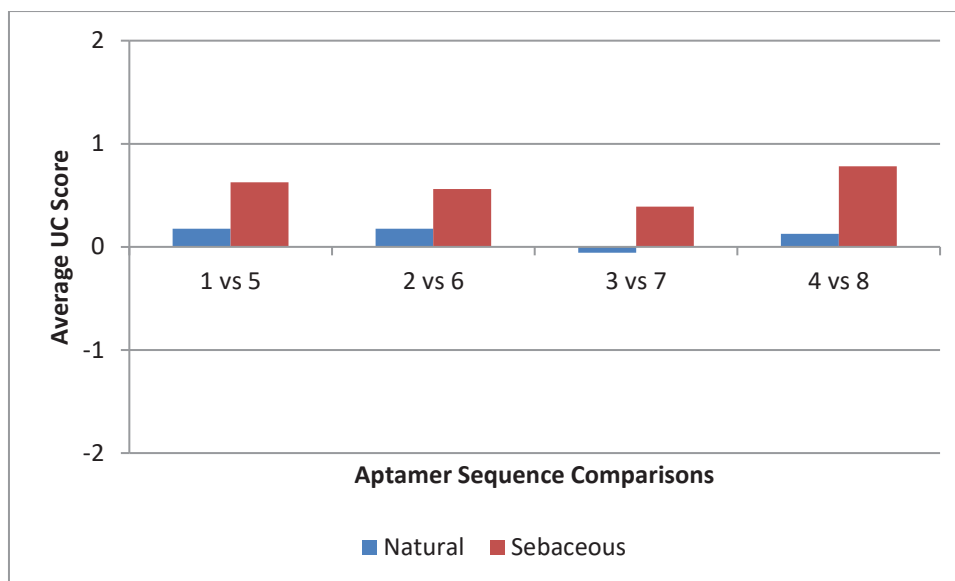


Figure 4-5 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the comparison between working solutions containing the corresponding short (i.e., 1, 2, 3, 4) and long (i.e., 5, 6, 7, 8, respectively) aptamer candidate sequences. Positive values correspond to an increase in enhancement with the working solution containing the longer aptamer candidate sequence when compared to that with its corresponding shorter aptamer candidate sequence.

For fingerprints on PVDF, it was clear that the working solutions containing the longer sequences (those with the primer regions included) provided stronger development and luminescence than working solutions with their corresponding shorter sequences (random regions only) (Figure 4-6). There was good contrast between the white PVDF and pink ridge detail visible to the naked eye. Under luminescence conditions, there was no background interference. Therefore, this reagent has potential to be a dual reagent such as DFO and IND-Zn on light-coloured substrates. Of the 48 fingerprints that were deposited on PVDF, only six – all from the poor fingerprint donor – were not observed under white light, but were under luminescence conditions. Although the differences were not significant, stronger luminescence indicated that shorter exposure times could be utilised, which may be important in an operational setting.

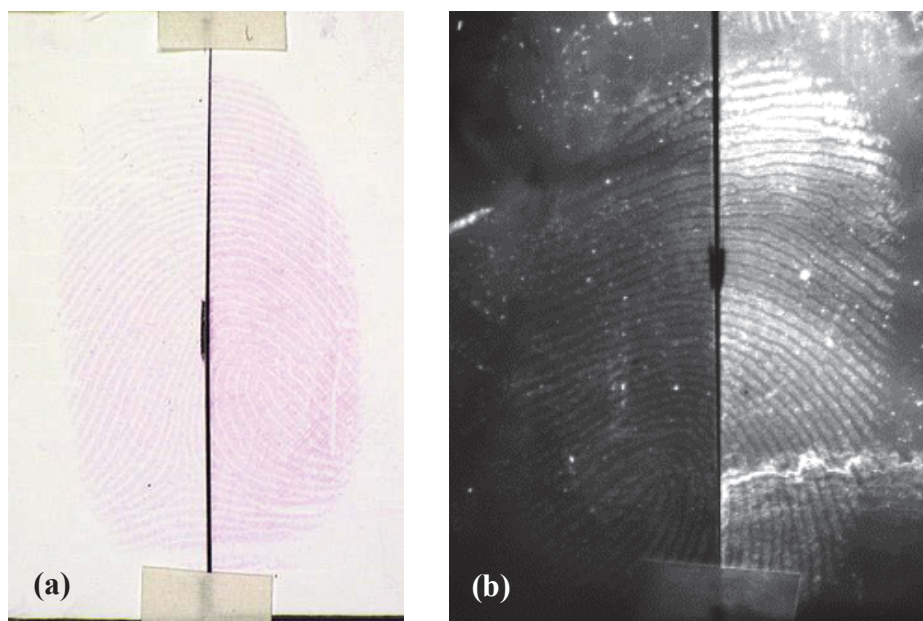


Figure 4-6 Development obtained on PVDF with working solutions containing corresponding short (left) and long (right) aptamer candidate sequences under: (a) white light and (b) luminescence conditions (530 nm with 590 nm bandpass filter).

The garbage bags resulted in a few fingerprints that were not detected; fingerprint locations being observed, but no ridge detail present; spotty development; or nice clear ridge detail present (Figure 4-7). Over three quarters of the fingerprints on the Australian garbage bag showed evidence of reverse development; there was also a lot of background staining. At first this was unexpected, as the goal was to have the aptamer candidates specifically bind to fingerprint residues, not the substrate. On the other hand, it was also not surprisingly, as the issue of background noise or interference is a major problem for any fingerprint detection method. A few possible explanations could be that the negative selection step (incubation of the substrate without fingerprints) in the SELEX process was not as effective as hoped; the aptamer candidates could be interacting with components of the substrate (Subsection 3.6.3); there could be an excess of fingerprint residues, such that they actually repel the working solution onto the substrate, which is then stained; or the substrates could have some sort of coating, plasticisers, or other additives during manufacturing which interferes with the working solutions performing as anticipated. Using substrates that would typically be encountered in casework during this experimental phase helps to identify other research questions that need further exploring. Since there was more

substrate than fingermark residues present, even a small interaction between the novel reagent and background could overpower or quench the desired signal.

As mentioned, the working solutions performed the worst on the porous substrates (87% of fingermarks not detected). Under luminescence conditions, there was a lot of background interference in the form of overall luminescence, but also fibres visible in the Australian copy paper (Figure 4-7). Additional tests were performed with much shorter time intervals and a different working solution formulation, which resulted in visible ridge detail (Appendix XI).

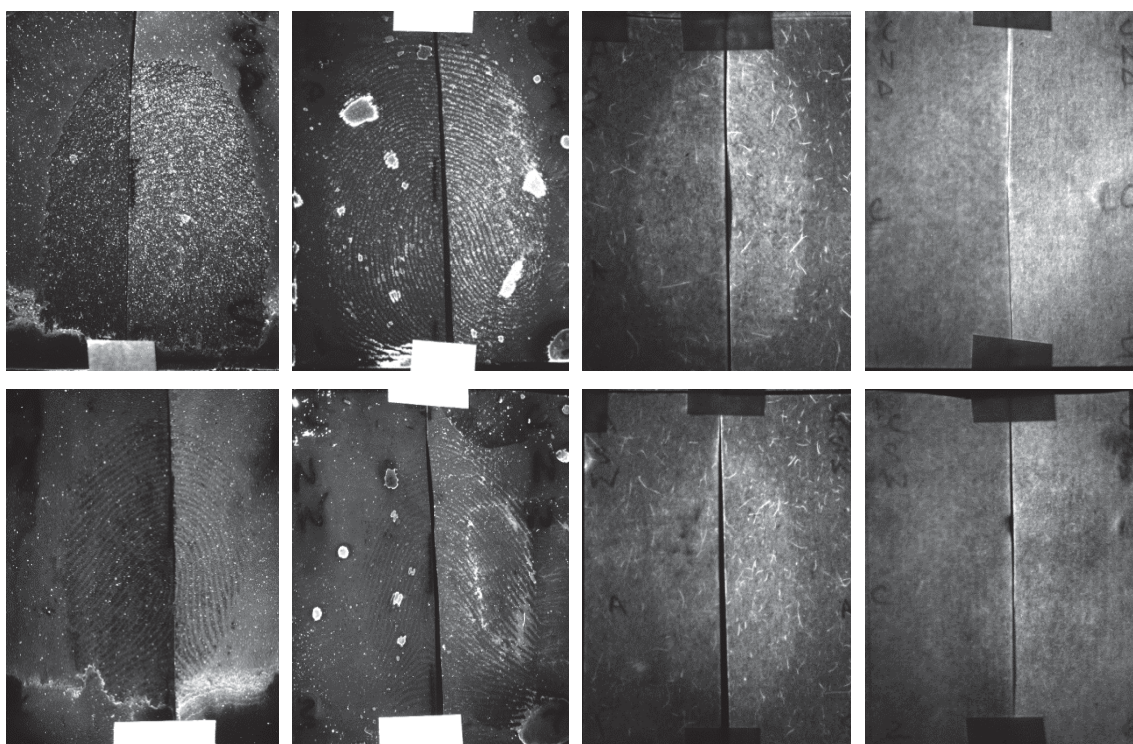


Figure 4-7 Three-day-old (top row) and 12-day-old (bottom row) fingermarks developed and observed under luminescence conditions (530 nm with 590 nm bandpass filter) on (from left to right) Australian garbage bag, Canadian garbage bag, Australian copy paper, and Canadian copy paper.

When working solutions containing the longer DNA sequences were directly compared to each other, no significant difference was observed (Figure 4-8). All fingermarks from the good donor were detected, while 20% and 58% of the average and poor donors' fingermarks, respectively, were not detected at all. The detection

rates for the good and average donor increased, while that for the poor donor decreased compared to fingerprints previously collected from the same donors and processed with the short and long sequences. The overall decrease in the amount of undetected fingerprints could be due to the choice of substrates; no porous substrates were used. Relatively ideal results can be obtained from the aluminium foil. There was excellent contrast between the luminescent ridge detail and this substrate. Background staining was again observed with the Australian garbage bag and with the ziplock bag, but not to the same extent. Reverse development was also prominent on these two substrates (Figure 4-9). Again, possible explanations could be non-specific binding, excess sebaceous secretions that were repelling the solution, and the surface manufacturing composition, which may be the key element to consider.

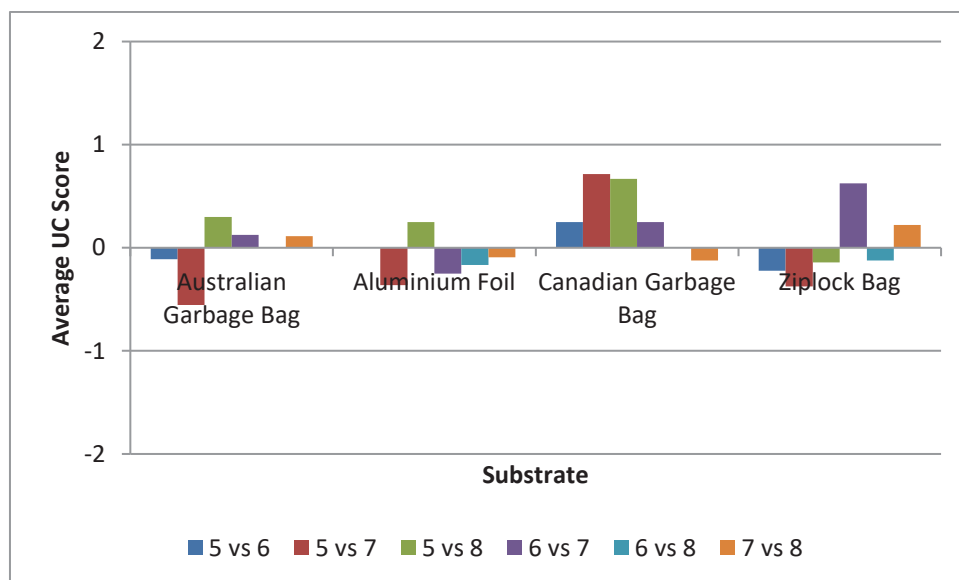


Figure 4-8 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of pairs of longer aptamer candidate sequences. Positive values correspond to an increase in enhancement with the aptamer candidate sequence listed second in its pairing when compared to the aptamer candidate sequence listed first.

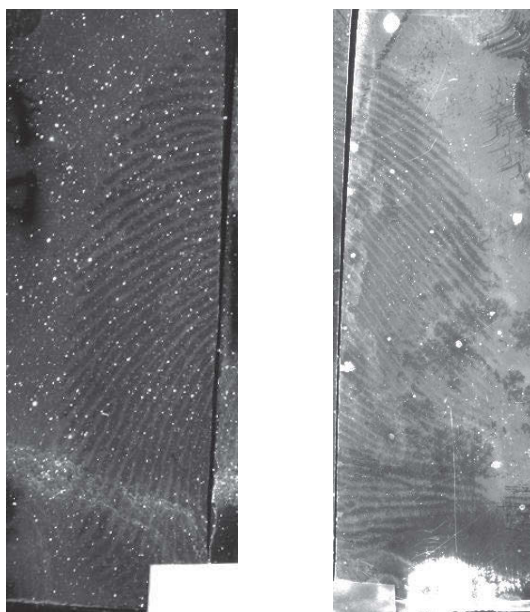


Figure 4-9 Examples of reverse development on Australian garbage bag (left) and ziplock bag (right) observed under luminescence conditions (530 nm with 590 nm bandpass filter).

While the DNA sequences were chosen for different reasons – target-specific, substrate-specific, “universal” – the resulting development appeared comparable. There are a few possible explanations for this. Firstly, the partitioning step in SELEX is not 100% effective. Therefore, some sequences that should be removed are not. For example, one of the “universal” aptamer candidates could be a target- or substrate-specific sequence rather than a truly “universal” one due to imperfect partitioning. Secondly, as the “fingerprint” aptamer candidates are not targeting one specific fingerprint residue constituent, it is possible that there is a cumulative abundance of targets to which they will bind. They are not restricted by the presence of a particular target. In the case of the possible squalene-specific “fingerprint” aptamer candidate, it is possible that the decrease observed in the sequencing data did not correspond to the decrease of squalene in the GC-MS data, meaning that the selected aptamer candidate could bind to a different target. Squalene has an unfavourable structure for aptamers, so this is likely. Thirdly, as this was a proof-of-concept experiment, no optimisation was performed. The formulation and protocol for previously performed work with aptamers for fingerprint detection at UTS may not be the best or appropriate for “fingerprint” aptamer candidates. For example, the dye amount may need to be adjusted or the processing time needs to be decreased to avoid overdevelopment.

Selectivity and Sensitivity of Novel Reagent

Each of the working solutions showed comparable results for the developed chemical spot tests. Under white light, visible pink spots were observed with no diffusion. It appeared that the aptamer sequences had a stronger affinity to the sebaceous components than to the eccrine components (Figure 4-10). Initially, there were concerns about contamination with the amino acid spots, as they appeared quite small and had similar results to the water control, which was the main reason to repeat the experiment. The colour of the developed spots was more intense with the fresh working solution. This was expected as the available aptamer candidates in the working solution would have depleted after being used to successfully develop other samples. The amino acid spots were not able to diffuse enough into the PVDF to have a larger spot circumference; many of the spots had dried and some crystallisation was visible to the naked eye prior to processing. Generally, the lowest concentration of the amino acid spots (0.001 M) was visualised. Alternatively, the lowest concentration of the sebaceous components was the weakest in terms of visibility. The strongest luminescence came from oleic acid, followed by squalene. Oleic acid was suspected to be present in Round 4 of fingerprint-SELEX, but could not be positively identified through GC-MS. Squalene was thought to be an unusual target for aptamer candidate Sequence 1KjJqCJD – due to its unfavourable chemical structure – as this compound’s presence in the GC-MS analysis decrease corresponded to a decrease in count during the sequencing analysis.

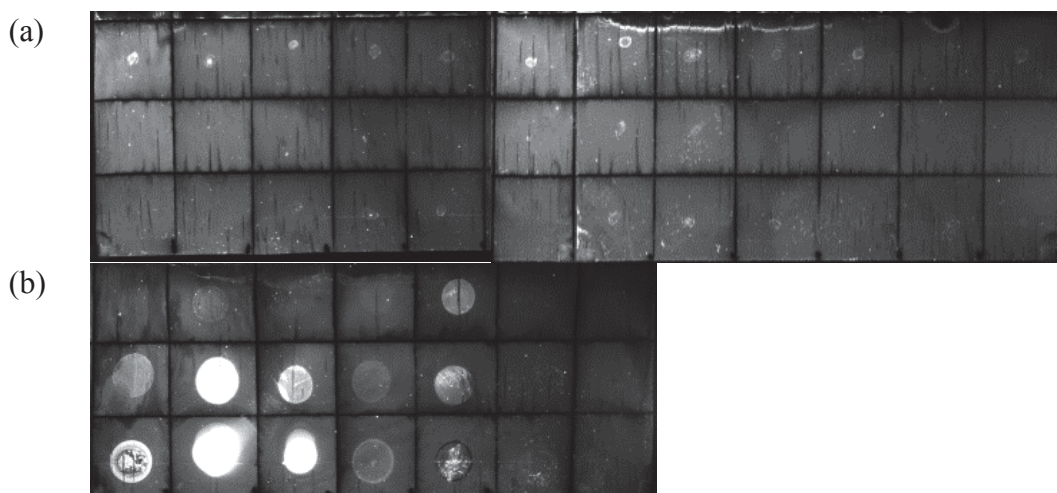


Figure 4-10 Chemical spot test results for fresh working solution (Sequence 5) by Method 1 (top to bottom): 0.001 M, 0.01 M, and 0.1 M for (left to right): (a) L-serine, glycine, L-ornithine, L-alanine, L-threonine, L-histidine, L-valine, L-leucine, L-isoleucine, L-lysine, and L-phenylalanine with water control; and (b) stearic acid, oleic acid, squalene, palmitic acid*, and cholesterol* with dichloromethane and blank substrate controls. *Denotes chemical solutions prepared to 0.05 M instead of 0.1 M.

While being able to develop aptamer candidates for a variety of targets, their binding affinities may have suffered due to the ease of undergoing an induced fit to interact with multiple targets [141]. It may be possible to develop aptamer candidates for a class of fingerprint residue constituents such as amino acids rather than a variety of structurally different targets. When there are multiple targets closely related to each other, they will continue to be effective competitors during the selection process, even at high affinities [141]. It has already been shown that RNA aptamers with high affinity to similar amino acids – L-citrulline and L-arginine, the former not common in fingerprint residues – can be obtained from different, but related sequences [204].

In Chapter 2, one of the issues with the multiplex solution was that it might have been too specific, i.e., antibodies and aptamers only binding to their cognate targets. In a pooled target approach, specificity may not be as an important factor as initially thought. Although we wish to avoid non-specific binding to the substrate, we ought to ask the question: as long as the aptamer-based reagent is developing ridge detail, does it matter what fingerprint constituent(s) it is targeting at this point? In the infancy of

method development, this aptamer-based reagent may be interacting with a single or multiple constituents, be they sebaceous or eccrine.

Of the 384 fingerprints processed with the increased donor population, 97% were detected. Those that were undetected were on grocery bag (eight fingerprints) and garbage bag (two fingerprints). The undetected fingerprints were from three known poor (four fingerprints), one average (four fingerprints), and two good (two fingerprints) fingerprint donors. All four “fingerprint” aptamer candidate-containing working solutions performed relatively similarly per substrate (Figure 4-11), however, the resulting development varied (Figure 4-12). Of the three substrates used, aluminium foil had the most fingerprints with CAST scores of 3s and 4s across all sequences. One known poor fingerprint donor had relatively good development due to their hands being contaminated with duck fat. This was done even before eating their lunch and illustrates how we cannot always control what the fingerprint donors will have touched prior to deposition. Reverse development was observed 76% of the time on grocery bag and 24% on garbage bag. Grocery bag typically had spotty positive development or good reverse development, with some background staining. Garbage bag probably had the most varied range of development. There was reverse development with some luminescent spots filling in the dark ridges. The spots tended to be relatively uniformly spaced, indicating the pore locations. Other spotty development appeared more like static along the ridges even though continuous ridges could be seen with the naked eye, at a wavelength of 530 nm without a filter or at 530 nm with an inappropriate filter (e.g., 555 nm). Excess sebaceous material was present along some of the friction ridges, therefore resulting in some of the furrows appearing more luminescent than the ridges at quick glance.

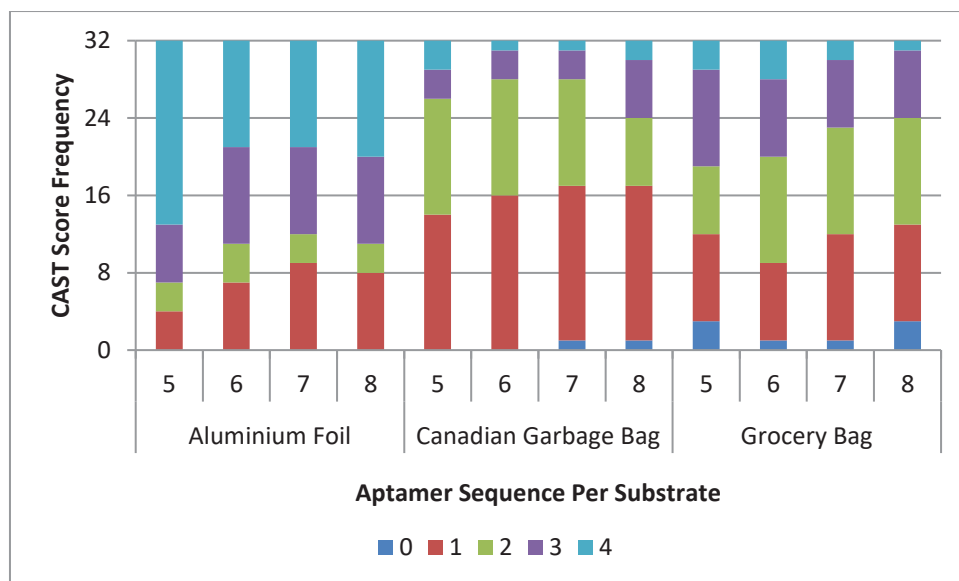


Figure 4-11 Distribution of CAST scores for larger donor population organised by aptamer candidate sequences per substrate.

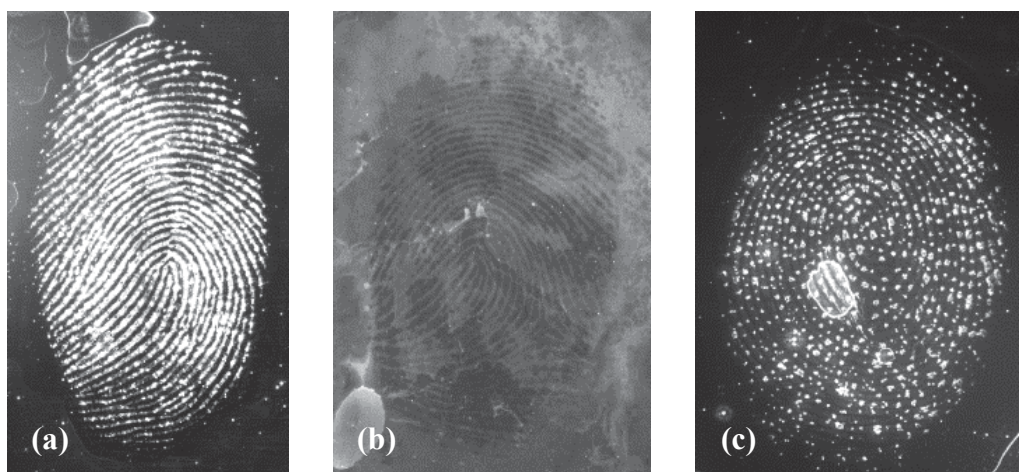


Figure 4-12 Natural fingerprints from the larger donor population study illustrating various types of development: (a) 2-week-old fingerprint aluminium foil with continuous ridge detail; (b) 3-day-old fingerprint on plastic grocery bag with reverse development; and (c) 2-week-old fingerprint on aluminium foil with spotty development indicating pore locations. All fingerprints were visualised at 530 nm with 590 nm bandpass filter.

All fingerprints were detected with the four different working-rinsing solution combinations. Reverse development was still observed on the Australian garbage bag, grocery bag, and ziplock bag with Method 1. Two instances of reverse development were observed on ziplock bag, one each for Methods 2 and 3. The combination of an

absence of surfactant in the working solution and the presence of organic moieties of the realistic substrates, which most likely are competing for binding, is believed to contribute to reverse development occurrences. Despite this, better ridge detail was observed with Method 1. The Method 2 working solution contained Tween® 20, a non-ionic surfactant that is routinely used in ELISA to minimise non-specific binding of antibodies [208] and used in fingerprint detection reagents to decrease background staining [209]. While Method 2 did minimise or eliminate the background staining, development of ridge detail was not as visible as those with Method 1 when viewed under the same luminescence conditions (Figure 4-13(a)). Peterson et al. suggest that they can control surface binding preferences of aptamers using surfactants; hydrophilic analytes would be favoured while the hydrophobic ones would be enclosed in micelles [210]. Their selected aptamers – each capable of binding to multiple small molecules (e.g., steroids) – were able to retain their function in the presence of up to 4% surfactant; only slight response attenuation was observed with Tween® 20 [210]. The addition of a surfactant into an antibody-based reagent would have an unfavourable effect, as the antibodies would denature due to the interactions between the hydrophobic portions of the antibodies and surfactant [210]. The rinse solution of Method 3 appeared to have been detrimental and washed away the ridge detail (Figure 4-13(b)). Method 4 was significantly better than Method 1 on aluminium foil in terms of luminescent enhancement (Figure 4-13(c)). That being said, aluminium foil was the only substrate to have significant background staining for Method 4, when the opposite for Method 1 was true. The background staining on Method 4 was not distracting or interfering with visualising ridge detail. Methods 1 and 4 had comparable enhancement on the three other substrates (Figure 4-14).

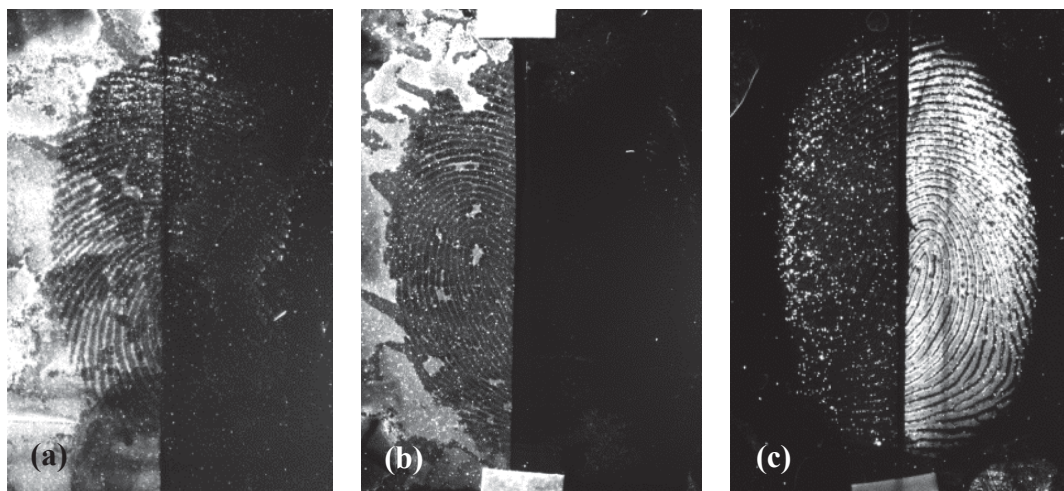


Figure 4-13 Three-day-old natural fingermarks developed with Method 1 (left halves) and: (a) Method 2 on Australian garbage bag; (b) Method 3 on Australian garbage bag; and (c) Method 4 on aluminium foil. All fingermarks were visualised at 530 nm with 590 nm bandpass filter.

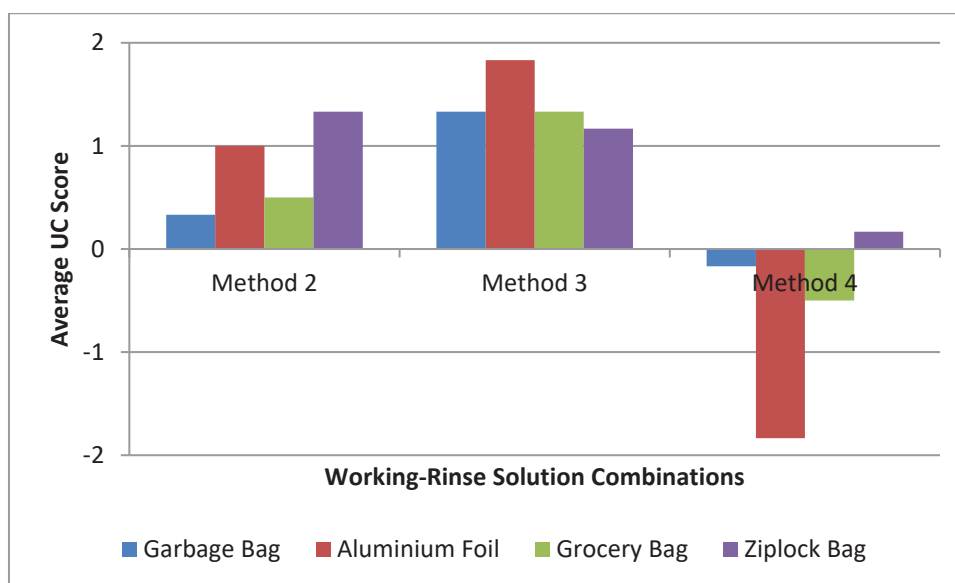


Figure 4-14 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of Method 1 to three other working-rinse solution combinations. Positive values correspond to an increase in enhancement with Method 1 when compared to the others.

Based on some of the spotty development results obtained with the larger donor population, additional instructions were provided to three donors during fingermark

collection. First, the donors were asked to deposit fingerprints without rubbing their hands together. Then, for the next set of fingerprints they deposited, they were asked to rub their hands together prior to deposition. This was an attempt to see whether the observed development differed when purposely trying to homogenise the fingerprint residues to get continuous ridges as opposed to spotty development. The expected outcome was not observed across all substrate-working solution formulation combinations. Fingerprints were anticipated to have spotty or uneven luminescence without rubbing, but the ridge detail would appear continuous and more evenly luminescent with fingerprints where the donor rubbed their hands together before deposition. To make these observations, two separately developed fingerprints were compared; no direct split comparisons could be made. Therefore, the fingerprint residue composition could be different so true comparisons could not be made. Since the fingerprints were photographed at separate times, the exposure times may also not be the same for true comparisons. At lower exposure times, development could look spotty. However, at higher exposure times, luminescence could be pushed to its limits to make it appear as if there are continuous ridges.

Surprisingly, there was no significant difference in enhancement when the “fingerprint” aptamer candidate concentrations were varied between 10 μM and 100 μM . A possible explanation could be due to an excess of dye still being present after purification (Appendix VIII). A total of 98% of deposited fingerprints were detected; only one fingerprint deposited by the poor donor was undetected on the Australian garbage bag. Positive development tended to be clear continuous ridge detail. Reverse development and/or background staining was only observed with the two bag substrates, 75% of the time in one or both corresponding fingerprint halves processed with the different Method 1 formulations (43% of all fingerprints processed with this method). As there did not appear to be a difference in aptamer concentration, the best Methods 1 and 4 formulations were not compared to each other directly. Also, with reverse development still being observed with Method 1 formulation, it was decided to continue using Method 4 with an aptamer concentration of 50 μM .

4.5.2 Comparisons to Routine Fingermark Enhancement Reagents

The routine sequence was able to detect all its fingermark halves, with the best development visualised on the grocery bag (i.e., all CAST scores of 3s and 4s; see Figure 4-15). The majority of CA → R6G-treated fingermark halves had continuous ridges with good clarity of minutiae. The novel reagent was able to detect all fingermark halves on aluminium foil, but not on the three different plastic bag substrates (eight undetected in total). The fingermark halves treated with the “fingermark” aptamer candidate-containing working solution once again had variable development. There was excellent continuous ridge detail that was highly luminescent visible on aluminium foil, the substrate on which the novel reagent obtained more CAST scores of 4 than the routine sequence. If continuous ridge detail was visible on the other substrates, it was faint or difficult to see as there were luminescent spots along the ridge edges.

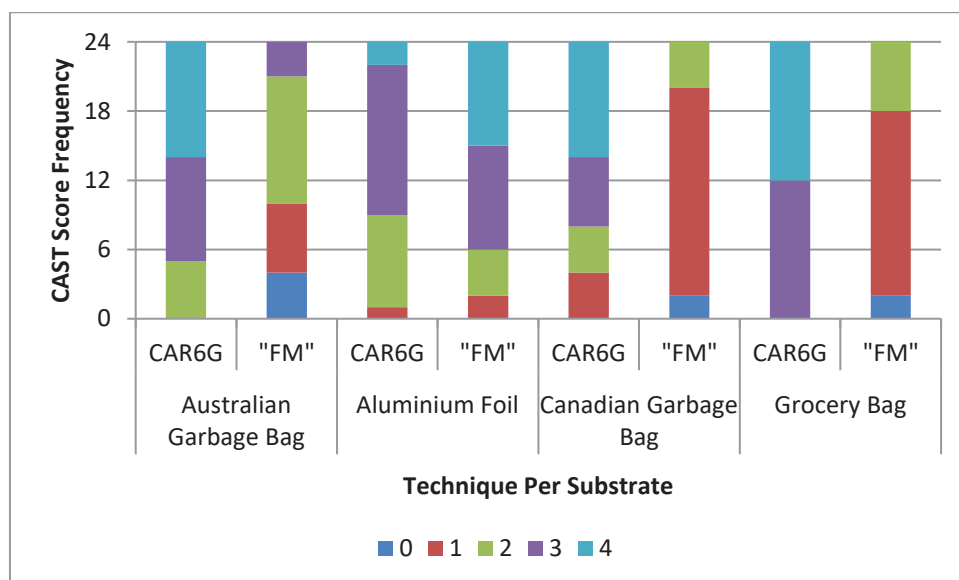


Figure 4-15 Comparison of CAST score frequency distribution between CA → R6G and working solutions containing “fingermark” aptamer candidates (“FM”) per substrate.

The routine sequence of CA → R6G had significantly better enhancement when compared to the “fingermark” aptamer candidate-containing working solutions for three out of four substrates tested (Figure 4-16). The only substrate where the two

techniques showed comparable results was aluminium foil. Both the novel reagent containing Atto 550 and the routine dyestain, R6G, are viewed at the same wavelength. When viewing conditions were optimised with the adjustment of exposure time for each fingerprint half, luminescence was comparable. That being said, the exposure times required for the fingerprint halves treated with the novel reagent were drastically longer than those for the routinely treated fingerprint halves (e.g., 40 s compared to 800 ms, respectively at an aperture setting of f/5.6). While a forensic identification practitioner would do whatever it took to get a good image of a fingerprint, these long exposure times are not practical. With such long exposure times, it is unlikely that the fingerprints treated with the novel reagent would be visualised during a preliminary search or in high volume and high throughput examinations. If time were not an issue, and if a large surface area could be examined in a single instance, then the long exposure times with the Poliview imaging system may be disregarded.

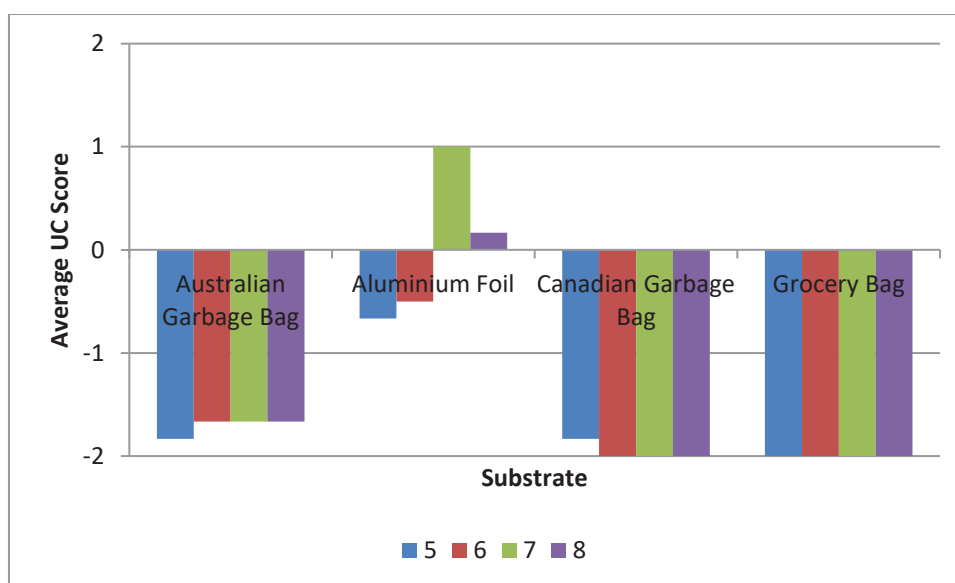


Figure 4-16 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of working solutions containing “fingerprint” aptamer candidates to CA → R6G. Negative values correspond to a decrease in enhancement with the working solutions when compared to the routine sequence.

4.5.3 Comparisons to Single- and Multi-Target Biomolecular Recognition Approaches

4.5.3.1 Comparisons to Single-Target Aptamer-Based Reagents

Aluminium foil was the only substrate on which one-week-old fingermark halves were assigned CAST scores of 4 (Figure 4-17). All eleven of said fingermark halves were developed with one of the “fingermark” aptamer candidate-containing working solutions. Not only were first and second level details observed, but so were third level details on this substrate (Figure 4-18). The second best substrate for these reagents to develop ridge detail on seemed to be the Australian garbage bag. Interestingly enough, this substrate was the worst for one-week-old ridge detail recovery for the three single-target aptamers, cathepsin, IgE, and lysozyme. The cathepsin and IgE aptamers seemed to have the same trends, where they performed their best on the grocery bag, then on aluminium foil, and then the Canadian garbage bag, followed by the Australian garbage bag. The lysozyme aptamer-based reagent performed the worse with one-week-old fingermarks, as its target has a fast degradation rate and is not ideal for universal fingermark detection [101].

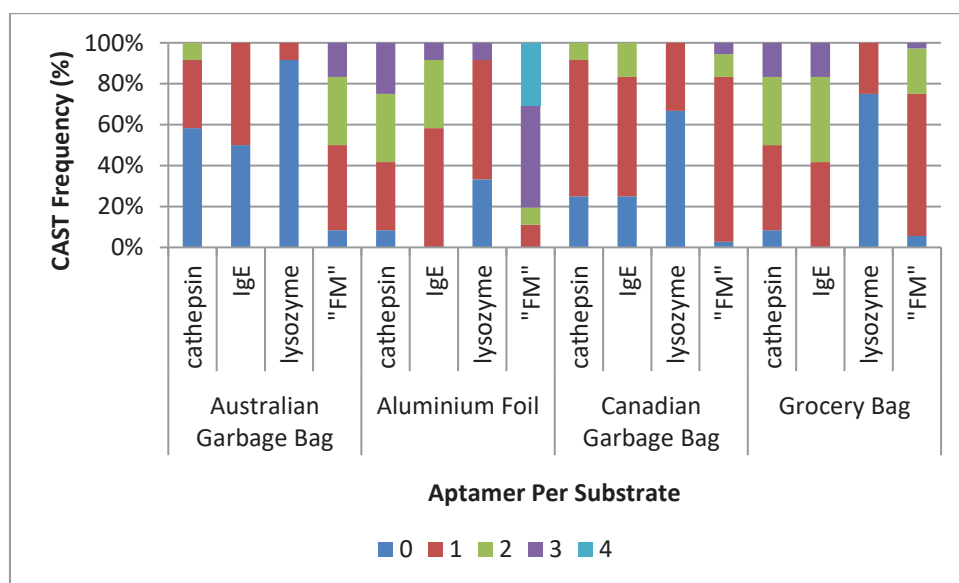


Figure 4-17 Distribution of CAST score frequency as percentages for each working solution per substrate, due to additional samples processed with “fingermark” aptamer candidate-containing reagent (“FM”) and lysozyme aptamer-based reagent.



Figure 4-18 One-week-old natural fingerprint half deposited by a male donor on aluminium foil, developed with the working solution containing Sequence #5, and visualised at 530 nm with a 590 nm bandpass filter.

Both the novel reagent and lysozyme aptamer-based reagent performed better on the two-day-old samples. The increase in performance of the lysozyme aptamer-based reagent with fresher fingerprints was expected, as its target would be present at a higher concentration than in the one-week-old fingerprints. CAST scores of 4 were assigned to fingerprint halves developed with a “fingerprint” aptamer candidate-containing working solution on aluminium foil and the Australian garbage bag, while only the former for the lysozyme aptamer. The results for the lysozyme aptamer were also not as good as expected when seeing the results in Wood et al. [195], however, those results were on an ideal substrate (i.e., PVDF). Worse results would be expected when trying to develop fingerprints on more realistic substrates as performed herein this study. While no stability tests were performed, it would be interesting to see if the lysozyme aptamer-based reagent would have performed better if the aptamer was freshly synthesised as opposed to stored in the fridge for over three years. There is the

possibility that the aptamers began to degrade over time if microbes or DNAses were somehow introduced into the tubes.

While the major downfall of the working solutions containing the “fingerprint” aptamer candidates was the luminescence intensity when compared to the routine technique sequence, it played to their favour when compared to the single-target aptamers. When the fingerprint halves were each imaged at their optimal viewing conditions, those developed with the novel reagent only provided slightly better enhancement than the three single-target aptamers (Figure 4-19). However, when the exposure time was the same, the enhancement and luminescence from the fingerprint halves treated with the novel reagent were significantly better Figure 4-20. This could be because of the pooled target approach as opposed to only a single known fingerprint residue constituent being targeted at once.

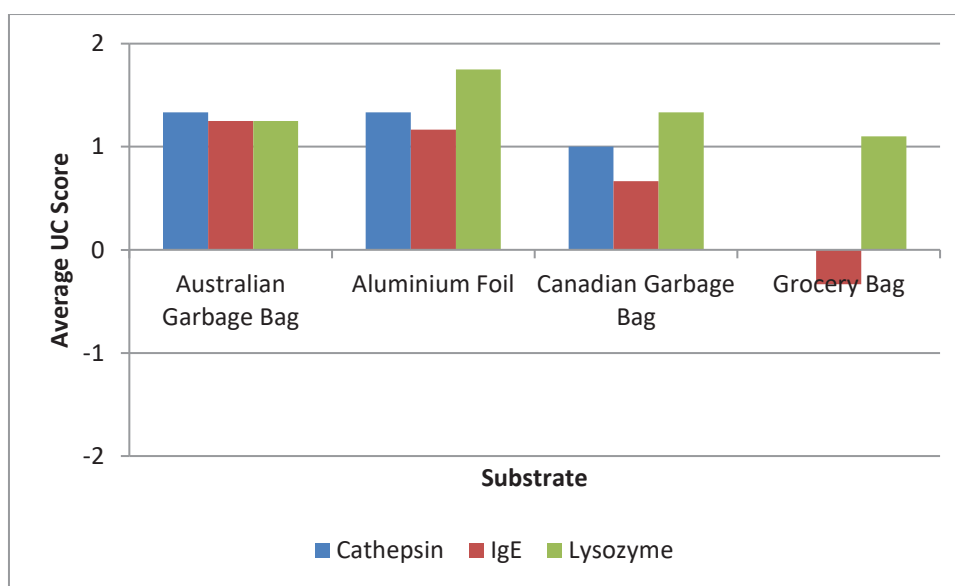


Figure 4-19 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of working solutions containing “fingerprint” aptamer candidates to those containing aptamers against cathepsin, IgE, or lysozyme. Positive values correspond to an increase in enhancement of the novel reagent when compared to the single-target aptamers.

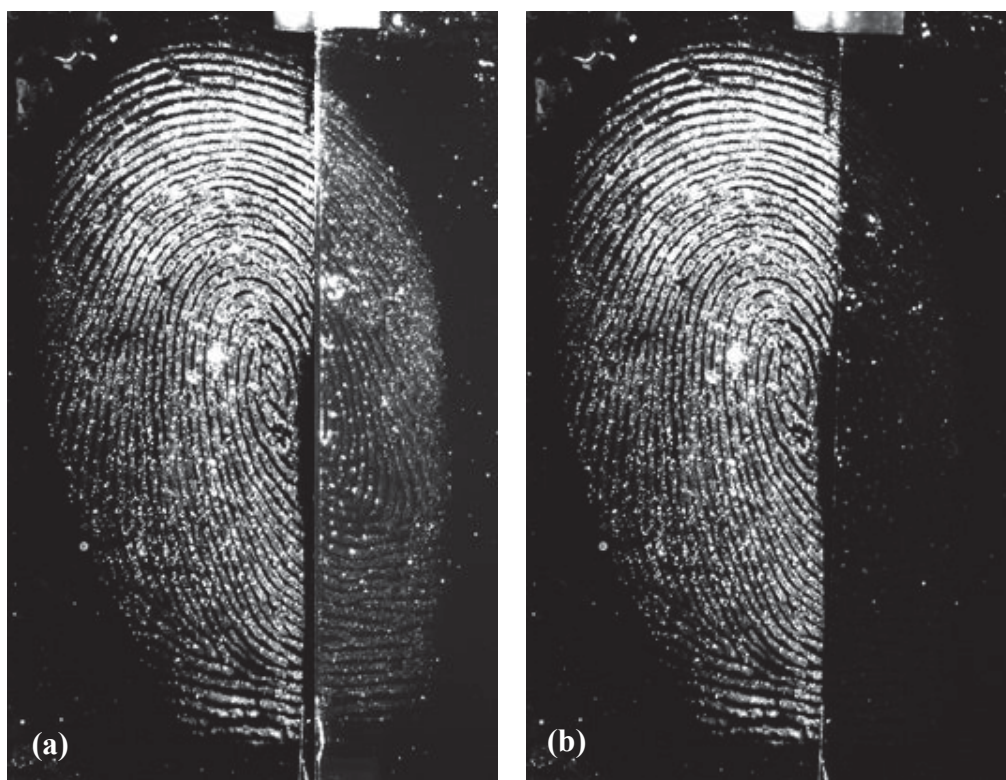


Figure 4-20 One-week-old fingerprint on aluminium foil with both halves imaged: (a) under optimal viewing conditions (e.g., exposure time adjusted) for both halves; and (b) at the same exposure time. (left half = working solution with “fingerprint” aptamer candidate; right half = working solution with single-target aptamer)

For the single-target aptamer PBS working solutions, background staining was observed frequently on the Australian garbage bag and grocery bag substrates. Some background staining was present while conducting the experiments in Chapter 2, but not as strongly. A possible explanation is that a different garbage bag was used. The manufacturing process and composition of the garbage bags (e.g., coating, additives) could affect the interaction between the substrate and the reagent. In particular, the different organic moieties present in the substrates could vary the degree of interference and/or competition for binding with the aptamer candidates. Prior to operational use, more detailed research needs to be conducted to propose explanations as to when the reagent will work and why.

Aptamers are more likely to be prepared in tris than in PBS buffer. Therefore, the effectiveness of the single-target aptamer reagents could possibly be improved if the

working solution formulation was modified again. It is interesting to note that despite the possibility of excess dye present in the working solution, background staining was not observed with the tris buffer formulation. As a primary amine buffer, tris would compete for NHS ester crosslinking reactions. Tris buffers may still be used in working solutions, but the NHS ester crosslinking reactions should be performed in phosphate, bicarbonate, or borate buffers [211].

4.5.3.2 Comparisons to Single-Target Antibody-Based Reagents

In general, the anti-cathepsin antibody detected more ridge detail than the cathepsin trimer aptamer. This may not be a true indication as to which biomolecular probe is superior, as the antibody and aptamer were not directly compared to another; they developed fingerprint halves deposited at different times. The novel reagent was also able to detect more ridge detail on the samples for this subsection than in the previous subsection (4.5.3.1). As the “fingerprint” aptamer candidate-containing reagent used in both experiments was prepared in the same manner, this demonstrates the impact of fingerprint composition on the effectiveness of a technique. Out of the single-target antibodies, anti-serotonin performed the worse, with only two fingerprint halves being assigned a CAST score of 4 and the reagent with the most no development (i.e., CAST score 0) (Figure 4-21).

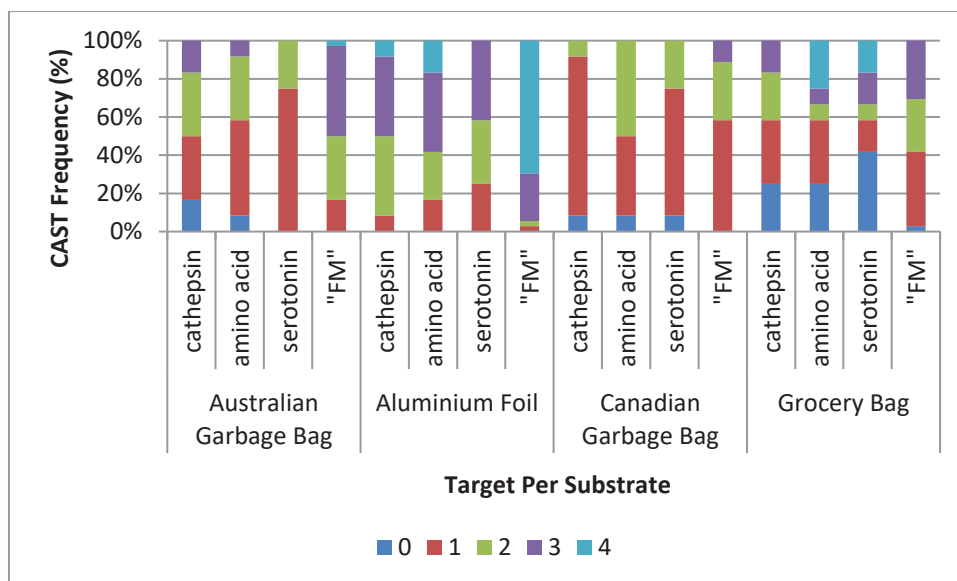


Figure 4-21 Distribution of CAST score frequency as percentages for each working solution per substrate since “fingermark” aptamer candidate-containing reagent (“FM”) processed all corresponding halves to the three single-target antibodies.

The anti-L-amino acid antibody was able to develop ridge detail, even without the AuNPs included in Chapter 2 or in [70]. There was positive development on aluminium foil – a substrate also used by Spindler et al. [70]. The fingermarks depicted in that article were ranging from two-week- to one-year-old. They were more luminescent than the fingermark halves visualised in this experiment, but the present work seemed to have more continuous ridge flow. This could be due to a higher amino acid concentration found in the fingermark residues. It could also be because the working formulation was further optimised since that publication and no AuNPs were included here; Spindler et al. had also used a secondary tagging method [70]. The age of the anti-L-amino acid antibodies could have also been a likely factor; their binding affinity could have been decreased due to denaturation. It is possible that the use of AuNPs in the reagent could have assisted with preferential development. It is thought that negatively charged AuNPs are attracted to the positively charged molecules in the fingermark residues [212]. Without the AuNPs, the free antibodies should still have been able to preferentially bind to their cognate targets.

The substrate on which the most development was observed was aluminium foil, while the Canadian garbage bag had the least amount of development. There was also nice clarity of ridge detail on the grocery bag substrate; however, it was reverse development. The Australian garbage bag exhibited reverse development and background staining.

Not only did the working solutions containing the “fingerprint” aptamer candidates perform better than the single-target aptamers, but also better when compared to single-target antibodies (Figure 4-22). It is however recognised that, as mentioned in Chapter 2, it may not be a fair assessment comparing the anti-L-amino acid antibody on non-porous substrates to another technique that has been determined to target sebaceous components of latent fingerprints, as eccrine secretions are not normally targets on non-porous substrates. This research still demonstrates that a major eccrine secretion component can still be detected well on non-porous substrates. No reverse development was observed with the TBS-based working solution, but it was observed with the PBS working solution (Figure 4-23). When corresponding fingerprint halves both had normal luminescent ridge detail development, and visualised under optimal conditions, the performances seemed comparable or only slightly different. However, when viewed at the same exposure time, the fingerprint halves treated with a working solution containing a “fingerprint” aptamer candidate were significantly more luminescent. While this could be an indication that the “fingerprint” aptamer candidates bind better than the antibodies do to their respective targets, another possible explanation could be that the antibodies were no longer stable and near expiry, if not already. These antibodies were from the same stock as used in Chapter 2, for at least 3 years, stored in the freezer. This is an advantage for aptamers which can denature and re-nature, i.e., they have a longer stability as long as they are in a DNase-free environment. If the antibodies were completely ineffective, we would not expect to see CAST scores of 3 and 4 consistently assigned to their treated fingerprint halves.

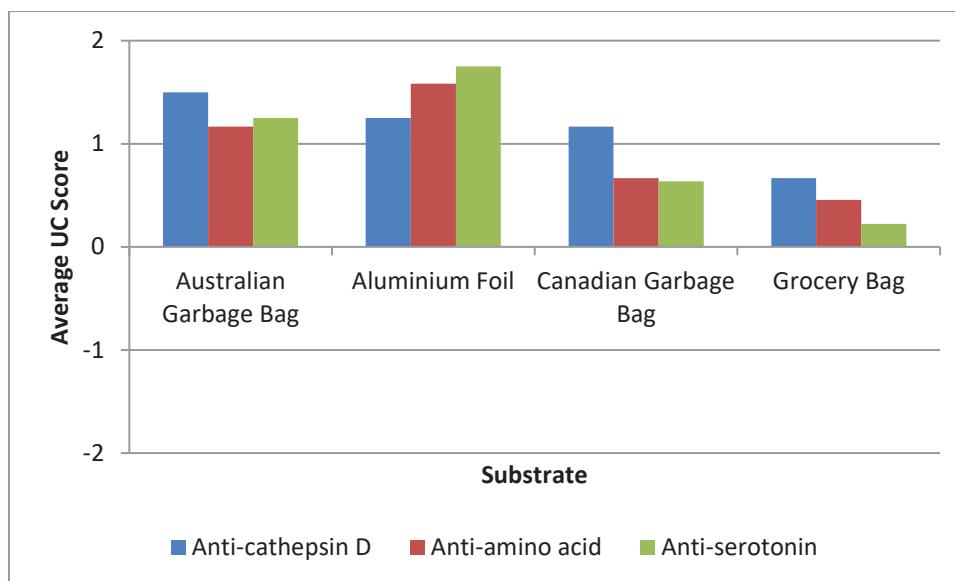


Figure 4-22 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of working solutions containing “fingermark” aptamer candidates to those containing anti- cathepsin D, anti-L-amino acid, or anti-serotonin. Positive values correspond to an increase in enhancement of the novel reagent when compared to the single-target antibodies.

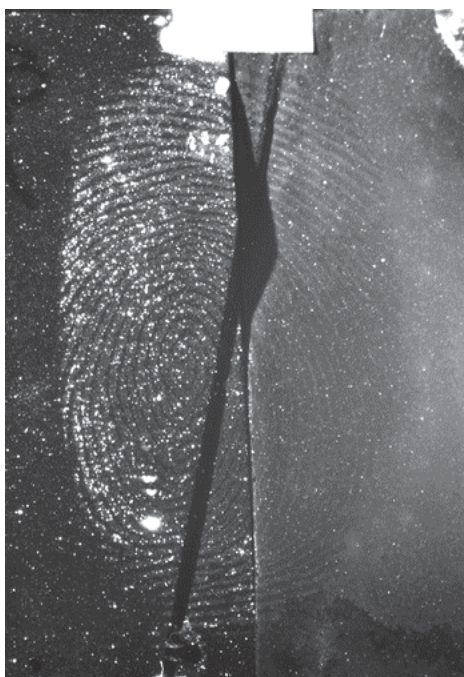


Figure 4-23 Normal development with working solution containing tris buffer (left) compared to reverse development with working solution containing PBS with non-fat milk (right). Fingermark visualised at 530 nm with 590 nm bandpass filter.

4.5.3.3 Comparisons to Multi-Target Antibody-Based Reagents

The multiplex solutions of four and seven antibodies, although developing different fingermarks, were relatively comparable, with the most noticeable difference on aluminium foil; the multiplex of seven antibodies had four times more the number of fingermark halves with CAST scores of 4 than the multiplex of four (Figure 4-24). As the two multiplex solutions were not directly compared, this does not indicate that one multiplex solution was better than the other; there could have been different fingermark residue constituents present in different concentrations. In descending order of amount and quality of detail developed by the multiplex solutions, the substrates were aluminium foil, Canadian garbage bag, grocery bag, and then Australian garbage bag. With the exclusion of aluminium foil, as it is the most ideal realistic substrate tested, the multiplex solutions’ comparable performance in this experiment supports the findings in Chapter 2, where the number of components within the multiplex solution did not play a significant role in the reagent’s performance when tested on realistic substrates.

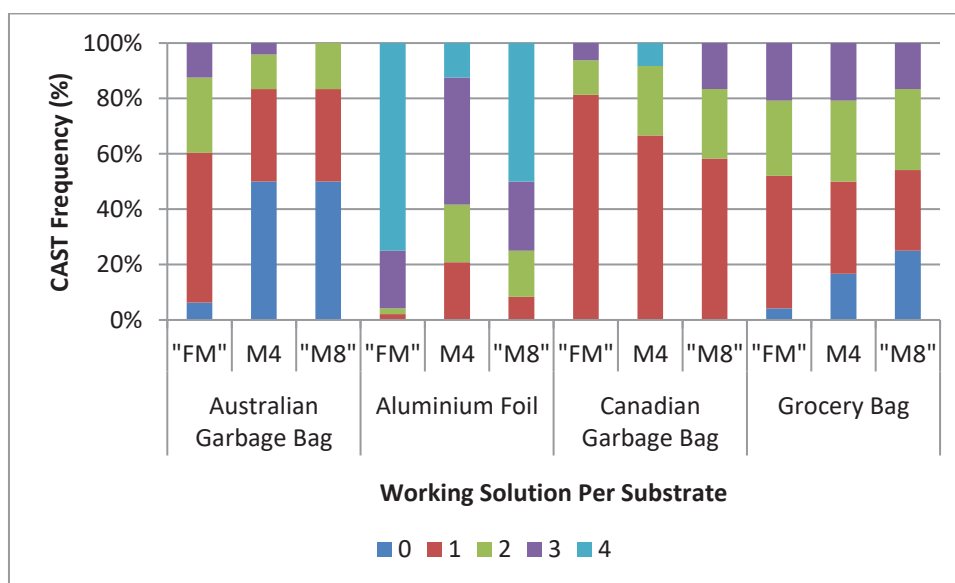


Figure 4-24 Distribution of CAST score frequency as percentages for each working solution per substrate since “fingermark” aptamer candidate-containing reagent(“FM”) processed all corresponding halves to the two multiplex solutions. (M4 and “M8” = multiplex solutions of four and seven antibodies, respectively)

All three reagents performed the best on aluminium foil. While the two multiplex solutions performed relatively well on the Canadian garbage bag, this substrate was the worst for the novel reagent to develop ridge detail. The multiplex solutions tended to result in background staining with reverse development or spotty development, except on aluminium foil which had continuous luminescent ridge details. Some spotty development was observed with the “fingerprint” aptamer candidate-containing reagent as well, but no reverse development was present on these fingerprint halves.

While it was expected that the novel reagent would perform better than the single-target aptamers and antibodies because the former target multiple fingerprint residue components at once, it was uncertain how it would compare to a multiplex solution. Only antibodies were used in the multiplex solution for this experiment, as there were not enough stock aptamers left to include in both multiplex solutions. After corresponding fingerprint halves were evaluated using the UC scale, it was found that they were relatively comparable, or the “fingerprint” aptamer candidate-containing reagent with slightly better enhancement, on three of the four substrates tested when viewed under their respective optimal conditions (Figure 4-25). There were instances where the reagent containing a “fingerprint” aptamer candidate developed the whole half while the multiplex solution barely developed any ridge detail (Figure 4-26(a)). The “fingerprint” aptamer candidate-containing working solutions possessed significantly better enhancement than the multiplex solutions on aluminium foil. While the multiplex solution had some background staining and reverse development, the reverse development was still good quality (Figure 4-26(b)). Unfortunately, this was not expected as the antibodies should have been binding to their respective targets. It is possible that the multiplex solutions could perform better in the future if fresh antibodies were used. Also, the choice in antibody selection could be reviewed, as it is unknown which are best to include in a multiplex solution as mentioned in Chapter 2. That is one advantage for the “fingerprint” aptamer candidates; they were selected to target pooled fingerprint residues from multiple donors to account for some of the inherent inter-donor variability.

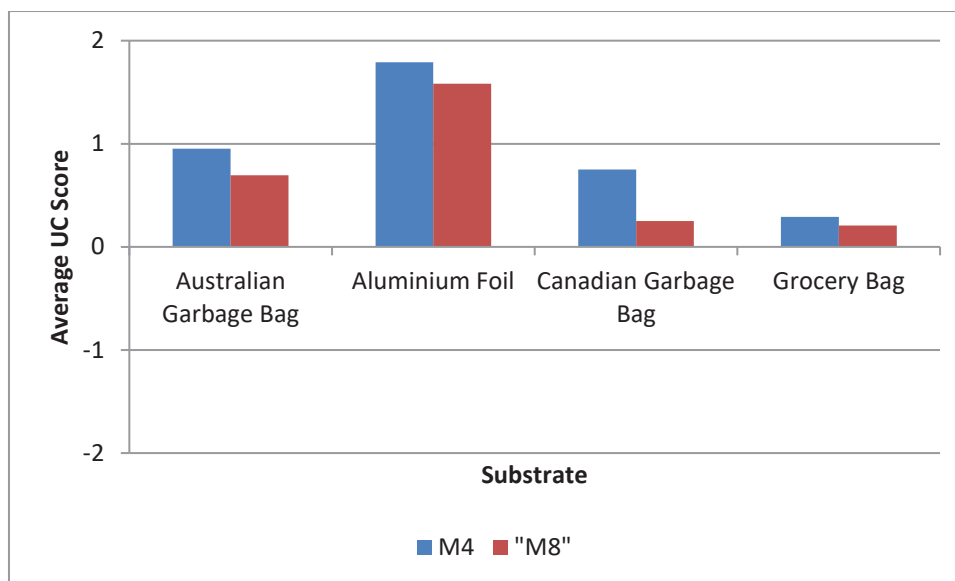


Figure 4-25 Average enhancement scores (for a comparative scale between -2 and +2) resulting from the direct comparison of working solutions containing “fingermark” aptamer candidates to a multiplex solution of four (blue columns) and of seven (red columns) antibodies. Positive values correspond to an increase in enhancement of the novel reagent when compared to the multiplex solutions.

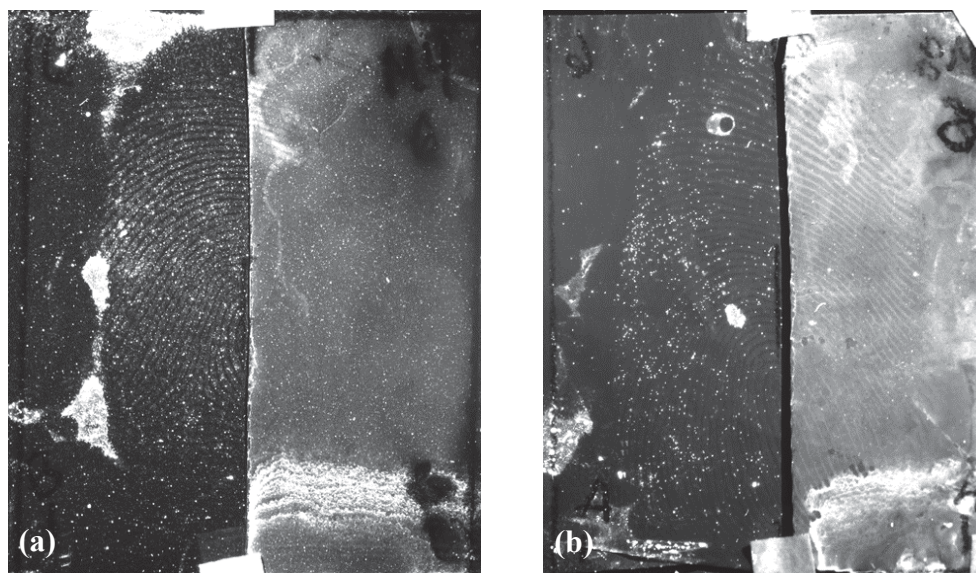


Figure 4-26 One-week-old fingermarks developed on: (a) Australian garbage bag with working solution containing “fingermark” aptamer candidate (left half) and multiplex of four antibodies (right half); and (b) grocery bag with working solution containing “fingermark” aptamer candidate (left half) and multiplex of seven antibodies (right half). Fingermarks visualised at 530 nm with 590 nm bandpass filter.

Although there seems to be a trend that there is a larger difference between the working solutions containing the “fingerprint” aptamer candidates and the multiplex solution of four antibodies compared to that with seven antibodies, this does not necessarily mean that the multiplex of seven is better at developing fingerprints than the multiplex of four. The difference between the two multiplex solutions is marginal, which means their performances are comparable. Both multiplex solutions developed different fingerprints, which could have varying concentrations of fingerprint residue constituents, which in turn could affect the amount of ridge detail development. On the other hand, if this data was taken in isolation, it is not unexpected that there was less difference between the multiplex of seven antibodies and working solutions containing “fingerprint” aptamer candidates compared to the multiplex of four antibodies because if more residue constituents were being targeted, it would be expected that there would be an increase in development.

4.5.4 Advantages and Limitations

In 2016, Bécue wrote that there were many limitations associated with immunodetection, holding it back from operational use. Those limitations included the fragility of antibodies, storage conditions, cost, limited volume of reagents, and long and multiple immersion steps [55]. As previously mentioned in Section 3.2.1, aptamers have greater stability than antibodies and are not temperature-sensitive like antibodies. Antibodies have shelf-lives (which may be one of the reason for their poor performance in this chapter), while aptamers can be stored long-term (e.g., years). Aptamers can also be produced on kilogram scales; therefore, the costs are significantly lower. Their sequence information can easily be shared for manufacturing purposes, and if no modifications (e.g., fluorescent tags) are needed, then their synthesis and delivery are as simple as those for primers [166]. Hundreds of aptamers can be produced with a highly parallel, automated process, which means that each aptamer will function in the same conditions. This drastically decreases the time required compared to antibodies that could take months to discover or produce with the use of animals or cell lines [213].

Many researchers who published immunodetection studies stated that they used a hydrophobic pen to create a barrier in which to contain a few microliters of solution over the fingerprint samples; this is not realistic of actual casework. While still not at the hundreds of millilitres scale desired by Bécue [55], the research presented in this chapter, as well as in Chapter 2, used larger volumes than other studies. Working solutions varying between 5 mL to 40 mL were prepared, the difference in volume depended on the size of the processing tray being used at the time while maintaining a solution height of around 0.75 cm. The “fingerprint” aptamer candidate-containing reagent presented in this chapter is easy to prepare and does not have multiple immersion steps. Preparation of the reagent could even be simplified further with by ordering pre-tagged aptamers as done by Wood [101]. This, however, is dependent on the selection of luminescent probes commercially available that are compatible with current FLSs (Appendix IX). The processing time required for this reagent was not optimised, but as there are already routine methods with processing times up to 45 minutes, this seemed reasonable at this time.

Aptamers are the logical next step in immunodetection. Their range of possible targets is expansive. As with the multiple single-target antibodies in Chapter 2, multiple aptamers could be combined into a single reagent if they were each developed through different SELEX experiments. For example, at a mass fatality incident, a portion of an item may have been burnt while another portion of it happened to be protected from damage. By combining aptamers developed for latent fingerprint residues as well as pyrolytic compounds, more information (i.e., ridge detail) could be recovered and assist in the identification of human remains and their property.

Over 25 years since their discovery, there are very few Food and Drug Administration-approved clinical diagnostic tests using aptamers [214], even though they have been shown to be comparable or superior to antibodies. While it seems that aptamers are a ‘no-brainer’, people do not like change; antibodies have been the gold standard for diagnostic applications for years. Another reason is that within the aptamer community, there is a “thrombin problem” [213]. Researchers continue to focus on the well-known thrombin aptamer [215] itself or designing novel detection methods with it as the target [183], rather than developing assays for more clinically relevant targets. Likewise, there seems to be a “lysozyme problem” within aptamer-based fingerprint

detection research. After the publication by Wood et al. [195], other researchers jumped on the lysozyme aptamer for fingerprint detection yet they ignored one major factor as to why it is not an ideal target (i.e., its fast degradation rate). One of the motivations of the present research was to address this challenge. Fingerprint residue is a complex mixture of eccrine and sebaceous secretions, with an abundance of targets from which to choose. This research demonstrates yet again – after Wood [101] – the potential of aptamers for fingerprint detection.

The biggest issue encountered with the "fingerprint" aptamer candidate-containing reagent presented in this chapter was its poor luminescence, resulting in long exposure times for imaging purposes. It is believed that a different method in which the oligonucleotide and the luminescent probe interact/attach would resolve this issue. While a 1:1 tagging method is not feasible with antibodies due to the presence of multiple lysine side chains, ordering a pre-modified aptamer sequence would achieve this. Caution needs to be taken during post-modification steps, as these could negatively affect aptamer activity [125]. Unreacted fluorophore should be removed and while size exclusion chromatography is commonly used, the fluorophore often interacts with the separation matrix causing poor recovery and separation [216]. If excess fluorophore is present in the working solution, it could be hindering the targets from fitting into the aptamer candidates active binding structures. The presence of additional fluorophores could also result in fluorescence quenching.

The issue of inconsistent ridge detail development could be attributed to the inherent variability of fingerprint residues, but also to the fact that these "fingerprint" aptamer candidates may not be the best candidates for an aptamer-based fingerprint reagent. Recall that only a fraction of the sequencing data was accessible when choosing which aptamer candidate sequences to test. There was also no obvious indicator as to when the reagent was losing its effectiveness, as was seen with the multiplex solution in Chapter 2.

4.5.5 Conclusions

The work presented in this chapter revealed that the resulting “fingerprint” aptamer candidates from Chapter 3 could be incorporated into a working solution that develops latent fingerprints. As a result, the idea of using the *in vitro* selection process to develop more aptamers against targets relevant to fingerprint detection is very promising. Development was still variable between donors; however, with only one fingerprint donor in common between fingerprint-SELEX and this work, the concern of donor specificity may be ignored and the resulting aptamer candidates could potentially be incorporated into a “universal” reagent. Issues of background staining and reverse development were resolved by modifying the formulation of the previously used working solution and eliminating the rinse solution all together.

While still in the early stages of conception, the relative performance of working solutions containing the “fingerprint” aptamer candidates were compared to that of the sequential processing of CA followed by R6G. The routine detection sequence outperformed the novel reagent; however, with additional optimisation, this could be re-evaluated as an alternative method. The relative performance of the “fingerprint” aptamer candidate-containing working solutions was also compared to that of single- and multi-target approaches previously designed by the same research group. The results indicated that a pooled target approach, as used for fingerprint-SELEX and resulting aptamer candidates, may be a better approach. Aptamers, again, were shown to have greater stability than antibodies, which were less effective in their working solutions due to degradation over the years.

***Chapter 5: General Discussion,
Recommendations, and
Conclusions***

Chapter 5 General Discussion, Recommendations, and Conclusions

5.1 General Discussion

There is currently an arsenal of routinely used fingerprint detection methods available to practitioners. Despite this, improvements to existing techniques or the development of novel methods need to be undertaken to increase fingerprint recovery at crime scenes and on exhibits. The overall aim of this research was to further explore the potential use of biomolecular recognition to address some of the existing sensitivity and selectivity issues related to current fingerprint detection methods, in addition to rectifying some of the identified issues within immunodetection research.

While immunodetection may sound like a good idea in theory, it is far from operational use; much work needs to be done in this space. Targeting specific molecules of interest demonstrates high selectivity, which in turn should result in efficient detection of that particular secretion residue; however, it seems counter-intuitive. In casework, we often develop partial fingerprints. Why would one only want to target a particular portion of that partial fingerprint? The end goal should be to recover as much ridge detail and as many fingerprints as possible.

This is why a multi-target approach is better than a single-target approach, but then comes the questions of which and how many fingerprint residue constituents should be targeted. A pooled target approach would be even better, as it would target a mixture of fingerprint residues in their natural concentrations. By also using a variety of fingerprint donors, one can account for inter-donor variability of fingerprint residues. A multi-target or pooled target approach should be designed so that targets that would result in spotty development and those that would result in continuous ridges are both covered.

In agreement with Wood et al. [43], if this immunodetection/biomolecular recognition approach to fingerprint detection was to continue, aptamers should be pursued more; it

was apparent that the stability of the antibodies was an issue. While it may be simpler to use existing single-target antibodies and aptamers or combining them, the benefits associated with aptamers developed for real fingerprint constituents in their relative concentrations would outweigh unfavourable aspects (e.g., time) of the *in vitro* process. As demonstrated in Chapter 4, fingerprint researchers interested in biomolecular recognition do not need to be reliant on published aptamer sequences to design a fingerprint detection method. The possibilities with SELEX seem endless; not only could a fingerprint detection reagent be designed for routine use, but niche applications as well, such as challenging substrates and scenes. Future research may be able to achieve such vision.

5.2 Recommendations

The majority, if not all, recently published immunodetection methods involve an antibody- or aptamer-containing working solution. From Chapter 2, it was shown that the application of working solutions had detrimental effects, especially on semi-porous substrates. It also prevented effective CA development when placed in sequence prior to the routine method. Therefore, the application mode of these biomolecular reagents should be re-investigated. It may be possible that contact sheets that have been pre-treated with an aptamer-based reagent may be used in a similar “sandwich” fashion as amino acid-sensitive reagents and dimethylaminocinnamaldehyde for latent fingerprint detection on thermal paper via a dry transfer process [8, 217]. Some work into NIN- or antibody-impregnated membranes also show promise [218, 219]. Chen et al. demonstrated a microfluidic platform capable of specific interactions with targeted cells on an aptamer-coated flat substrate through the deposition of avidin and immobilisation of biotin-aptamer [220]. An “information” gathering method to simultaneously detect friction ridge detail and an explosive substance also modified a surface on which the reaction would occur [221]. Making a liquid-free reagent or reactive membrane should reduce the amount of background staining observed with porous substrates. Also, as a liquid-free reagent, this method could be used earlier in the general technique sequencing and may have less detrimental effects on subsequent techniques.

This was the first time SELEX was utilised to develop aptamers specifically against fingerprint residues (see Chapter 3). No optimisation was performed due to the limited time of the overseas research placement. If possible, it would be beneficial to perform another SELEX experiment targeting fingerprint residues. Using the same aged fingerprints for several rounds before using another set of aged fingerprints would allow for better interpretation of the stringency effects. By controlling the fingerprint age parameter more in the *in vitro* selection process, and analysing the sequencing data for resulting DNA pools, aptamers may be discovered for particular age ranges. This may be an area to further investigate, as there are researchers interested in developing methods to determine the age of a fingerprint left at a crime scene or on an exhibit, rather than increasing the fingerprint recovery rate with a more “universal” reagent as presented here. Different aptamers identified for different age ranges could be tagged with different luminescent probes in a single fingerprint detection reagent. If performing another fingerprint-SELEX experiment is not feasible, reviewing the inaccessible sequencing data may still lead to the identification of other potential, more strongly binding, “fingerprint” aptamer candidates to test in a fingerprint detection reagent. Once optimisation of the novel aptamer-based reagent has been achieved, not only could direct comparisons to conventional detection methods be performed, but technique sequencing as well, similar to what was conducted in Chapter 2.

Similar to the IFRG guidelines [96], Cho et al. suggested some standards for both the aptamer community and journal editors to consider when deciding whether a research paper should be accepted for publication [183]. Most have been covered in this research: the sequences used were reported; the full sequences and not just the random regions were tested; atypical analytes of interest were targeted; and the same buffer conditions during fingerprint-SELEX were also used to reconstitute the synthesised “fingerprint” aptamer candidates upon delivery. While chemical spot tests may be sufficient to determine specificity for fingerprint detection research, different controls are required for aptamer development research. Therefore, it is advisable to also perform experiments with two negative controls: one with a non-cognate sequence (i.e., scrambled version of aptamer sequence) [222] and one with a non-cognate protein, preferably a related protein, but not BSA [183]. Proper characterisation and functional verification need to be conducted prior to stating that a new aptamer has

been discovered [179, 223]. During this phase, binding affinity can be calculated through multiple tests. Truncation experiments to determine the shortest possible sequence required for binding activity could be performed [224]. It will be likely that the small molecule targets will result in lower affinity aptamers compared to larger molecules [225].

Finally, but most importantly, aptamer-based fingerprint detection should only be pursued if expertise in aptamer development, discovery, and applications is available to fingerprint researchers, and vice versa. During the course of this PhD project, it was very apparent that an open exchange of knowledge was needed to successfully conduct this interdisciplinary research. As Cho et al. stated in 2009, without the basic knowledge of aptamer biochemistry or technical requirements, serious application difficulties will be encountered [183].

5.3 Conclusions

As a forensic science community, we need to keep abreast of new technologies and how we could effectively implement these methodologies into real-world applications. While SELEX and aptamers have been around for over 25 years, it has only been in the past five years or so that these biomolecular recognition probes have been used for proof-of-concept fingerprint detection studies. This PhD project is the first known instance of SELEX being used for the forensic application of detecting fingerprints. This is also the first known instance where novel aptamer candidates have been incorporated into a fingerprint reagent rather than using sequences found in literature.

It may take many years before an aptamer-based fingerprint enhancement reagent is fully validated for operational use. This is based on the fact that it took around 20 years for aptamers to come of age and will probably take just as many before aptamer-based systems can convince researchers, clinicians, and industry of their clear advantages over traditional macromolecules (e.g., antibodies) [166]. That being said, with such a versatile tool at our disposal, fingerprint researchers – and forensic scientists in general – should start to appreciate the full potential of SELEX and resulting aptamers for the development of new reagents with improved specificity.

Appendices

Appendix I Routine Fingerprint Enhancement Method Formulations and Applications

Routine Techniques	Formulations		Applications
Acid yellow 7 (AY7)	20 g 5-sulfosalicylic acid 700 mL deionised water 250 mL ethanol 50 mL acetic acid 1 g acid yellow 7		<ul style="list-style-type: none"> - Working solution was applied to substrate and left for 3 to 5 minutes. - Substrate was rinsed well with deionised water and allowed to air dry.
Amido black (AB)	<u>Stain Solution:</u> 2 g amido black 900 mL methanol 100 mL acetic acid	<u>Wash Solution A:</u> 900 mL methanol 100 mL acetic acid <u>Wash Solution B:</u> 50 mL acetic acid 950 mL deionised water	<ul style="list-style-type: none"> - Stain solution was applied to the substrate and left for 30 seconds to 1 minute. - Substrate was rinsed in wash solution A. - Substrate was rinsed in wash solution B. - Solution was rinsed in deionised water and allowed to air dry.

Basic yellow 40 (BY40)	2 g basic yellow 40 1 L methanol	- Sample was immersed in stain solution for 10 to 20 seconds. - Sample was rinsed thoroughly with deionised water and allowed to air dry.
Cyanoacrylate (CA)	20 drops	- Sample was placed in CA fuming chamber with following conditions: 120 °C, 80%RH, 20-minute fuming cycles
1,2-Indanedione-zinc chloride (IND-Zn)	<u>IND Stock Solution:</u> 2.3 g 1,2-indanedione 480 mL ethyl acetate 20 mL acetic acid <u>Zinc Chloride Stock Solution:</u> 8 g zinc chloride 200 mL absolute ethanol	<u>IND-Zn Working Solution:</u> 130 mL IND stock solution 870 mL HFE7100 4 mL zinc chloride stock solution - Sample was immersed in the working solution long enough to soak through. - Sample was allowed to air dry. - Sample was sandwiched between clean sheets of absorbent paper and heated for 10 seconds in the dry heat press.
Rhodamine 6G (R6G)	<u>Stock Solution:</u> 0.2 g rhodamine 6G 200 mL isopropanol 300 mL methyl ethyl ketone	<u>Working Solution:</u> 250 mL stock solution 750 mL deionised water - Sample was immersed in stain solution for 20 seconds. - Sample was rinsed thoroughly with deionised water and allowed to air dry.

Physical developer (PD)	<p><u>Maleic Acid Solution:</u></p> <p>25 g maleic acid 1 L deionised water</p> <p><u>Detergent-Surfactant Stock Solution:</u></p> <p>1.5 g n-dodecylamine acetate 1.5 g Tween® 20 500 mL deionised water</p> <p><u>Silver Nitrate Solution:</u></p> <p>10 g silver nitrate 50 mL deionised water</p>	<p><u>PD Working Solution:</u></p> <p>30 g ferric nitrate 900 mL deionised water 80 g ammonium ferrous sulphate 20 g citric acid 40 mL detergent-surfactant stock solution 50 mL silver nitrate solution</p>	<ul style="list-style-type: none"> - Sample immersed into tray of deionised water for a minimum of 20 minutes. - Sample immersed into tray of maleic acid solution for at least 15 minutes, until effervescence had ceased and substrate developed a uniform opacity. - Sample was placed into tray of deionised water for 10 minutes, with constant gentle agitation. - Sample was immersed into tray of PD working solution, with constant gentle agitation, for 5 to 40 minutes until adequate development was observed. - Sample was placed into tray of deionised water for 10 minutes, with constant gentle agitation. This step was repeated two more times and then the samples were allowed to air dry.
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Appendix II Fingerprint-SELEX Donor Information

in brackets = order for fingerprint deposition on paper substrate
 - = information not provided

Donor	Sex	Age	Origin	Cosmetics	Diet	Coffee	Gum	Smoke	Diseases
1	F	-	African	-	-	-	-	No	-
2 (3)	F	27	Caucasian	Make-up	No restrictions	2	No	No	No
3	F	29	Caucasian	None	No restrictions	0	No	No	No
4	M	21	Caucasian	None	No restrictions	0	No	No	No
5 (7)	M	23	Caucasian	None	No restrictions	2	No	No	No
6 (6)	M	24	Caucasian	None	No restrictions	0	No	No	No
7	F	39	Caucasian	Hand cream, facial cream, make-up	No restrictions	3	Yes	No	No
8 (9)	M	24	Caucasian	None	No restrictions	0	No	No	No
9	M	24	Caucasian	None	No restrictions	2	No	No	No
10 (1)	F	23	Caucasian	Facial cream, make-up	No restrictions	2	No	No	No
11 (5)	F	-	African	-	-	-	-	No	-
12 (4)	F	24	Caucasian	Hand cream, facial cream, make-up	No restrictions	3	No	No	No
13 (8)	F	21	Caucasian	Facial cream, make-up	No restrictions	1	No	No	No
14	M	-	Asian (Indian)	-	-	-	-	No	-
15 (10)	F	30	Asian (Chinese)	Hand cream	No restrictions	0	No	No	No
16 (2)	F	25	Caucasian	Hand cream, make-up	Vegetarian	1	No	No	No

Appendix III Primer Sequences

Forward and reverse primer sequences included in the PCR master mix

Primer	Sequence (5' to 3')
Forward	/5Atto550N/ATCCAGAGTGACGCAGCA
Reverse	AAAAAAAAAAAAAAAAAAAAAA/iSp18/ACTAAGCCACCGTGTCCA

Forward and reverse primers used for sequencing

Primer	Sequence (5' to 3')
F1	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTATCCAGAGTGACGCAGCA
F2	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCCTCCGATCTNATCCAGAGTGACGCAGCA
F3	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNATCCAGAGTGACGCAGCA
F4	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNATCCAGAGTGACGCAGCA
R1	CAAGCAGAAGACGGCATACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTAAGCCACCGTGTCCA
R2	CAAGCAGAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTACTAAGCCACCGTGTCCA
R3	CAAGCAGAAGACGGCATACGAGATGCCTAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNACTAAGCCACCGTGTCCA
R4	CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNACTAATCCACCGTGTCCA
R5	CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNACTAAGCCACCGTGTCCA
R6	CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNACTAAGCCACCGTGTCCA
R7	CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNACTAAGCCACCGTGTCCA
R8	CAAGCAGAAGACGGCATACGAGATTCAAGTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNACTAAGCCACCGTGTCCA

Appendix IV Phenol Chloroform and Ethanol Precipitation

The following phenol chloroform and EtOH precipitation steps were only performed with the positive elutions from SELEX Round 1. Since the amount of DNA recovered was very low (0.17 nmol when 1 nmol was required for SELEX Round 2), this step was deemed unnecessary for the subsequent rounds and omitted. It was confirmed by PAGE that this step was optional.

Phenol Chloroform Extraction

The positive elutions were split into two 1.5 mL microcentrifuge tubes. Equal amounts of phenol chloroform (470 μ L) was added to each tube, vortexed, and then centrifuged at 14,800 rpm for two minutes. The top aqueous layer – where the DNA was – was separated from the bottom organic layer into a new 1.5 mL microcentrifuge tube.

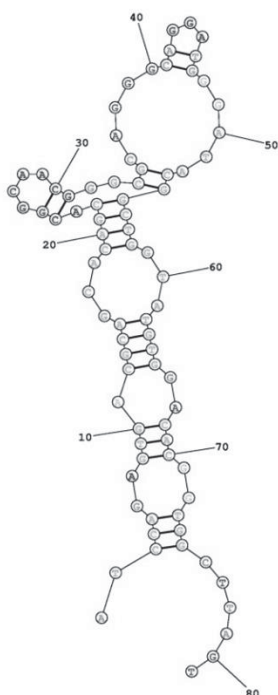
Ethanol Precipitation

3 M NaCl (50 μ L) was added to 1 mL anhydrous EtOH and chilled on dry ice. DNA (100 μ L) was added to this chilled NaCl/EtOH tube and placed on dry ice for one hour. Normally, centrifugation would be done at 14,800 rpm at -9 °C for 30 minutes followed by ten minutes on dry ice, and these steps repeated twice. However, there were delays in changing the rotor for the centrifuge with temperature control. Therefore, in the meantime, the tubes were placed into the centrifuge without temperature control at 14,800 rpm for 15 minutes. The tubes were then placed on dry ice for ten minutes. The tubes were centrifuged for ten minutes, placed into dry ice for ten minutes, and then these two steps were repeated again. The rotor for the centrifuge with temperature control was finally changed and the proper EtOH precipitation protocol was followed. Typically, a pellet would start to form after the first centrifuge round; however, it was possible to not see pellet formation at all. The majority of the EtOH was collected into a tube. There was less than 0.1 mL left in each tube. The resulting tubes were placed in the speedvac with the cryopump option activated and left overnight.

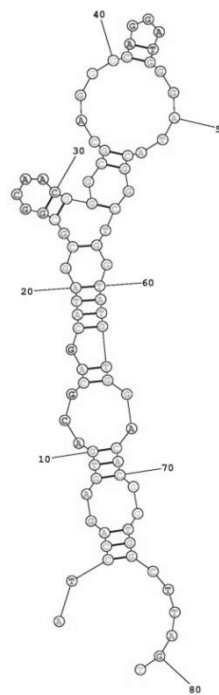
Appendix V Predicted Secondary Structures

Predicted secondary structures for the longer sequences only (i.e., random regions including primers) at 298 K by using RNAstructure software [203].

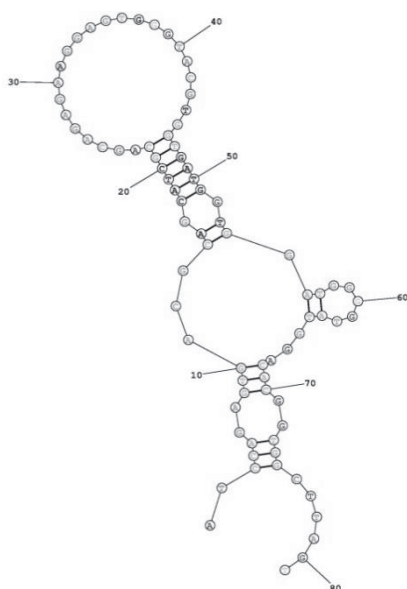
Sequence #5 - long 1BwJHFIHZ



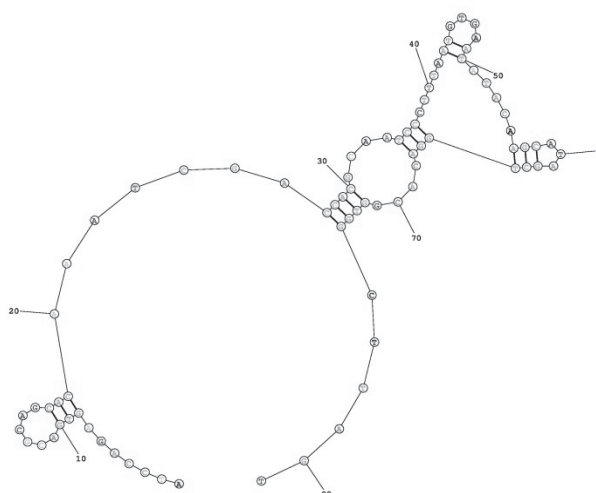
Sequence #6 - long IHDelHA



Sequence #7 - long 1KjJqCJD



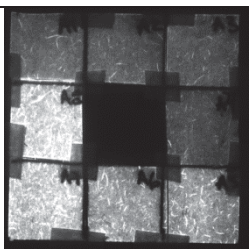
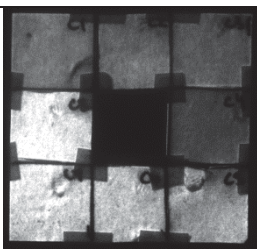
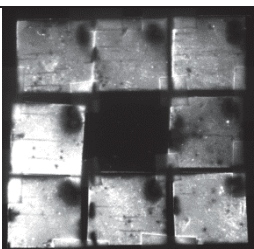
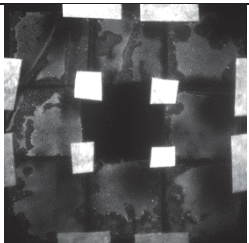
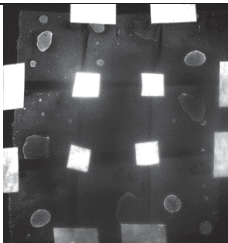
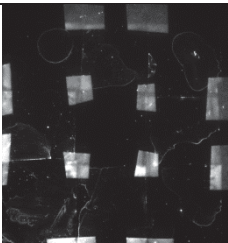
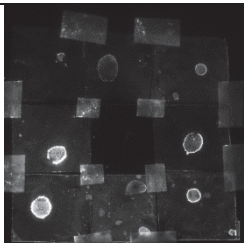
Sequence #8 - long GiAGIIGI



Appendix VI Observations on Background Fluorescence and Non-Specific Binding

Two porous (Australian and Canadian white copy papers) and five non-porous (PVDF, Australian and Canadian garbage bags, aluminium foil and ziplock bag) substrates were used to gauge the amount of background staining or non-specific binding there was with realistic surfaces. Blank 1 cm² pieces of substrates, without any deposited fingermarks, were subjected to the eight different working solutions (those used in Subsection 4.4.2.1), each containing one of the short or long aptamer candidates. A blank piece of substrate was also left untreated for comparison purposes (Table VI-1). Both the processed paper substrates and processed PVDF had the highest background fluorescence. This was not unexpected for the paper substrates as they would be able to absorb and retain more of the working solution than non-porous substrates. The longer sequences luminesce slightly more strongly than the shorter sequences, which is a similar observation to fingermarks processed with both length of aptamers. One of the paper substrates also had fluorescent fibres already embedded into its composition. The aluminium foil, ziplock bag, and Canadian garbage bag had the least amount of background fluorescence. The Australian garbage bag had a lot of background staining. However, it continued to be used in the subsequent experiments, as this research was being conducted in Australia and therefore relevant realistic substrates should be included.

Table VI-1 Background fluorescence visualised at 530 nm with 590 nm bandpass filter for substrates processed with, from top left (clockwise): Sequences #1 to #8. The middle piece is untreated substrate.

			
Australian copy paper	Canadian copy paper	PVDF	
			
Australian garbage bag	Canadian garbage bag	Aluminium foil	Ziplock bag

Appendix VII Substrate Suitability

One male and one female, good and poor fingermark donors, respectively, each deposited two natural and two sebaceous fingermarks onto three non-porous substrates: Canadian garbage bag (Kirkland Signature), pop can (Coca Cola), and plastic grocery bag (Woolworths). These fingermarks were left to age for 24 hours before being cut in half and processed by Method 1, one half for each of the longer sequences.

To the naked eye, some ridge detail was visible for both natural and sebaceous fingermarks deposited by the good donor, but only in sebaceous fingermarks for the poor donor. Observations under luminescence conditions can be found in Table VII-1. Development appeared better (e.g., less background staining) on this garbage bag purchased in Canada compared to the garbage bag used during the fingermark-SELEX process. The multi-coloured background of the pop can substrate was unable to be suppressed at the given visualisation conditions, and was therefore not used for subsequent experiments. Cleaning the pop can as was done in Chapter 2, seemed to have been too abrasive and may have altered the surface composition.

Table VII-1 Observations of processed natural and sebaceous fingermarks under luminescence conditions.

Substrate	Poor Fingermark Donor	Good Fingermark Donor
Grocery bag	<ul style="list-style-type: none"> - Natural: evidence of touch; splotchy background even where fingermark was deposited - Sebaceous: continuous ridge detail; splotchy background limited to non-fingermark area 	<ul style="list-style-type: none"> - Natural: ridge detail present, but unclear; splotchy background - Sebaceous: continuous ridge detail; not as splotchy where fingermark deposited
Garbage bag	<ul style="list-style-type: none"> - Natural: random fine luminescent spots in background - Sebaceous: continuous ridges visible; similar amount of fine luminescent spots 	<ul style="list-style-type: none"> - Natural: nice continuous ridges; some fine luminescent spots within fingermark, but also in background - Sebaceous: similar to natural fingermark results
Pop can	<ul style="list-style-type: none"> - Natural: evidence of touch; substrate still visible - Sebaceous: small amount of ridge detail visible; substrate design not suppressed at particular viewing conditions 	<ul style="list-style-type: none"> - Natural: some continuous ridges visible; substrate pattern interfering with visualisation - Sebaceous: similar to natural fingermark results

Appendix VIII **Assessment of Aptamer Candidate-Dye Filtration Effectiveness**

The eight “fingermark” aptamer candidates (50 μ M, 45 μ L) were each combined with 15 μ L Atto 550 dye, left to incubate in the fridge overnight, and filtered by spin column following the method previously described in Subsection 4.4.2.1. A 40x dilution sample (200 μ L) was then prepared for each aptamer candidate-dye mixture with milliQ water, as this was used for the baseline run on the UV-vis spectrophotometer (Cary 30, Agilent Technologies). One at a time, the 40x dilution samples were run; the quartz microcuvette was rinsed out with milliQ water three times in between each sample. The absorbance values at 260 nm (for DNA) and at 554 nm (for Atto 550) had to be between 0.1 and 1.0 to be deemed reliable. The undiluted aptamer candidate-dye mixture was then centrifuged again to see if additional dye would be removed. A 40x dilution sample (200 μ L) was then prepared with each of the two-time filtered aptamer candidate-dye mixtures and run on the UV-vis spectrophotometer. This process was repeated again after the remaining undiluted aptamer candidate-dye mixtures were filtered for a third time. Aptamer candidate sequences 3 and 7 were also prepared to 40x dilutions and run on the UV-vis spectrophotometer, in addition to an 80x dilution of the dye, as the absorbance value at 554 nm was too high with a 40x dilution.

Some excess dye was visible at the bottom of the microcentrifuge tube as expected after the first filtration. However, more dye was also being filtered out even after the third filtration by centrifugation. The UV-vis data showed that there was less absorbance at 554 nm – the peak attributed to the Atto 550 dye – after the third filtration. This correlated to the removal of excess dye. The absorbance value at 260 nm could not solely be attributed to the DNA sequences, as Atto 550 dye also had an absorbance peak there.

Appendix IX Atto 550 NHS Ester Compatibility

As the working solutions containing the “fingerprint” aptamer candidates appeared to develop all chemical spot tests in Subsection 4.5.1, and it was later discovered that there was an excess of dye that may be hindering any differences between working solutions from being observed (Appendix VIII), further investigation was performed to determine how much influence the dye in the working solution would have on the resulting fingerprint development. To do so, two fingerprint donors (one male – good donor, one female – poor donor) each deposited three natural and three sebaceous fingerprints on aluminium foil. These fingerprints were left to age and processed the following day using a working solution 20 μ L of dye in 10 mL tris buffer as prepared for Method 4; no “fingerprint” aptamer candidates were included.

All fingerprints were visualised under luminescence examination. This means that some of the previously observed fingerprint development may be the result of the dye and not the “fingerprint” aptamer candidates. As mentioned before, the Atto 550 dye was selected based on previous work performed by Spindler [164]. The particular dye product ordered included a NHS ester, which would be reactive to some fingerprint residue constituents such as amino acids and proteins, hence the observed luminescent ridge detail. This does not mean that the dye alone – Atto 550 without the reactive functional group – would develop fingerprints. Unfortunately, this product was not available to confirm. However, Spindler had previously performed some non-binding control tests by attaching the dye to BSA, an inert protein, and diluting it in the same manner of the reagent (in this case, 5 mL PBS with 1% w/v skim milk powder) [164]. Spindler stated that Atto 550 outperformed all other commercially available dyes and had excellent specificity.

Appendix X Lysozyme Aptamer Tests

Prior to comparisons between the “fingermark” aptamer candidate-containing working solutions and single-target aptamers, tests were conducted to ensure that the lysozyme aptamers (Table X-1) used by Wood [101] were still effective and the protocol was reproducible.

Table X-1 Lysozyme aptamer sequences used in Wood’s PhD research [101].

Aptamer	Sequence
Aptamer 1 (Cox and Ellington [200])	ATCAGGGCTAAAGAGTGCAGAGTTACTTAG
Aptamer 2 (Tran et al. [201])	AGCAGCACAGAGGTCAGATGGCAGCTAAGCA GGCGGCTCACAAAACCATTCGCATGCGGCCCT ATGCGTGCTACCGTGAA

The two DNA aptamers were synthesised by Biosearch Technologies Inc. (USA) in 2012. They were tagged with CAL Fluor® Orange 560 during synthesis. The aptamers were received lyophilised and stored in the fridge at 4 °C until required. As there were no markings on the product vials in 2017, it was assumed that they were unused.

According to product information found in Wood’s lab notebook, there was 258 µg (20 nmol) of DNA in a vial of Aptamer 1 and 333.5 µg (10 nmol) of DNA in a vial of Aptamer 2. Each was made into a 100 µM stock solution in their respective selection buffers. Aptamer 1 was reconstituted in 200 µL of 20mM tris, 100 mM NaCl, 5 mM MgCl₂ at pH 7.5, while Aptamer 2 in 100 µL of 25 mM Trizma® base, 192 mM glycine, 5 mM K₂HPO₄ at pH 8.3. Wood’s aptamer-based reagent formulation involved 200 µL of 10 µM aptamer in 30 mL tris buffer (25 mM tris, 150 mM NaCl, 2 mM KCl, pH 8) [101].

Three donors (one male, two female) each deposited one natural and two sebaceous fingermarks onto PVDF. These nine fingermarks were left to age for two days before

being split in half; one half processed with Aptamer 1-based reagent and the other half with Aptamer 2-based reagent. The fingerprint halves were placed face down on top of the working solutions in petri dishes and covered in the shaker (60 rpm, 24 °C) for two hours. The fingerprint halves were left to air dry without a prior rinse treatment.

The sebaceous fingerprints were visibly purple to the naked eye. Some of the natural fingerprints were also visible, but the purple coloration was faint. Under luminescence conditions – 505 nm with 555 nm bandpass filter with the Poliview – all fingerprints showed ridge detail development (Figure X-1). As the relative performance of the two lysozyme aptamer-based reagents were comparable in terms of amount of development and luminescence, Aptamer 1 was used in the comparison study against the working solutions containing the “fingerprint” aptamer candidates. There was also more product vials still stored in the fridge compared to Aptamer 2, which may have been useful if additional experiments needed to be performed.

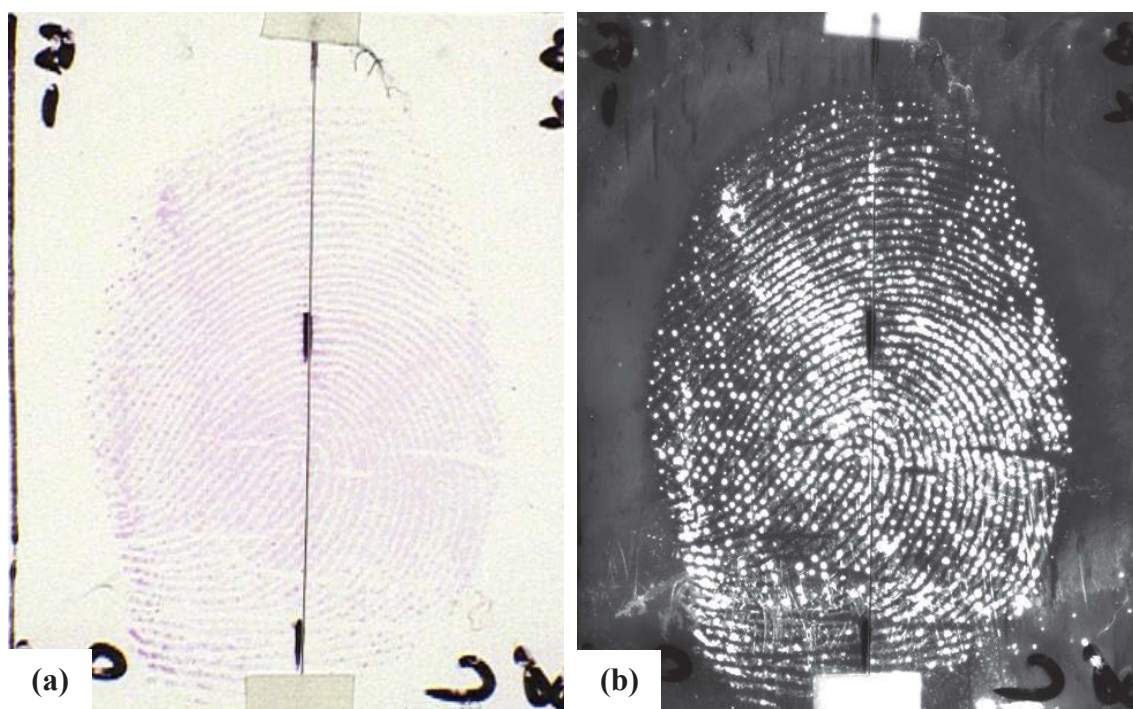


Figure X-1 Two-day-old sebaceous fingerprint on PVDF developed with working solution containing Aptamer 1 (left) and Aptamer 2 (right) viewed under (a) white light and (b) 505 nm with 555 nm bandpass filter.

Appendix XI Fingerprint Development on Porous Substrates with Revised Working Solution Formulation

During the initial proof-of-concept experiments, results on the porous substrates were not desirable (Subsection 4.5.1). However, once the working solution formulation was changed from PBS-based to TBS-based, the porous substrates were re-visited.

One female donor deposited four natural and three sebaceous fingerprints onto the Australian and Canadian copy paper used. Once aged for three days, the fingerprints were split in half and processed with two time intervals, differing in five seconds. The shortest time interval was five seconds, while the longest was 25 seconds. The working solution used was 135 μL of 50 μM Atto 550-labelled Sequence #6 in 20 mL tris buffer. The samples were not rinsed and left to air dry.

The corresponding fingerprint halves were viewed with the Poliview. Sebaceous fingerprints at the 15- and 20-second intervals had the best ridge detail (Figure XI-1). Unfortunately, the development was reverse with dark ridges and light furrows and background. It is possible that the processing times of the PBS-based reagent were far too long. These results support the decision to focus on the non-porous substrates at the moment.

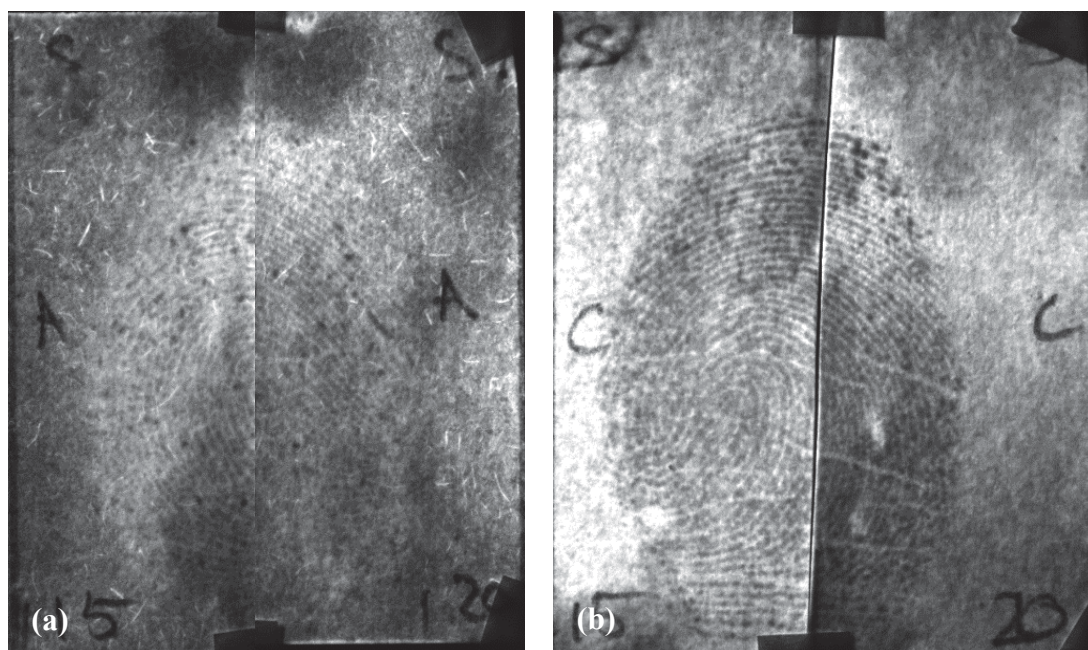


Figure XI-1 Three-day-old sebaceous fingerprints deposited on (a) Australian and (b) Canadian copy paper and subjected to the working solution for 15 (left halves) and 20 (right halves) seconds. Fingermarks were visualised at 530 nm with a 590 nm bandpass filter.

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