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Developing DNA Friendly Fluorogenic Methods for Detecting, Enhancing, and Preserving Bloody and Proteinaceous Impression Evidence

Final Technical Report
(2013-DN-BX-K026)

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Abstract

Impressions are commonly found as evidence associated with crime scenes. Current fluorogenic enhancement methods for impression evidence are problematic for DNA preservation and are often impractical for crime scene use. This may lead to an either or decision made during evidence collection based on whether to enhance the impression and potentially damage the DNA evidence, or to gather the DNA evidence, which may destroy impression evidence. Zar-Pro™ Fluorescent Blood Lifters have been successful in lifting, enhancing, and preserving blood impressions; however, this technology had not been explored to determine effectiveness in the recovery of non-blood proteinaceous impressions. This project focused on optimizing the detection, enhancement, and preservation of impressions deposited in blood, semen, saliva, eccrine/sebaceous sweat, and non-human oil using Zar-Pro™ Fluorescent Lifters and three novel Fluorogenic Enhancement Sprays which were developed in the course of the project. The first set of trials were devised to test the effectiveness of these enhancement methods across a broad range of substrates, titled the Substrate Trials. In the Substrate Trials impressions were deposited in the above mentioned biofluids on a series of fifteen substrates of varying porosity and were enhanced after one hour and one day. A subset of substrates from the Substrate Trials were used for the Aged Trials. In the Aged Trials impressions were deposited in the five biofluids and were enhanced after one month, three months, six months, and one year. Zar-Pro™ Lifters and one of the developed Fluorogenic Enhancement Sprays were found to effectively enhance and preserve blood and semen while having more limited success with saliva, eccrine/sebaceous sweat, and non-human oil. Based on preliminary results, the enhanced impressions remain fluorescent and can be preserved long-term. In attempt to create DNA friendly fluorogenic enhancement methods, DNA extraction, amplification, and analysis was conducted on each of the enhancement methods utilized in this project. It was found that amplifiable DNA capable of producing full STR profiles can be extracted from Zar-Pro™ lifted semen and blood impressions, but not from impressions in any biofluid treated with the Fluorogenic Enhancement Sprays. In simplifying the collection and preservation of impression evidence, while expanding the utility of enhancement methods to include DNA friendly approaches, both impression evidence and DNA can be recovered from a single evidentiary item.

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Executive Summary

The goal of this project was to produce simple, time and cost effective, non-toxic methods for recovery and enhancement of impression evidence that are safe for use at crimes scenes and provide opportunities for subsequent DNA recovery at the laboratory. Zar-Pro™ Fluorescent Blood Lifters effectively lift, enhance, and preserve blood impressions across substrates of varying porosity. They are highly sensitive and inherently fluorogenic in combination with proteinaceous materials when visualized with alternate lighting making them an ideal tool in the collection of proteinaceous impressions for use in forensics science. One of the reasons the Lifter are effective is that they physically remove the impression from the substrate, affixing it to the Lifter. Lifted reddish colored blood impression are often visible on the white background of the Zar-Pro™ Lifter under normal lighting conditions. They can also be fluorogenically enhanced without any additional chemicals by simply visualizing the lifted impression under alternate lighting, creating a fluorescent impression on the darkened Lifter. The ability to lift and fluorogenically enhance impressions from substrates is beneficial as this process helps remove substrate variables, such as background colors and patterns that impede visualization of impression details. They ability to lift the impression also allows for impressions deposited on large or immovable objects to be brought back to the laboratory for analysis in the form of a Lifter, helping alleviate storage concerns associated with large items. Furthermore, fluorescent capable Zar-Pro™ lifted impressions are fixed and preserved on the Lifter allowing for repeat visualization over long time intervals. The fluorescent properties of Zar-Pro™ Lifters are unique and not completely understood.

The first experiments performed in this project were conducted to assess the contribution of each components comprising the Zar-Pro™ Lifters as a means to investigate the Lifters fluorescent phenomenon, specifically associated with the enhancement of blood. It was proposed that by understanding the fluorescent mechanism of Zar-Pro™ Fluorescent Blood Lifters that this technology could be developed into a spray form. Initial efforts to define the fluorescence phenomenon seen in the Zar-Pro™ formulation followed two strategies. One, to determine the fluor in blood that was being fluoresced, and two, to determine what components in the Zar-Pro™ formulation were necessary to create the phenomena. Initial experiments were limited to examination of Zar-Pro™ fluorescence in the solid phase of the Lifters as attempts to replicate the phenomenon in a liquid phase were unsuccessful. Eventually, it was discovered that the addition of butanol and/or sulfosalicylic acid to the extract allowed for monitoring of fluorescence in a liquid phase and the resulting fluorescence could be measured in terms of intensity using a spectrofluorimeter. Further tests indicated that purified hemoglobin was capable of fluorescing when whole blood was exposed to conditions present in the Zar-Pro™ formulation. In response, purified hemoglobin was then ultra-filtered through a 10 kD cutoff filter and the red flow-through filtrate was fluorescent when combined with butanol. The fluorescent flow-through confirmed that the fluor is less than 10 kD and the red coloration suggests that the fluor is most likely the protoporphyrin-IX component of the heme. The

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fluorescent spectral profile of protoporphyrin-IX excitation and emission is generally at shortened excitation wavelength with a longer emission wavelength than the wavelengths observed in this research project. This result could be an artifact of the combination of solvents used in this study, resulting in an increase in the intensity of obtained fluorescence.

To further understand the fluorescent role of each component in the Zar-Pro™ formulation derivatives of the product were evaluated for both impression recovery and fluorescence yield. The preliminary results of these studies hinted that there were likely two sources of fluorescence present in the Zar-Pro™ formulation. An absolute requirement for the more intense fluorescence was the presence of acid incorporated into Zar-Pro™ formulation. A secondary fluorescence appeared to be a result of the presence of surfactant in the Lifter. The novel fluorescence mechanism in blood that contributes to the overall fluorescence observed in Zar-Pro™ Lifters has not been previously reported in literature and is an exciting discovery that warrants further exploration.

Information obtained by understanding the fluorescence properties associated with Zar-Pro™ Lifters were utilized to develop three novel Primary Fluorogenic Enhancement Sprays and a secondary Enhancement Spray. The effectiveness of the Primary Enhancement Sprays (Spray #1, Spray #2, and Spray #3) and Zar-Pro™ Lifters in the enhancement of blood, semen, saliva, eccrine/sebaceous sweat, and non-human oil on a broad range of substrates (Substrate Trials) with a subset tested over a one year interval (Aged Trials). Within the Substrate and Aged Trials the use of a Secondary Enhancement Spray was applied to the Zar-Pro™ lifted impressions after 2 days to determine if the onset of fluorescence could be decreased after activation and whether fluorescence intensity could be improved.

Prior to the Substrate and Aged Trials optimal deposition parameters were established for the five biofluids on fifteen substrates. In an effort to minimize human and environmental factors associated with the deposition of impression evidence, optimal deposition parameters were utilized to create consistent reproducible impressions for analysis. The deposition parameters defined in this study provide a guideline for producing optimal impressions. Optimal impressions are crucial in impression based research as variation in the deposited impressions could adversely affect the outcome of the project.

Preliminary results of examiner ratings for blood impressions in the Aged trials at the one year, one week interval indicated that enhancements using Zar-Pro Lifters were highly effective, as was expected since the Zar-Pro™ Lifters were initially created to lift and preserve blood impressions. Blood impressions are also traditionally red in color making them more easily visible under normal lighting. Visualization of semen or saliva cannot be visualized on the Lifter under normal lighting, due to the absence of color, which was expected since these impressions are latent. However, due to the fluorescent capabilities of the Lifters the visualization of impression details in semen, and partial ridge detail in saliva were visible under alternate lighting. The visualization of eccrine/sebaceous sweat or non-human oil were not visible on the Lifters, even when visualized under alternate lighting.

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Blood impressions were enhanced with Spray #1, but the other biofluids were not effectively enhanced with this method. Spray #2 enhanced blood impressions, but only on ceramic tile and glossy paper under normal lighting, but this method did not produce fluorescence when visualized under alternate lighting. Spray #3 enhanced impressions deposited in all biofluids under normal and alternate lighting. The resultant fluorescence was intense on all substrates, increasing the quality of ridge detail when visualized under alternate lighting

Spray #3 was effective on most substrates for blood when visualized under normal light and very effective at fluorescent enhancement under alternate lighting. Results were similar for semen with the exception of the porous substrates. This is consistent across the Aged Trials, again being affected by substrate variables such as background, color, pattern, and porosity. These substrate variables create similar issues for saliva, vegetable oil, and eccrine sebaceous with vegetable oil producing slightly decreased visualization on some of the non-porous and semi-porous substrates. However, this spray does not preserve and enhance ridge detail on porous substrates. It was the only method of those examine in this study that had a modicum of success in enhancement of eccrine-sebaceous impressions.

The best performing enhancement methods were the Zar-Pro™ Lifters and Spray #3. Zar-Pro™ Lifters were able to effectively lift and enhance four of the biofluids across many substrates. It was effective with blood across all substrates and across all non-porous and semi-porous substrates with semen, with some stochasticity in the porous substrates. Spray #3 was the only method that effectively enhanced eccrine-sebaceous impressions but this was across fewer substrates due to the limitations of substrate variables associated with performing enhancement on the substrate.

Impression quality ratings are subjective and are often based on the examiners education, training, and experience. In order to ensure the results of this impressions based research were valid, inter-observer and intra-observer errors are being calculated for the trial ratings, to include impression quality visualized under normal and alternate lighting and the fluorescent intensity of the enhanced impressions visualized under alternate lighting. The Cohen's Kappa statistical model was used to calculate agreement between examiners (inter-observer variability) for the Aged Trials, specifically for blood at the 1 year, 1 week interval and amongst a blind set of second ratings of the same sample set (intra-observer variability) for the Aged Trials at one year, one week interval, and one year, one week, and one day interval. The inter-observer agreement levels and intra-observer agreement levels revealed at least fair agreement for all impression ratings from the 1 year, 1 week interval, thus the results can be deemed significant and valid. There was variation amongst examiners in their spectrum of agreement, yet this slight variation was expected due to individual examiner differences in education, training, and experience.

Both the DNA IQ system (Promega) and the Biosystems PrepFiler® BTA Forensic DNA Extraction Kit were optimized for use extracting DNA from blood and semen impressions lifted from glass. A sample 9 – 16 mm^2 in size is optimal for DNA extraction and can easily be removed from the lift without causing large scale damage to the impression evidence. DNA extracted from enhanced impressions lifted using Zar-Pro™ Fluorescent Blood Lifters was tested

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using the PowerPlex ESX 16® Fast System (Promega) to ensure the viability of generating reliable STR profiles suitable for forensic casework. DNA analysis was considered successful for the optimized impressions in both blood and semen. The optimized protocol was then applied to a subset of the impressions lifted from various substrates over the Aged Trials time intervals to assess the effectiveness of the protocol for detection, lifting, extraction, quantification, STR amplification, and profile analysis. These experiments led to an understanding that amplification success was contingent upon the amount of time that DNA was on the lifter.

Full STR profiles could be generated from lifted blood and semen impressions using the PowerPlex ESX 16® Fast System and the GlobalFiler™ PCR Amplification Kit meaning that the methods are applicable across both Applied Biosystems and Promega systems, which are commonly used in forensic science laboratories. Techniques to extract DNA from evidence "fixed" by Zar-Pro™ Lifters have the potential to greatly improve the evidentiary value of impression and DNA evidence, thus alleviating the either-or determination often made between using the detected evidence for impression enhancement or DNA analysis.

This project has the potential to not only improve impression evidence collection methods utilized in forensic science, but also to provide safe, non-toxic methods for detecting, enhancing, and preserving both blood and non-blood impression and DNA evidence. The methods developed during this research are currently being field tested by the Oakland County Sheriff's Office Forensic Science Laboratory through validation studies aimed at approving this new technology for incorporation into their protocols. Thus, this technology has the potential to directly influence standard practice in law enforcement.

Main Body

I. Introduction

Statement of the Problem

Blood and proteinaceous impression evidence is often problematic for law enforcement, with no universally-accepted procedures for detection, enhancement or preservation. Current detection and enhancement methods for this type of impression evidence are often time-consuming and require the use of flammable or toxic chemicals, which can only be used safely in a controlled laboratory setting. These chemicals are primarily applied in a liquid form, either through spray application or dipping/soaking. Enhancement procedures vary in effectiveness based on substrate porosity, background pattern, and/or texture, with fluorogenic methods often preferred over non-fluorogenic methods. Fluorogenic enhancement methods provide increased sensitivity with improved contrast for analysis on a larger variety of substrates, but they are not without limitations. Most of these methods lack reliability and reproducibility, as well as vary in intensity of fluorescence. The effectiveness of fluorogenic enhancement chemicals is dependent, among other factors, on the substrate porosity and degree of background interference, both of

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which affects the overall fluorescent sensitivity and contrast for analysis. Immediate photography is recommended with most fluorogenic enhancement methods, as fluorescence is often short-lived and the impression can be altered or destroyed in the enhancement process. In addition, DNA may be degraded in evidence processed with these methods creating situations where investigators must make an either-or decision during evidence collection.

Zar-Pro™ Fluorescent Blood Lifters provide an affordable, durable, easy-to-use, and a non-toxic method for lifting, enhancing, and preserving blood impressions with long term fluorescent capabilities. Impressions can be recovered from various substrates, regardless of the degree of porosity or the background patterns pertaining to the substrate in which the impression is deposited [68]. This is an important determination, as substrate porosity and background patterns are often a limiting factor with other blood-based enhancement methods. The Lifters are also highly sensitive to proteinaceous materials, allowing for the recovery of faint or even invisible impressions which cannot be visualized under normal lighting conditions. The fluorescent properties of the Lifters when combined with blood and visualized under alternate lighting, create brightly fluorescing, high-contrast impressions for analysis [68]. They are additionally valuable, as fragile or perishable impression are stabilized upon contact with the Lifters, allowing for the preservation of impressions in a "fixed" state while maintaining their fluorescent capabilities for future use. The ability to expand the use of the Zar-Pro™ Lifters to fix and recover other proteinaceous impressions, outside of blood had yet to be explored. Furthermore, the ability to extract DNA from impressions fixed onto the Lifters has not been studied and if the DNA is indeed viable it could provide an additional avenue of identification, outside of just the impression itself.

Given the success of the Zar-Pro™ Lifters, it should also be theoretically possible to utilize the fluorescent technology of Zar-Pro™ to develop novel fluorescent enhancement sprays. Small particle enhancement sprays are often comprised of molybedenum disulfide or titanium dioxide mixed with a surfactant and are used to enhance non-blood impressions on wet and non-porous substrates [27]. The molybedenum disulfide darkens impressions, which is ideal for light colored substrates, whereas titanium dioxide whitens impressions and is recommended for use on dark colored substrates. The contrast of the enhanced impression on the substrates is important when utilizing sprays as it will affect the visualization of the impression details for analysis.

Fluorophores naturally present in proteinaceous secretions, positioned in close proximity to metals in their smallest particle form, exhibit highly enhanced fluorescence when visualized with alternate lighting [15, 21, 46, 57]. This is an area of research being explored by fluorescence spectroscopists for its various applications in the biotechnology industry. The premise of utilizing inherent fluorescent capabilities naturally found in blood and other proteinaceous impressions to enhance and preserve impression evidence, instead of the traditional methods of oxidation reactions or protein staining may have broad implications in forensics.

The composition of metals used to create fluorescence, combined with titanium dioxide or other metals in their smallest particle form, suspended in a surfactant solution and dispersed in

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an aerosol form may serve as an effective enhancement spray. Preliminary trials conducted at the Madonna University Forensic Science Research Facility suggest that this combination has the ability to fluorogenically enhance blood impressions. Thus, the development of a fluorogenic enhancement spray could result in a novel detection method for locating, enhancing, and preserving impression evidence with long lasting fluorescent capabilities without having to first differentiate between blood and non-blood evidence. It would be ideal, if the enhancement spray was also suitable for the preservation and recovery of DNA. This is an important determination, as many enhancement methods currently used for forensic crime scene investigation either degrade or contaminate DNA and /or inhibit its amplification and analysis [7].

Literature Review

It is hypothesized that the effectiveness of the Zar-Pro™ Fluorescent Blood Lifters are due, in part to the high affinity of titanium dioxide for bonding with proteinaceous materials. Previously, titanium dioxide has been successfully used in small particle form in spray or paste-like composition as an enhancement chemical for non-blood latent impressions on both the adhesive and non-adhesive sides of dark-colored tape [50, 64, 65], and other dark-colored non-porous substrates [6, 64]. Blood impressions have also been enhanced with the use of titanium dioxide, but to a lesser degree with effectiveness limited to non-porous and some semi-porous substrates [6]. The use of titanium dioxide powder as an enhancement chemical is preferable to many other methods because it is a non-toxic and non-flammable white powder, which results in a consistent and reliable enhancement method for dark-colored non-porous substrates however, it does not provide the contrast needed to visualize impressions on light-colored or porous substrates [6, 50, 64, 65]. In order to expand the effectiveness of this technology to include light-colored, porous, blood, and proteinaceous impression evidence, a number of other metals could be utilized in concert with nigrosin to produce a dark field of contrast.

Blood, when combined with titanium dioxide, fluoresces despite the fact that neither blood [39, 49] nor titanium dioxide [64] is inherently fluorescent when visualized with alternate lighting. Even though blood contains some intrinsic fluorophores [15, 21, 57], it has a tendency to absorb light, darkening impression details and limiting contrasts [47, 59]. This darkening under alternate lighting provides an effective contrast on some fluorescent substrates without the need for subsequent chemical enhancement methods [20, 39]. When this contrast cannot be visualized, extrinsic fluorophores found in blood enhancement chemicals may be used [44].

Fluorogenic enhancement methods create fluorescence through either oxidation reactions or protein staining after spraying or soaking the impression area deposited on the substrate. Some of these chemicals target all protein groups, whereas others are blood-specific and target only the heme protein group. Known fluorogenic protein stains are Hungarian red [6, 59], diazofluorenone (DFO) [6, 40, 53, 59], and ninhydrin [53], all of which are non-specific, and acid yellow 7 [52, 53], which is blood-specific. Chemicals known to create fluorescence through oxidation reactions are leucocrystal violet (LCV) [54], leucorhodamine (LeuR6G) [8, 18, 47, 50], merbromin [6, 49, 59], luminol [18, 20, 47, 53], and fluorescein [35, 47, 53], although not all are suitable for use with impression evidence.

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Fluorogenic enhancement chemicals are often preferred over non-fluorogenic chemicals for blood impressions due to the increased sensitivity, visual contrast, and reduction of background interference provided by fluorescence. Many of these methods are limited by the following factors: fluorescent sensitivity, contrast for visualization, substrate porosity, safety of the enhancement chemicals, ease of preparation and application, duration of fluorescence, and preservation of the impression evidence.

The most sensitive blood enhancement chemicals currently known are luminol and fluorescein. Luminol has the ability to detect blood in water in dilutions as low as 1:5,000,000 [47]. Luminol is also chemiluminescent, thus alternate lighting is not required to produce fluorescence. However, the resulting fluorescence is often weak, short-lived, and difficult to visualize on dark-colored substrates [53]. Unlike luminol, fluorescein does require alternate lighting to visualize fluorescence [35]. While both luminol and fluorescein are sensitive to blood, neither is recommended for enhancement of impression evidence. This is because neither is compatible with acidic fixatives and thus will degrade and diffuse ridge details, altering or destroying the impression [18, 35, 53].

Alternate lighting (with the appropriate barrier filter) is necessary to visualize and capture fluorescence with most blood enhancement chemicals. Acid yellow 7 [52, 53] and DFO [40, 53] are highly sensitive and allow for the visualization of faint or invisible impression details not visible under normal lighting conditions. However, acid yellow 7 is only effective on non-porous substrates, and DFO is conversely only effective on porous substrates. Other chemicals such as merbromin [59] and LeuR6G [18, 50] are fluorescent, but often produce distracting background interference. Hungarian red [59], ninhydrin [53], and LCV [54] exhibit fluorescence, but not with the sensitivity of the other methods, and they often produce background interference. Furthermore, with several of these chemicals the resulting fluorescence is diminished as the blood volumes are increased [35, 52, 53, 59].

Blood enhancement chemicals are selected based on their level of fluorescence along with their ability to minimize background interference. Substrate porosity will affect the resulting background interference, even with enhancement chemical producing increased levels of fluorescence. Substrates of varying porosities behave differently in regards to the deposition of blood impressions, subsequently impacting the effectiveness of blood-enhancement processing. In most cases an enhancement method suitable for non-porous substrates will be of inferior quality for porous substrates. Acid yellow 7 [53], Hungarian red [20, 52, 59], and LCV [40, 50, 53, 59] are effective for enhancing blood impressions on non-porous substrates, but due to excessive background staining are ineffective on porous substrates. Conversely, suitable methods for porous substrates are inferior for enhancement on non-porous substrates such as ninhydrin [41, 53, 59] and DFO [40, 53].

A very important consideration with these methods is chemical toxicity. While ease of preparation and application are important, safety of personnel at the crime scene and end users after the scene has been processed is of vital concern. The working solutions for acid yellow [53], LeuR6G [18, 67], DFO [59], and ninhydrin [59] are flammable, and merbromin [49, 59]

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and LCV [50] are toxic. Methanol, often present in working solutions, also presents flammability concerns [51]. Due to the hazardous nature of these chemicals, they are not recommended for use at crime scenes and must be used in the controlled laboratory setting with proper protective equipment and safety precautions. It is critical within the forensic sciences to explore chemical enhancement options that improve the safety and general health of forensic personnel.

Safety concerns aside, many of these methods are also time-consuming and impractical for use at crime scenes. The preparation of blood-enhancement working solutions require various chemicals and must often be pre-made with multi-step procedures, including fixing the impression; this is done with chemical or heat fixatives prior to application of enhancement chemicals, and is followed by lengthy dry times. Acid yellow 7 can take hours to stain blood impressions depending on the volume of blood, while yellowing the impression in the process [53]. Hungarian red [20] and LCV [53] are fragile during dry times, with Hungarian red requiring an extended period to dry compared to similar methods. Care must be taken during this time to prevent the impressions from being altered or destroyed. Merbromin [59] has a lengthy preparation time and requires refrigeration prior to use, which is often not practical for crime scene use. LCV [53] and fluorescein [35] are most effective when made fresh, with fluorescein having a short shelf-life. Ninhydrin [53] requires time or heat and humidity, and DFO [53] requires heat. Blood impressions do tend to develop faster than non-blood impressions, but the process is still lengthy [53].

The enhancement of impression evidence using extrinsic fluorogenic chemicals create short-lived fluorescence while frequently altering or destroying the impression evidence in the process, thus the preservation of impressions after such enhancement is often not possible. LCV is light-and heat-sensitive and even when stored in a dark environment will continue to react, eventually eliminating contrast and obliterating the impression in the process [40, 41]. Luminol creates a fast-acting and irreversible reaction that must be captured immediately via photography [53]. Fluorescein is not as time sensitive. Photography is recommended within hours [35], but fluorescent capabilities have been reported to last for a month [47]. In general, fluorogenically-enhanced impressions require immediate photography to preserve details. While noted that these are the most sensitive fluorogenic chemicals, neither luminol nor fluorescein is recommended for use on impression evidence because they alter and/or destroy the impression during the enhancement process.

The loss of coloration in blood impressions occurs as a result of age and other environmental conditions, therefore, mimicking impressions deposited with other non-blood media. Therefore, aged blood impression evidence may be misidentified as a latent non-blood impression due to the loss of iron in the hemoglobin of blood that gives it its characteristic red color. As a result, the most suitable blood enhancement methods may not be selected for the analysis of these impressions. In addition, non-blood latent impressions that are not readily visible may also be overlooked or not optimally enhanced in favor of preserving and enhancing visible blood impression evidence.

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Blood enhancement methods often alter or destroy non-blood impression evidence during chemical processing. This is especially problematic with enhancement chemicals that are blood-specific, such as the acid yellow 7 [53], LCV [40, 53], luminol [20, 53] and fluorescein [35, 53]. Due to the ineffective nature of blood-specific enhancement chemicals on non-blood impressions, many potentially important latent non-blood impressions are altered or destroyed while enhancing the blood impressions. Some research has been conducted in this regard. The use of cyanoacrylate (CA) fuming prior to the application of blood enhancement stains has been used to preserve both blood and non-blood impression evidence. This method was reportedly conducive to enhancement with protein stains after CA fuming. Its effectiveness was limited, however, due to complete destruction of blood impressions on metal, glass and textured substrates [37, 50]. Others did not recommend the use of CA fuming prior to the application of the blood enhancement stains because the method inhibited subsequent staining [53]. Thus, forensic science practice would greatly benefit from the development of effective methods to preserve both blood and non-blood impression evidence, avoiding the destruction of either.

The fluorogenic properties of Zar-Pro™ Fluorescent Lifters have previously been attributed to a phenomenon known as metal-enhanced fluorescence. Blood and other non-blood proteinaceous secretions contain fluorophores, components of a molecule that cause the molecule to be fluorescent. These fluorophores are predominately found in proteins and amino acids such as tryptophan, tyrosine, and phenylalanine [15, 21, 57]. When these fluorophores are immobilized within close proximity to certain metals, such as titanium, they can be excited with alternative lighting [15, 18, 46, 57]. The relationship between the protein-intrinsic fluorophores and metal create a high-intensity fluorescence. The effect resulting from metal-enhanced fluorescence has been reported to increase intrinsic fluorescence up to a million times [21]. Additional candidates for improving contrast in metal-enhanced fluorescence applications include aluminum [15] and silver [3] nanoparticles both of which are known to create metal-enhanced fluorescence.

As discussed, oxidation reactions and protein staining enhancement methods create fluorescence through the application of extrinsic fluorophores. The biotechnology industry has provided methods of metal-enhanced fluorescence to magnify the fluorescent capabilities of some of the extrinsic blood enhancement chemicals, such as LeuR6G and fluorescein [3, 21, 57], but none of these applications have been incorporated into practice in forensic science. Developing a method that can rapidly, safely, and cost effectively fix and enhance both blood and non-blood impression evidence with long lasting fluorogenic properties has the potential to impact a wide variety of casework.

By providing the ability to successfully lift blood impressions from a wide variety of substrates, the Zar-Pro™ Lifters, eliminate some of the major drawbacks of current chemical enhancement methods. These drawbacks specifically include incompatible substrate porosity, low visual contrast, and high levels of background interference that have been deemed problematic in lifting blood impression evidence [26, 39, 44, 50, 55, 59]. It is common practice in forensic science to use fingerprint dusting powders to detect non-blood latent impressions and

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to lift the impression from the substrate using various lifting media. However, lifting impression evidence is not always an efficient, cost-effective choice. As an alternative, traditional small particle reagent (SPR) sprays provide a versatile, cost-effective method for fixing and enhancing impressions on the substrate. Adding the qualities of metal-enhanced fluorescence to SPR Sprays would further expand the options for forensic science practitioners.

The processing capability provided by Zar-Pro™ technology is notable, and advancing that technology to include the detection, enhancement, and preservation of blood and non-blood impression evidence using a fluorogenic spray would expand the opportunity for analysis of impression evidence in forensic science. Following that one-step further by ensuring that the fixed biological evidence is suitable for DNA analysis would mean that the biological evidence would have evidentiary value regardless of whether or not the impression minutiae was identifiable.

In the last three decades, the use of DNA evidence in forensic science has grown immeasurably. Polymerase chain reaction (PCR) analysis methods have made it possible for forensic scientists to develop reliable crime scene DNA profiles from progressively smaller biological samples. However, the viability of DNA and the utility of evidence are often compromised by the collection methods employed [7]. The analysis of DNA extracted from forensic human source materials is complicated by four major factors: 1) the presence of contaminating human DNA, 2) the presence of non-target DNA whether exogenous or endogenous to the sample, 3) co-extracted polymerase chain reaction (PCR) inhibitors, and 4) the degree to which template molecules have been damaged or chemically modified post-mortem or from the time of deposition of the biological material. These associated problems make the authentication of DNA profiles from low copy number (LCN) and degraded samples particularly problematic [1, 2, 4, 11, 19, 28, 63]. LCN DNA samples encountered in forensic investigations may represent those that begin from an initial deposition of only a minute amount of biological material as may be the case with impression evidence, i.e. “trace DNA” or “touch DNA” [34, 61]. However, trace samples can also be considered LCN if found in a chemically degraded state with regard to strand length [25].

In forensic DNA analysis, both false positives (originating from contamination) and false negatives (arising, for example, from allelic drop-out) can compromise the strength of profiles recovered from such samples. While numerous methods have been developed and validated to improve extraction and downstream analyses of LCN and/or forensic DNA [e.g. 5, 12, 13, 23, 24, 56], there is continued need to develop methods that increase the yield and purity of genetic material extracted from forensic sources. A goal of this project was to develop methods to extract amplifiable DNA from impressions enhanced and fixed by Zar-Pro™ Lifters or novel Fluorogenic Enhancement Sprays thus providing multiple lines of evidence from the same sample. The ability to capture both impression and DNA evidence in a single sample would be an invaluable tool that would act to simplify evidence collection and processing as well as to boost the evidentiary value of each impression submitted for analysis in a forensic laboratory.

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Statement of Hypothesis or Rationale for Research:

A better understanding of the fluorescent and fixative properties associated with Zar-Pro™ Lifters may make it possible to develop methods to optimize the enhancement of blood and proteinaceous impression evidence on a wide variety of substrates that are suitable for subsequent DNA analysis. The development of novel Fluorogenic Enhancement Sprays that can improve fluorescent enhancement long-term while preserving impression details would be beneficial as a forensics tool.

This project focused on exploring the fluorescence phenomenon associated with Zar-Pro™ Lifters in order to develop better performing fluorogenic enhancement sprays than what is currently available for forensic use. The Lifters and developed Fluorogenic Sprays were studied to determine their overall capabilities for use on blood, and other biofluids, such as semen, saliva, eccrine/sebaceous sweat, and non-human oil. The studies included a diverse set of substrates over a series of Substrate and Aged Trials, to provide insight into their performance for adoption into traditional forensic case work. Lastly, enhanced impressions were studied to determine the ability to preserve DNA post-enhancement. The ongoing research associated with this project aims to simplify the collection and preservation of impression and DNA evidence, thus preventing the either or decisions often associated with this type of evidence.

II. Methods and Results

1. Development of a Fluorescent Standard Scale

A solid phase fluorescence standard scale was developed to rate fluorescent intensity (Figure 1). This was deemed an important part of this project, as a common problem with the visualization of fluorescence is the inability to quantify the intensity. Although it is recognized that the rating of fluorescence is somewhat subjective, the scale served as a standard for comparison throughout these trials.

The scale was prepared by using serial dilutions of fluorescein in a 1% Photoflo™ and 10% methanol solution applied to the Zar-Pro™ Lifters in a linear array. A 4 mg/ml stock solution of fluorescein was prepared and then diluted one part to one part in a series of twofold dilutions. A six point rating scale was made using in 5 µl drops from the following dilutions: 0.5 mg ml (highest), 0.125 mg/ml, 0.0313 mg/ml, 0.00781 mg/ml, 0.00391 mg/ml, 0.00195 mg/ml.

The fluorescence of the scale must be excited with an alternate light source and appropriate barrier filters to be able to visualize the fluorescence. The primary light sources used in these research trials was the Rofin Polilight Flare Plus at UV and 50 5nm wavelengths and the Flare Plus II at 450 nm, 505 nm, and 544 nm wavelengths using orange, yellow, or red barrier filters.

Additional alternate light sources were also examined but the Rofin Polilight Flare Plus and Flare Plus II at 505 nm with an orange barrier filter produce superior fluorescence. Other light sources lacked the intensity of the Rofin model, but hinted the fluorescence obtained using a narrower wavelength source at shorter wavelengths were also capable of exciting fluorescence. By using LED bulbs and filters in the 400 -500 nm range it was estimated that 460 nm is an optimal excitation frequency for blood fluorescence observed on the Zar-Pro™ Lifters. It also

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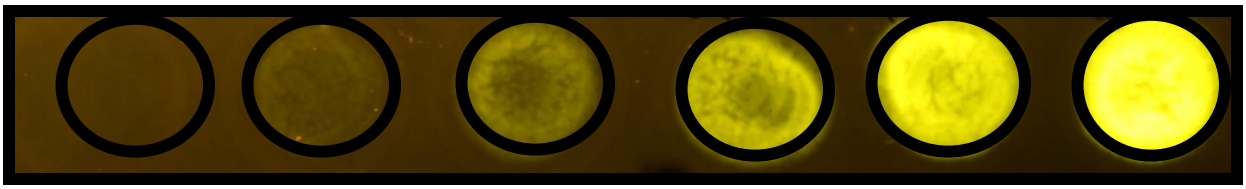
suggests that the Rofin Polilight spans a much wider excitation range than the manufacturers reported range.



1 **2** **3** **4** **5** **6**

Figure 1: Fluorescence Scale Standard

2. Mechanisms of Fluorescence

One of the initial phases of this project was to define the fluor or fluorophore responsible for the fluorogenic properties of Zar-Pro™ lifted impressions. It was previously assumed that the use of titanium dioxide in the Zar-Pro™ Lifters may be associated with metal-enhanced fluorescence (MEF) [68]. Several indicators suggested that if this was truly MEF, it would be a novel phenomenon in this area, as most examples of MEF occur with excitation in the ultraviolet (UV) frequencies with resulting emissions only shifted to slightly longer frequencies in the UV spectrum [15, 21]. The wavelengths used to excite the Zar-Pro™ Lifters were outside the UV spectrum, generally around 505nm with emissions in the yellow-orange frequencies between 530 nm and 610 nm. In addition, the rutile titanium dioxide used in Zar-Pro™ Lifters was spin coated with aluminum which does have the ability to carry out MEF in the UV range of excitation and emission [15]. Thus, the fluorescent phenomenon achieved with Zar-Pro™ was deemed not to be associated with MEF. Still, the intensity observed in the longer wavelengths suggested that this was a novel phenomenon yet to be defined.

The fluorescence phenomenon is also strongly dependent on the solvent context involved, thus an immediate determination of the molecules could not be made simply by looking at published fluorescence spectra as they are in different chemical contexts. Biological fluorescence can be observed in cellular molecules such as proteins, flavins, cholecalciferal, nicotinamide adenine dinucleotide (NADH), lipofuscin, and riboflavin [17, 22, 69]. Of these molecules, flavins, lipofuscins, and porphyrin contain known fluorophores, which can emit fluorescence when excited with alternate light sources. Protein based fluorescence as the basis for Zar-Pro™ fluorescence however was eliminated, as known examples of this type of fluorescence are excited in the middle UV range (260-310 nm) with subsequent emissions in the near UV range (300-400 nm). The fluorescence observed in Zar-Pro™ Lifters is excited at around 505 nm with emission from 530-610 nm.

Derivatives of the Zar-Pro™ formulation were made by eliminating ingredients one at a time with titanium dioxide the first to be omitted. It was discovered that that the fluorescence seen in Zar-Pro™ Lifters was titanium dioxide independent. This led to a search for an inherent biological fluor responsible for the observed fluorescence. These efforts were hampered by the

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inability to replicate fluorescence in a liquid phase which prevented accurate quantization of the intensity and wavelengths associated with excitation and emission. The titanium dioxide was replaced with metal salts of aluminum, zinc, and silver using both nitrate and sulfate forms. Various reducing agents were utilized to reduce the metal salts in order to obtain small particle metal forms in the Lifters derivatives. None of these derivatives produced an enhancement of the existing fluorescence when compared with the original formula. Therefore, it was suggested that the fluorescence of blood on Zar-Pro™ Lifters was likely inherent fluorescence (autofluorescence). Preliminary results hinted that there were likely two chemicals responsible for inducing the fluorescence present in the Zar-Pro™ formulation. An absolute requirement for the more intense fluorescence was the presence of acid incorporated into Zar-Pro™ formulation. A secondary minor fluorescence appeared to be a result of the presence of surfactant in the lifter. The inherent fluorescence is believed to be normally quenched by an inner filtering effect of some component within blood or by the fluor itself not obtaining a fluorescent mode until it is exposed to the chemical milieu within the Lifter.

Further tests indicated that purified hemoglobin was capable of fluorescing when whole blood was exposed to conditions present in the Zar-Pro™ formulation. In response, purified hemoglobin was then ultra-filtered through a 10 kD cutoff filter and the red flow-through filtrate was fluorescent when combined with n-butanol. The fluorescent flow-through confirmed that the fluor is less than 10 kD and the red coloration suggests that the fluor is most likely the protoporphyrin-IX component of the heme. A previously published fluorescent spectral profile of protoporphyrin-IX excitation and emission [48] displayed a shortened excitation wavelength and a slightly longer emission wavelength when compared to the ones obtained in the current study. This could be an artifact of the solvents used in the current study, resulting in an increase in the intensity of obtained fluorescence.

In addition to the conversion of the porphyrin into a fluorogenic state, it was proposed that there may be a conversion of the salicylate fixative into a fluorescent molecule by formation of a methyl-salicylate through Fischer esterification. Previous studies looked at methyl salicylate under far UV excitation and observed fluorescence in the blue 430 – 470 nm range [33]. In this study, when methyl-salicylate was excited with an intense alternate light source (wide range around 505 nm) it fluoresced in the yellow wavelengths between 540-590 nm. The conversion appeared to be pseudoenzymatic in nature, as it required blood and acid as a catalyst but persisted after denaturing conditions for enzymes.

In pursuing the identity of the fluor in blood, the blood was fractionated and it was discovered that a fluor localized to the cytoplasm of red blood cells (RBCs) was fluorescing. Subsequent fractionation of cytoplasmic extract showed that an element found in the 40% ammonium sulfate cut fraction was responsible for generating the bulk of the fluorescence. This fraction retained the red color associated with heme. Further fractionation of this material by ultra-filtration though a 10 kD cutoff filter suggested that the fluor was less than 10 kD in size.

When a purified hemoglobin preparation was subjected to ultrafiltration through aYM10 kD cut off filter (Amicon), the resulting red filtrate demonstrated similar fluorescence

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enhancement. Leading to the belief that the porphyrin component of the heme is becoming fluorogenically enhanced. It is speculated that in an unaltered state, fluorescence of a protoporphyrin-IX molecule is quenched. The solvent/detergent treatment relieved the quenching effect, either by relief of inner filtering effects or by allowing the heme to adopt a conformation change conducive to fluorescence. Further studies will be focused on the purified porphyrin to see if the effect can be further enhanced. Because fluorescence can be detected in extremely old and color faded blood impressions that have been lifted with Zar-Pro™, it is suspected that the iron in the heme is not required for this fluorescence. Due to the structural similarities of protoporphyrin-IX and chlorophyll, chlorophyll extracts were blotted onto Zar-Pro™ Lifters resulting in intense fluorescence when observed with the 505 nm Rofin Polilight and were observed to be shifting into red wavelengths (600nm or above). Future experiments will examine if intensity or emission spectrum are altered when iron is chemically chelated from the protoporphyrin.

3. Primary Fluorogenic Enhancement Spray Development

A project goal was to develop a novel non-toxic, easy to use, fluorogenic spray for use during evidence collection at crime scenes that is suitable for both blood and non-blood impression evidence. The initial strategy was to replicate the Zar-Pro™ formulation in a liquid form independent of the Zar-Pro™ membrane. To this end, a spray was prepared using ingredients in the Zar-Pro™ Lifters. The initial formulation was weakly effective in imparting fluorescence to blood impressions, but was ineffective for most of the other fluids examined (semen, saliva, eccrine-sebaceous, and vegetable oil). Preliminary efforts demonstrated the spray derivatives of Zar-Pro™ were ineffective across all biofluids thus the decision was made to explore alternative fluorogenic spray methods. Additional formulations of the Zar-Pro™ sprays may still be explored in the future.

The following spray formulations are considered to be generally non-toxic, as the concentrations of components are well below the LD_{50} values in their concentration ranges and are unlikely to be ingested or inhaled when used as directed. The sprays were also carried in a 50% methanol to water composition determined to be optimal in fixing impressions, with the added benefit that in this concentration is nonflammable.

The Enhancement Sprays were developed in the project study design phase and are novel for use in forensic science. These sprays were deemed effective in enhancing impression deposited in blood and other biofluids in preliminary studies and were therefore selected for use in the research trials.

Novel Dye Stain Spray #1 consisted of: 2% 5-sulfosalicylic acid, 0.5% salicylic acid, 20% Photoflo™ (triton x-114), 3 mg/ml methylene blue, 6% PEG-8000 (polyethylene glycol average molecular weight 8000), 5 mM EDTA, 50% methanol. The spray was prepared and then filtered through a Millipore 0.45μm polysulfone filter. Subsequent to the Aged Trials, methylene blue in this formulation was scaled back to 0.5 mg/ml to enhance contrast in subsequent trials.

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Spray #1 was based on initial attempts to replicate the chemistry found in the Zar-Pro™ formulation. It included the component, 5-sulfosalicyclic acid that was found to be necessary for the primary fluorescence yield in the Lifters. After examining multiple concentrations of this fixative, it was determined that 2% was optimal for yielding fluorescence. A second salicylate was included that was found to crystallize at low concentration to potentially highlight ridges where crystallization might nucleate. Another component found to be responsible for inducing a minor fluorescence of blood was the Photoflo™ surfactant (triton X-114). PEG-8000 was included in the recipe to provide viscosity to allow the formulation to persist on the impression and react longer. The addition of ethylenediaminetetraacetic acid (EDTA) was included to chelate any divalent cations in the impression that might lead to DNA breakdown. A basic stain, methylene blue, was added to Spray #1 to allow immediate enhancement and visualization of an impression. A precedent indicating that this stain could interact with blood was provided by its use in Wright's blood stain [31]. In addition, the fluorescence of methylene blue was explored when excited using a Rofin 505 nm alternate light source. Although lifted impressions were not fluorescent in the 505nm wavelength, the spray did impart some fluorescence in the red wavelengths above 620 nm.

Novel Fluorogenic Dye Stain Spray #2 consisted of: 2% 5-sulfosalicylic acid, 0.5% dinitrosalicyclic acid, 9 mg/ml Coomassie brilliant blue R-250, 1.6 mg/ml bismark brown, 0.5 mM EDTA, and 50% methanol prepared and filtered through a Millipore 0.45μm polysulfone filter, and was an attempt to create a multipurpose broad protein enhancement reagent. The use of 5-sulfosalicylic acid was included in Spray #1 to react with blood and to help to yield fluorescence. The secondary salicylate, dinitrosalicyclic acid was added as it exhibited a more intense fluorescence yield in previous studies, but tended to stain the Lifters yellow. This would be less of an issue at lower concentration independent of the lifter. The primary protein tagging reagent in this formulation is Coomassie blue R stain. This stain has a long history of binding to proteins [58] under acidic conditions. The additional stain, Bismark Brown was used to prevent Coomassie blue from interacting nonspecifically with substrates by forming an ion-pairing complex with excess Coomassie blue [14]. The recent discovery that Coomassie blue can be fluorescent in the near IR when excited by orange light [10] also provided impetus for its inclusion in this formulation. Ethylenediaminetetraacetic acid (EDTA) was added to help preserve DNA by inhibiting nucleases that require Mg as a cofactor. Preliminary experiments with Spray #2 showed it to be highly effective in binding impressions with high levels of protein such as blood and semen.

Novel Fluorogenic Dye Stain Spray #3 consisted of: 2% 5-sulfosalicyclic acid, 3 mg/ml Eosin Y, 0.0006% TiO_2, 20% Photoflo™ (Kodak) and 50% methanol prepared and then filtered through a Millipore 0.45μm polysulfone filter. Spray #3 was designed to be a dye stain capable of binding blood impressions and potentially other protein based impressions. It contains 5-salicylic acid as a fixative and acidifying agent to generate positive charges on neutral pKa proteins. The choice of dye for this formulation is Eosin Y. Eosin has traditionally been used as a blood stain [32], but its use in forensics had not been reported. Chemically, Eosin Y is a

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brominated derivative of fluorescein. Initial experiments used fluorescein as a dye stain because it had been reported to be effective in enhancing latent blood impressions [36], but it was not effective in this instance. It was surmised that Eosin might have an advantage over fluorescein because it has an established affinity for blood [32], and it retains its fluorescence down to pH 1.5 (unpublished data) whereas fluorescein loses its fluorescent nature below pH 4.0. A small amount of suspended TiO_2 in what was assumed to be nanoparticle form was included in this formulation as a potential contrasting agent to reflect emitted yellow wavelengths from the Eosin Y. This dye stain has demonstrated the ability to bind all of the body fluids examined in this study or alternately bind to the substrate and provide an impression negative.

Significant Results

The information obtained by studying the fluorescent phenomenon of Zar-Pro was used to develop three novel Fluorogenic Enhancement Sprays for use in the Substrate and Aged Trials. Spray #1 will be utilized in Trial 5 (T5), Spray #2 will be utilized in Trial 6 (T6), and Spray #3 will be utilized in Trial 7 (T7).

4. Secondary Fluorogenic Enhancement Spray Development

The study of solvents used to activate Zar-Pro™ Lifters outside of the manufacturer's Activator solution were explored. The percentage of alcohol in the activator solution was increased, resulting in a loss of clarity in the lifted impression, whereas when the alcohol in the activator was decreased, fluorescent intensity diminished, as it prolonged the developments of fluorescence. A series of other solvents, both more and less polar to include ethyl acetate, acetonitrile, acetone, n-butanol, petroleum ether, ethanol, isopropyl alcohol, and hexane were explored to increase the fluorescence output of activated Zar-Pro™ Lifters. The solvents close in polarity to methanol, ethanol and isopropyl alcohol, performed much like methanol but one solvent, n-butanol, demonstrated intensified fluorescence.

Butanol along with a variety of detergents were found to generate fluorescence in a liquid phase, thus a spectrofluorimeter could be employed to confirm the optimum excitation wavelength, previously determined to be centered at 460 nm. The broad emission spectrum peak spanned from 520 nm to 620 nm. Whereas normal blood and hemoglobin preparations containing heme exhibited minimal fluorescence when excited at 460 nm in the absence of butanol, salicylate, and detergent, however, these reagents alone or in combination exhibited a broad peak of fluorescence attaining a 200 fold increase of inherent fluorescence.

Based on above mentioned experimental trials, the use of n-butanol was deemed not be a suitable replacement for Zar-Pro™ Activator as it did not preserve the impression integrity but it may be useful as a Secondary Enhancement Spray to increase the fluorescence of Zar-Pro™ lifted impressions after the impression is recovered.

Secondary Enhancement Spray consisted of: 25% ethanol, 25 % n-butanol, and 2% -sulfosalicyclic acid and applied from an atomizing spray bottle. This Spray was applied to Zar-

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Pro™ Lifters after two days to determine if fluorescent intensity of the impression could be intensified to aid in visualization of impression details.

Significant Results

The information obtained by studying the fluorescent phenomenon of Zar-Pro™ was used to further the development of a Secondary Enhancement Spray which was used to increase the fluorescent output of the Zar-Pro™ Lifters and to expedite the peaking of fluorescence. The Spray was deemed effective in the study design phase of the project and was utilized in Trial 4 (T4) the Substrate and Aged Trials. The Secondary Enhancement Spray was used after 2 days and measured against Zar-Pro™ in accordance to the manufacturer's guidelines as used in Trial 3 (T3) to determine if fluorescent intensity of Zar-Pro™ lifted impressions was increased.

5. Impression Optimization

A number of factors can affect the quality of deposited impressions, making it difficult to create consistent and reproducible impressions for analysis in research trials. In order to determine the effectiveness of the Zar-Pro™ Fluorescent Lifters and the newly developed Fluorescent Enhancement Spays utilized in this project it was crucial that the impression quality remain consistent throughout the trials. This consistency allows for the rating of impression quality based on the chemical and physical enhancement methods not on variations of quality in the deposited impressions. In order to create consistency in the deposition of impressions the laboratory temperature was controlled, as well parameters associated with impression deposition; such as the ratio of friction skin surface area to the volume of biological fluid, pre-deposition waiting time, deposition pressure and angle, and deposition pressure time. These deposition parameters were standardized during the project study design phase and adopted for each biological fluid (eccrine/sebaceous sweat, non-human oil, blood, semen, and saliva) for the fifteen substrates utilized in the research trials.

The selection of the control substrate (glass) and the fourteen additional substrates selected for the Substrate and Aged Trials were also of importance in this project as many biofluids have the ability to hold impression evidence but the substrate onto which the impression is deposited strongly affects the impression quality, as well as the variables associated with the deposition. The most important factor when considering substrate is porosity, which is sub-divided into three main categories; non-porous, semi-porous, and porous. Non-porous substrates do not absorb biological fluid deposited with an impression, leaving the impression on the substrate surface. Semi-porous substrates absorb some of the biological fluid that is placed along with an impression onto the surface, but some of the impression may still remain on the surface of the substrate. Porous substrates completely absorb the fluid in which the impression is deposited leaving little to no residue on the substrate surface. Other factors outside of porosity were considered in the substrate selection, such as the background color(s), pattern(s), and degree of texture, all of which can inhibit visualization of impression details.

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Substrates of various degrees of porosity were chosen for this study based on their ability to hold impression evidence and their likelihood of being encountered at crime scenes.

Substrates

The non-porous substrates included clear glass (standard microscope slides, Carolina Biological Supply Company), silver aluminum (heavy duty foil, Reynolds Wrap), multi-colored grey and beige ceramic tile (Concerto, Menards), multi-colored grey stainless steel (peel and stick matted tile, Aspect Metal), black and white vinyl tile (Nexus, Menards), and black and white plastic (gentleman's shower curtain, Kikkerland). The semi-porous substrates included white glossy paper (standard gloss photo paper, Office Depot), red painted drywall (Zinsser Primer and Pittsburgh Paint, eggshell finish) black polyvinyl leather (JoAnn Fabrics), light brown finished wood (polyurethane, Minwax), and multi-colored wall paper (gourmet coffee border, Better Homes and Gardens). The porous substrates included blue poster board (Office Depot), light brown untreated wood (paint sticks, Home Depot), brown cotton (700 thread count sheet set, Better Homes and Gardens), and blue denim (JoAnn Fabrics). A diverse sample set of substrates were selected with various colors and background patterns (including some variations in texture) to replicate substrates that would require chemical or physical enhancement in order to detect impression details.

Substrates and any items that might come into contact with the impressions were treated with short wave UV light by exposure to germicidal mercury halide fluorescent lamps in a dedicated light box. The box utilized three GE G25T8 germicidal tubes arranged at 20 cm distance from the substrates to be treated. Substrates were exposed to three hours UV on each side at an intensity of 18 $\mu W/cm^2$. The substrate samples were then individually packaged to prevent cross-contamination.

Personal Protective Equipment

All laboratory personnel were dressed in proper personal protective equipment (PPE); such as disposable laboratory coats, hair nets, masks, and nitrile gloves when working in the research laboratory. Nitrile gloves were selected over latex gloves because latex contains proteins that may lead to contamination of the biofluids being tested. All laboratory personnel were dressed in proper PPE to protect the researchers from contact with human biofluids, as well as to minimize contamination issues while working with multiple biofluids in the laboratory. Gloves were changed at 20 minute intervals during experimentation and between biofluids to avoid cross contamination. All waste was disposed of according to institutional guidelines.

Work Stations

Separate work stations were established for each of the different trials and cleaned thoroughly with 20% bleach solution before use and between biofluids to minimize potential contamination issues. The separation of work stations was based on the impression enhancement trial, with each being conducted across all biofluids (blood, semen, saliva, eccrine/sebaceous

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sweat, and non-human oil) at that particular station. DNA cutting (Substrate Trial 1 and Aged Trials 1 and 2) was conducted in personal prep hoods, Zar-Pro™ enhancement (Substrate Trials 2, 3, and 4 and Aged Trials 3 and 4) was conducted on an isolated laboratory bench top, while spray enhancement (Substrate and Aged Trials 5, 6, and 7) was performed in a fume hood. Attention to contamination issues was essential to ensure the reliability of subsequent DNA analyses being conducted after enhancement in both the Substrate and Aged Trials. DNA analysis was performed in a UV sterilized PCR preparation station to protect the impressions from exogenous DNA in order to ensure authenticity of the DNA extracted from each sample.

In addition to workstation cleanliness, precautions were taken throughout the research trials to prevent fluorescent contamination from ink pens and sharpie markers, which are highly fluorescent leave fluorescent contamination when the samples are visualized under alternate lighting. Therefore, only pencils or liquid graphite pens were used in the research study.

Biofluids

The biofluids used in the study were eccrine/sebaceous sweat, non-human vegetable oil, blood, semen, and saliva. The blood used in setting the optimal deposition parameters was bovine blood obtained from a scientific supplier (VWR) and was replaced by human blood obtained from a scientific supply company (Innovative Research, Inc.) for the research trials. The following biofluids were used in setting the optimal deposition parameters and the research trials. The semen and saliva samples were obtained from human donors, either purchased from a scientific supply company (Innovative Research, Inc) or donated by the depositor. Eccrine/sebaceous sweat was obtained from the skin of a female donor. The non-human oil sample was obtained from 100% pure canola oil (Western Packaging Corporation). The oil sample was incorporated to replicate the likelihood of impression evidence recovered from non-human oils associated with food.

Prior to use the blood was refrigerated (approximately 36°F/2°C) and the semen and saliva were frozen (approximately 32°F/0°C). The blood, semen, and saliva samples were heated to the average core body temperature (98.6°F/37°C) using a mini dry bath (Benchmark) prior to depositions in order to simulate an impression deposited in biofluids shed from a living person. The non-human oil sample was stored and maintained at room temperature (70-75°F/21-24°C) to simulate an impression deposition being transferred from a food source while eating. The eccrine/sebaceous sweat was collected from the female donor's forehead an hour after her face was cleaned with an individually packaged antibacterial moist towelette (Wet Ones). The ingredients in the Wet Ones towelette were assessed to determine inherent fluorescent properties prior to use and did not exhibit any which could affect results in this study. The female donor's forehead temperature (93-95°F/34-35°C) was maintained throughout the deposition process to simulate a subject's average body temperature while depositing sweat impressions. An infrared digital thermometer (Cen-Tech) was utilized to measure temperatures throughout the trials.

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Surface Area Calculations

The depositor's friction skin area was calculated to determine the volume of biological fluid necessary to create optimal impressions for analysis. The deposition friction ridge surface area of the right thumb for two males was calculated by measuring the length and the width of the friction ridge area (length (in) x width (in) = depositor friction ridge surface area). The average surface area of Male A's thumbs was calculated at 1.313 in^2 and Male B's thumbs were calculated at 1.375 in^2. Given the similarity of the surface area, the optimal biological fluid volumes were not altered between donors. The depositor's fingerprint samples were collected with the informed consent of the subject's involved in this study.

Laboratory and Friction Skin Guidelines

The laboratory temperature was maintained between 70-75°F/21-24°C throughout the deposition process using an infrared thermometer (Cen-Tech) and airflow was minimized by working in a closed laboratory area. This is important as the ambient temperature and air flow can have a profound effect on the impression results, especially as they pertain to impression dry times. Prior to depositing impressions the male depositor's washed their hands with water (no soap) to help eliminate environmental contaminants from their friction ridge skin.

The depositor then placed their thumb into a beaker of crushed ice with a thin plastic wrap (Saran) barrier to prevent contact between the ice and friction skin. This minimizes moisture accumulation and keeps the friction skin dry, until the thumb temperature reached 70-71°F/21-22°C using an infrared thermometer (Cen-Tech). This temperature range provides ideal impressions by creating pronounced friction ridges while minimizing the production of eccrine sweat. Once the depositor's skin temperature was in the target range (70-71°F/21-22°C), the non-eccrine/sebaceous biofluids were pipetted onto the depositor's thumb using a P-10, 20 or 100 volume pipette (Gilson) depending on the optimal volume per biological fluid and substrate. Eccrine/sebaceous sweat, however, could not be collected in this manner as it was obtained directly from the female donor's face and forehead. Therefore, it was not possible to quantify eccrine/sebaceous sweat as was done with the other biofluids utilized in this study. The sweat was collected by loading the finger with eccrine/sebaceous material through rubbing the forehead in a circular motion for 5 seconds, to cover the entire friction ridge surface with sweat.

Pre-Deposition Waiting Interval

The pre-deposition waiting interval is defined as the period in which the biological fluid remains on the friction skin prior to deposition. The interval began after the biological fluid was pipetted or loaded onto the depositor's thumb. During this interval, the depositor would rub their index finger against their thumb in a circular motion to facilitate the coating of the entire friction ridge surface area with biological fluid, while keeping the thumb in anatomical position. This thumb position helps to keep the biological fluid on the friction skin while allowing the fluids to become slightly tacky prior to deposition. If the biological fluid is too wet, it will not leave clear ridge details for analysis, and if it is too dry, the biological fluid will not transfer to the substrate.

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The pre-deposition intervals, measured with a digital stop watch (Taylor), vary depending on the fluid and the substrate.

Deposition Pressure and Pressure Interval

The deposition pressure interval is the time in which the friction skin is applied to the substrate to transfer the impression from the friction skin. The male donor deposited the biological fluid covering the friction ridge surface area of their thumb to the various substrates which were placed directly onto a scale. The deposition pressures for the biofluids deposited on each of the fifteen substrates were measured with a digital scale (Taylor) in lbs/in^2 for the duration of the deposition contact. All the depositions were conducted with the depositor's thumb contacting the substrate in a vertical movement to minimize distortion of the friction ridges during deposition.

The deposition pressure interval describes the time the depositor's thumb remains in contact (while applying pressure) with the substrate during the deposition of the impression. These times vary based on the biological fluid and the substrate in which the impression is being deposited; some may have an immediate deposition pressure interval, whereas others may require extended time intervals to produce optimal impressions for analysis. The intervals in these trials were measured with a digital stop watch (Taylor).

Visualizing and Rating the Impression to Determine Optimal Parameters

A rating system was developed for assessing impression details to determine optimal quality impressions. The impression details of interest comprise the overall impression pattern, including ridge paths and deviations, such as bifurcations, enclosures, and ridge endings; without voids in the overall impression and having minimal distortion. Deposited impressions were visualized using normal (NL) and alternate lighting (AL) conditions to determine impression quality.

The visual examination of blood impressions deposited on light colored substrates, as well as the visualization of semen, saliva, eccrine/sebaceous sweat, and non-human oil impressions on light colored substrates was conducted using oblique lighting under normal lighting conditions. Biofluids on dark colored or patterned substrates required visualization with an alternate light source. The fluorogenically enhanced impressions were visualized and rated using a Rofin Polilight Flare Plus (505 nm wavelength) with orange, yellow, or red barrier filters.

Zar-Pro™ Fluorescent Lifters were used according to the manufacturer's directions to lift blood impressions from the substrate onto the white background of the lifter for visualization of blood impressions on dark or porous substrates, such as denim and cotton. The lifted impressions were analyzed under normal lighting and then illuminated with alternate lighting (Rofin Polilight Flare Plus) at 505 nm wavelength with an orange barrier filter to visualize impression details.

Some of the semen and saliva samples were spiked with fluorescein while setting the deposition parameters in this study. The semen and saliva aliquots (2 mL) were spiked with

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fluorescein (2 µL of 4 mg/ml) and then vortexed (Vortex Genie 2) to evenly distribute the fluorescein throughout the biological fluid. The fluorescein enhanced the semen and saliva so the impression details deposited in these biofluids could be visualized with an alternate light source (Rofin Polilight Flare Plus). The fluorescein added to the semen and saliva did not noticeably interfere with the viscosity of the biological fluid. This method was effectively used for all saliva impressions and most semen impressions with the exception of impressions deposited onto glass, aluminum, poster board, and wall paper, which were visualized under normal lighting without additional enhancement.

Methylene Blue, was utilized to provide color in semen and saliva samples while determining the optimal deposition parameters for finished wood. Semen and saliva aliquots (2 mL) for this substrate were spiked with Methylene Blue (6 µL of 2 mg/ml) and then vortexed (Vortex Genie 2) to evenly distribute the Methylene Blue throughout the biological fluid without interfering with the viscosity of the biological fluid. The dyed-blue semen and saliva impressions were visualized on the wood substrate under normal lighting conditions**.** It is important to note that no additives were possible for non-human oil because of insolubility and for eccrine/sebaceous sweat because it was loaded directly from the skin of the female donor.

Significant Results

Optimal deposition parameters can be utilized to create consistent, comparable and reproducible impressions for analysis. This was done by setting deposition parameters for each biological fluid on the various substrates selected in this study. The variables that were adjusted to create optimal quality impressions were volume of biological fluid, deposition pressure, pre-deposition waiting interval and deposition pressure interval. These parameters were optimized and set for fourteen of the fifteen substrates with five biofluids (Tables 1-5). The fifteenth substrate, denim, could only be optimized with blood and semen. The impression details for saliva, eccrine/sebaceous sweat, and non-human oil impressions were very difficult to visualize on this substrate, even with the use of chemical enhancement methods. Therefore, while the parameters set for denim with these three biofluids produced visible impression details on the substrate they did not meet the standards for optimal quality.

Pre-deposition waiting intervals set in this study ranged from 0 to 45 seconds, whereas the deposition pressure interval ranged from 0-10 seconds (Tables 1-5). It is important to note that as the volume of the biological fluid was increased, the pre-deposition waiting interval would also need to be increased to achieve proper drying prior to deposition. In addition, the non-human oil and eccrine/sebaceous sweat impressions did not fully "dry" even after the pressure deposition waiting interval and were therefore more susceptible to being altered or destroyed post deposition.

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Table 1: Deposition Parameters for Eccrine/Sebaceous Sweat Impressions

	Deposition Pressure (lbs/in^2)	Pre-Deposition Waiting Interval/Deposition Pressure Interval (seconds)
Non-porous Substrates:		
Glass	5-6	0/5
Aluminum	5-6	0/5
Ceramic Tile	8-10	0/5
Stainless Steel	8-10	0/5
Vinyl Tile	8-10	0/5
Plastic	8-10	0/5
Semi-porous Substrates:		
Glossy paper	8-10	0/1
Painted Dry Wall	7-8	0/5
Poly Vinyl Leather	8-10	0/5
Finished Wood	8-10	0/5
Wall Paper	5-6	0/5
Porous Substrates:		
Poster Board	8-10	0/5
Untreated Wood	3-4	0/1
Cotton	8-10	0/5
Denim	8-10	0/5

Table 2: Deposition Parameters for Non-human Oil Impressions

	Oil Volume (µl)	Deposition Pressure (lbs/in^2)	Pre-Deposition Waiting Interval/Deposition Pressure Interval (seconds)
Non-porous Substrates:			
Glass	2	5-6	15/5
Aluminum	2	5-6	15/5
Ceramic Tile	3	8-10	15/5
Stainless Steel	1	8-10	15/5
Vinyl Tile	2	8-10	15/5
Plastic	1	5-6	15/5
Semi-porous Substrates:			
Glossy paper	2	8-10	10/5
Painted Dry Wall	2	8-10	10/5
Poly Vinyl Leather	3	8-10	15/5
Finished Wood	3	8-10	10/5
Wall Paper	2	8-10	15/5
Porous Substrates:			
Poster Board	2	2-3	15/5
Untreated Wood	2	2-3	15/5
Cotton	10	8-10	15/5
Denim	15	8-10	10/5

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Table 3: Deposition Parameters for Blood Impressions

	Blood Volume (µl)	Deposition Pressure (lbs/in^2)	Pre-Deposition Waiting Interval/Deposition Pressure Interval (seconds)
Non-porous Substrates:			
Glass	14	8-10	25/10
Aluminum	12	8-10	25/10
Ceramic Tile	12	8-10	25/10
Stainless Steel	14	8-10	25/10
Vinyl Tile	14	8-10	30/5
Plastic	12	8-10	20/5
Semi-porous Substrates:			
Glossy paper	17	8-10	20/5
Painted Dry Wall	18	8-10	25/5
Poly Vinyl Leather	14	8-10	25/5
Finished Wood	18	8-10	30/5
Wall Paper	17	8-10	25/5
Porous Substrates:			
Poster Board	20	8-10	45/5
Untreated Wood	18	8-10	20/5
Cotton	20	7-8	45/5
Denim	20	5-6	30/10

Table 4: Deposition Parameters for Semen Impressions

	Semen Volume (µl)	Deposition Pressure (lbs/in^2)	Pre-Deposition Waiting Interval/Deposition Pressure Interval (seconds)
Non-porous Substrates:			
Glass	15	8-10	33/0
Aluminum	15	8-10	30/5
Ceramic Tile	12	8-10	20/10
Stainless Steel	16	8-10	20/10
Vinyl Tile	15	8-10	20/5
Plastic	12	8-10	25/5
Semi-porous Substrates:			
Glossy paper	17	8-10	20/5
Painted Dry Wall	18	8-10	25/5
Poly Vinyl Leather	16	8-10	25/5
Finished Wood	18	8-10	30/5
Wall Paper	25	8-10	45/5
Porous Substrates:			
Poster Board	30	8-10	45/5
Untreated Wood	18	8-10	20/5
Cotton	20	7-8	30/5
Denim	18	5-6	30/10

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Table 5: Deposition Parameters for Saliva Impressions

	Saliva Volume (µl)	Deposition Pressure (lbs/in^2)	Pre-Deposition Waiting Interval/Deposition Pressure Interval (seconds)
Non-porous Substrates:			
Glass	15	8-10	30/0
Aluminum	15	8-10	35/0
Ceramic Tile	14	8-10	20/5
Stainless Steel	14	8-10	20/5
Vinyl Tile	14	8-10	20/5
Plastic	14	8-10	20/5
Semi-porous Substrates:			
Glossy paper	15	8-10	20/5
Painted Dry Wall	16	8-10	15/5
Poly Vinyl Leather	14	8-10	20/5
Finished Wood	20	8-10	25/5
Wall Paper	14	8-10	20/5
Porous Substrates:			
Poster Board	14	8-10	20/5
Untreated Wood	14	8-10	20/5
Cotton	18	5-6	25/5
Denim	25	1-2	45/0

The biological fluid in which the impression was deposited was the most relevant factor in creating consistency of impressions, due to variations in composition and viscosity. This strongly affected the volume of fluid and the pre-deposition waiting and deposition pressure intervals. Substrate porosity was also an impactful variable affecting the consistency and reproducibility of the impressions in all biofluids. This was noticeable in general but also within each of the porosity ranges (non-porous, semi-porous and porous). The volume and deposition pressure were closely tied to the substrate porosity and texture.

The deposition parameters were specific to each biological fluid and could not be compared directly. When comparing eccrine/sebaceous sweat, non-human oil, blood, semen, and saliva on a non-porous substrate such as glass, in order to produce optimal impressions, the volume, deposition pressure, pre-deposition waiting interval, and deposition pressure interval are all different. The same parameters would not produce consistent impressions amongst these biofluids. Lighter deposition pressures appeared to prevent dissipation of the ridge details into the more porous substrates. Eccrine/sebaceous sweat and non-human oil impressions generally required less deposition pressure than the other biofluids. This variant seemed to be due to the dispersant properties of the oil-based biofluids, which often resulted in impression distortion as deposition pressure increased. In decreasing the deposition pressure, this problem was alleviated and optimal impressions were deposited. It was observed in this study that the volume of biofluids, as well as deposition pressure did create some tonally reversed impressions. This phenomenon was primarily observed in the deposition of blood impressions deposited on glossy paper.

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The level of porosity of substrates is highly variable even within the subcategories studied; this was further influenced by the texture of the particular substrates. The texture and porosity greatly influenced the volume of biological fluid and deposition pressure necessary to create optimal impressions regardless of the porosity subcategory. By increasing volume for some of these biofluids, the pre-deposition waiting intervals are also affected. These intervals were utilized to allow the biological fluid to dry just enough to transfer an optimal impression to the various substrates. It was determined that a longer pre-deposition waiting interval was preferred over a longer deposition pressure waiting interval, as it was evident that optimal quality impressions were achieved when the biological fluid was allowed to dry on the friction skin as opposed to during the contact with the substrate. This is most evident in the porous/textured substrates, cotton and denim.

One of the challenges encountered during optimization of deposition parameters was in visualization of impressions deposited on various substrates for some biofluids. Substrate background color and pattern had a large impact on the ability to visualize the deposited latent impressions. Impressions deposited on non-porous light colored substrates were easily visible for analysis, while porous and dark colored, and patterned or textured substrates were the most difficult to assess. This was due to the substrate absorbing the biological fluid rather than it merely sitting on its surface making the impression harder to visualize with oblique lighting under normal lighting conditions. And if impressions were visible, the impression details were often obscured due to the background pattern and color which concealed or mimicked the ridge details of the impression. Therefore, some of the optimal deposition parameters were set by using chemical or physical enhancement methods to visualize impressions and assess impression quality.

Denim was a problematic substrate in this study, as it was difficult to visualize impression details deposited in saliva, eccrine/sebaceous sweat, and non-human oil impressions. The porosity of the denim, as well as the dark colored and textured weave-patterned background made the visualization of impression details on the substrate surface difficult, even with the addition of the fluorescein. The eccrine/sebaceous sweat and non-human oil samples could not be spiked with Fluorescein or Methylene Blue due to solubility issues with the biological materials. Finished (polyurethane coated) wood was another difficult substrate in regard to the deposition of semen and saliva impressions, as the biological fluid was quickly absorbed into the substrate and the ridge detail was obscured, possibly due to the reflective properties of the polyurethane.

The optimal impressions being proposed for research purposes are unrealistic to actual evidence submitted for analysis in association with criminal cases. However, optimal quality impressions are necessary as a benchmark for the creation of depletion series, thus serving as the study control. A depletion series starting with an optimal (control) impression would provide a comprehensive series ranging from optimal to faint impressions. This method is best suited to test the effectiveness of new products, conduct chemical and physical enhancement trials for the comparison of existing methods, as well as to validate enhancement methods for laboratory use.

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Furthermore, the depletion series impressions can also be cut in half (depending on the substrate) after deposition to conduct a side by side, split series comparison of techniques. Dilution series can also be conducted by diluting some biofluids with water to replicate crime scene clean-up situations, while still maintaining consistency of impression quality throughout the trials.

Optimizing these parameters across a broad range of biofluids and substrates demonstrated the interaction between these variables, and that no single variable can be separated from the influence of the others examined. These results were replicated multiple times showing consistency across all trials. Given that the volume of biological fluid necessary to create an optimal impression is closely tied to the surface area of the friction skin of the donor, it is important to recognize that to change the volume will require that all other parameters be adjusted accordingly. Therefore, to implement these guidelines, changes can be calculated by keeping the relationships between the substrates and parameters consistent with those provided in Tables 1 – 5.

The optimal deposition parameters are necessary to assess the effectiveness of any physical and chemical enhancement methods associated with impression related projects. During the deposition phase of research trials, it is often not possible to visualize the deposited impression. Therefore, it is necessary to have optimal parameters that can be trusted to produce consistent impressions for research. The guidelines produced in the study design phase were applied to all the impression deposited in the Substrate and Aged Trials.

6. Substrate Trials

The premise of the Substrate Trials was to determine effectiveness of the Zar-Pro™ Lifters and the three novel primary Fluorogenic Enhancement Sprays (Spray #1-3) to enhance impression deposited in biofluids (blood, semen, saliva, eccrine/sebaceous, and non-human oil) across a broad range of substrates (14 in total). Zar-Pro™ Lifters are utilized in forensic science for the detection, enhancement, and preservation of blood impressions, but attempts had not been made to explore this methodology with other biofluids. The Substrate Trials were designed to test the ability of Zar-Pro™ Lifters to lift, enhance, and preserve semen, saliva, eccrine/sebaceous sweat, and non-human oil impressions from substrates of varying porosity, background color/pattern, and texture. The possibility of using a Secondary Enhancement Spray to increase fluorescence on Zar-Pro™ lifted impressions to determine if visualization of impression details could be improved for analysis was also explored. The second aim of the project was to determine the effectiveness of three novel Dye Stain Sprays (#1, #2, and #3) developed in the course of this project. The final aim was to determine whether DNA contained within the ridge details of the enhanced impressions would still be viable for analysis.

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Impressions were deposited using the optimal deposition parameters determined for blood, semen, saliva, eccrine/sebaceous sweat, and non-human oil on fourteen substrates (Tables 1-5). The Substrate Trial protocols are defined as follows:

Trial 1 (T1) *- DNA Substrate Control*
Trial 2 (T2) *- Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) after 1 hour (control)*
Trial 3 (T3) *- Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) after 1 day*
Trial 4 (T4) *- Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) after 1 day and treated with a secondary fluorescent enhancement spray after 2 days*
Trial 5 (T5) *- Novel Dye Stain Spray #1 (blue color)*
Trial 6 (T6) *- Novel Fluorogenic Spray #2 (blue color)*
Trial 7 (T7) *- Novel Fluorogenic Spray #3 (pink color)*

DNA Substrate Controls (T1)

An impression free substrate sample was packaged separately to provide a substrate control to eliminate the possibility of DNA contamination from the substrate itself.

Application of Zar-Pro™ Fluorescent Lifters (T2, T3, and T4)

The Zar-Pro™ Fluorescent Lifters and Zar-Pro™ Activator Solution were purchased from a forensic science supplier (Tri-Tech Forensics, Inc.). The Lifters were cut into 1”x3” pieces and a small diagonal cut was made in the upper left corner of the non-activation side to ensure the activation side would be applied to the impression area at the time of use. The Lifters were then re-sealed in their original packaging. The Zar-Pro™ Activator Solution was stored in the research laboratory.

The Zar-Pro™ Fluorescent Lifters were activated using the Zar-Pro™ Activator Solution which comes in a pump spray bottle. The Activator Solution was applied to the Lifters until completely saturated (approximately five pumps). After spray activation, the lifter was air dried for 30-45 seconds to allow for the evaporation of any pooled alcohol; excessive alcohol left on the Lifters could alter the impression. Evaporation happens quickly and can be visualized as it is occurring. After the lifter was activated without any observable alcohol pools, it was ready to be applied to the impression area. The Lifter was placed onto the substrate with the activation side on lifter contacting the impression (the diagonal cut corner of the lifter on the left). The user then applied 8-10lbs of pressure measured using a digital scale (Taylor) with their hand for 30 seconds to the backside (non-activation side) to completely affix the blood impression to the lifter. The Lifters were then carefully removed from the substrate for analysis.

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Application of Fluorogenic Dye Stain Sprays (T5, T6, and T7)

The Fluorogenic Enhancement Sprays (#1, #2, and #3) were freshly made in a 250 ml stock and stored in an atomizing spray bottle. A single full pump of spray (1.2 ml of the formulation) was applied to each impression area at a distance of 12 cm while the substrates were affixed in a horizontal position. After being sprayed, the substrates were then moved to a vertical position and allowed to incubate for one minute before being rinsed. The rinse solution consisted of a pre-made 50% methanol water mixture that was stored in an atomizing spray bottle. The rinse solution was sprayed onto the substrate twice (1.2 ml each spray) from 15 cm distance and allowed to dry in a vertical position for two minutes. The dried impressions were placed on trays for analysis.

Application of Secondary Enhancement Spray (T4)

The Enhancement Spray was applied to Zar-Pro™ lifted impressions, 2 days post enhancement for trial four (T4) in the Substrate Trials. The Secondary Enhancement Spray consisted of 25% ethanol, 25 % n-butanol, and 2% sulfosalicyclic acid. It was applied to lifted impressions affixed to the Zar-Pro using an atomizing spray bottle at a distance of 15 cm until visible wetness was observed on the Lifter.

Rating of Impression Detail and Fluorescence Intensity:

Impression details and fluorescent intensity were rated prior to enhancement (T2-T7), after one hour (T2), one day (T3-T7), two day (T2-T7), and one week (T2-T7) intervals under normal (NL) and alternate lighting (AL) conditions using the following guidelines:

Impression Details (ID)

0 = no visible proteinaceous material, no visible ridge detail
1 = visible proteinaceous material, no visible ridge detail
2 = visible proteinaceous material, visible ridge detail; such as a partial impression pattern with minimal ridge details
3 = visible proteinaceous material, visible ridge detail: overall impression pattern, ridge paths and deviations, such as enclosures, dots, ridge endings, bifurcations, etc.

Fluorescence Intensity (FI)

0 = no visible fluorescence
3 = moderate fluorescence
6 = intense fluorescence

One examiner rated the impression details of the five biofluids on the fourteen substrates prior to enhancement under both normal and alternate lighting. Two examiners then independently rated the impression details and fluorescent intensity after one hour (T2), one day

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(T3-T7), two day (T2-T7), and one week (T2-T7) intervals post enhancement under normal (NL) and alternate lighting (AL).

Visualization with alternate lighting

The alternate lighting used in the scope of this project was the handheld Rofin Polilight Flare Plus and 505 nm Cyan head, which provided a high-intensity light in the range of 505 nm and has the ability to generate fluorescence for the visualization of impressions with orange barrier filter goggles (Arrowhead). The pre-made solid phase fluorescence scale, ranging from 0-6 was used in the research trials to provide a standard of measurement for fluorescent intensity (Figure 1).

Biofluid impressions deposited on the fourteen substrates were visualized with alternate lighting prior to enhancement, helping to detect the impression area which was then marked on the substrate using a sterile pin. The marking of the impression area helped to direct the enhancement method to the impression area, this was especially important for the latent impressions deposited in semen, saliva, eccrine/sebaceous sweat, and non-human oil which were not visible under normal lighting conditions. The alternate light source and orange barrier filter goggles (Arrowhead) were also used to fluoresce the enhanced impressions for ratings conducted at the one hour, one day, two day and one week intervals.

Photography

Photographs were taken of all the Substrate Trials with a Canon EOS T5i digital SLR fitted with an EF 100 mm Macro lens 1:2.8 ratio at 12.5 inches away from the stage. Orange barrier light filtering was done with a 0.125 '' thickness orange acrylic plexiglass #2422 amber sheet. The 505nm light to excite impressions was provided by a Rofin Polilight Flare mounted nine inches away from the stage at an oblique angle of 30 degrees. The sensitivity was set to ISO 100 with F stop set to an aperture of 5.6. The shutter speed was set to open for 6 seconds for weakly fluorescent samples and 1 second, or $1/25^{th}$ second, for more strongly emitting samples, such as those deposited on glossy paper.

Subsets of impressions deposited on the fourteen substrates were photographed under normal and the alternate lighting prior to enhancement. A full set of photographs were taken under normal lighting conditions at the one day interval under normal lighting conditions, at the two day interval under alternate lighting condition, and again under alternate lighting at the one week interval. Standardization of photography was conducted in order to facilitate the photography of all samples at the above mentioned intervals in an efficient manner, however, this practice resulted in less than optimal photographs. In the future, optimal photography parameters will be set per substrate and biofluid to improve the quality of the resulting sample set.

The photographs were labeled and stored on an external hard drive in the research lab and have been archived as part of the raw data from the research trials in accordance with institutional guidelines.

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Storage

All impressions in the substrate impression trials were stored in paper envelopes labelled and affixed to a file folder which was then placed in a plastic sheath and stored in a three ring binder separated by the biofluid. The binders were then placed in to sterile plastic totes for storage in the research laboratory.

Significant Results

The Substrate Trial ratings have been graphed for two examiners (Appendix A: Data/Graph Sets 1-10). Based on the preliminary review of this data, the Zar-Pro™ Lifters utilized in T2, T3, and T4 were very effective for lifting blood impressions (Appendix A: Data/Graph Sets 1 and 6). The lifted blood impressions could be visualized under normal lighting (Figures 2, 4, 6, 8, 10, and 12) due to the red-brown color of blood, and exhibited intense fluorescence when visualized with alternate lighting (Figures 3, 5, 7, 9, 11, and 13). Semen (Figures 14 and 16), saliva, eccrine/sebaceous sweat, and non-human oil could not be visualized on the Zar-Pro™ Lifters under normal lighting (Appendix A: Data/Graph Sets 2-5 and 7-10), as they do not contain coloration to aid in visualization against the white background of the Lifter. Yet, when visualized under alternate lighting these fluids did exhibit fluorescence, allowing for the visualization ridge details in semen (Figures 15, 17-21) and some details in saliva (Figures 22-24) lifted impressions, whereas most of the eccrine/sebaceous sweat and non-human oil impressions merely indicated the presence of biofluids (Appendix A: Data/Graph Sets 2-5 and 7-10).

T2, T3, and T4 demonstrated that the Lifters effectively lifted and enhanced blood impressions from the majority of substrates tested in these trials (Figures 2-13) (Appendix A: Data/Graph Sets 1 and 6). Semen impressions were effectively lifted and enhanced from non-porous (Figures 14-15, 18-19) and semi-porous substrates (Figures 16-17, 20-21), as well as some porous substrates (Appendix A: Data/Graph Sets 2 and 7). The trials indicated that Zar-Pro™ Lifters could be used to effectively enhance blood and semen impressions across a broad range of substrates, which was primarily due to the Lifters ability to remove substrate variables that affect visualization by lifting the proteinaceous material from the substrates. The fluorescent properties of the Lifters were also important as biofluids used in this study were latent in nature, with the exception of blood. Thus the ability of the Zar-Pro™ Lifters to impart fluorescent properties to lifted impressions was valuable in the visualization of impression details for analysis.

The age interval between T2 (1 hour) and T3 (1 Day) prior to lifting the deposited impression was not a relevant factor in the enhancement of blood (Appendix A: Data/Graph Sets 1 and 6) or semen (Appendix A: Data/Graph Sets 2 and 7), but may have had an impact on the saliva impressions, as the ridge detail appeared to improve as the impression aged (Appendix A: Data/Graph Sets 3 and 8). The Secondary Enhancement Spray utilized in T4 after 2 days appeared to decreased fluorescence intensity of saliva lifted impressions but increased the fluorescence of blood, semen and non-human oil lifted impressions. However, the increase in

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fluorescence intensity did not correlate to the improved visualization of impression details as ridge details appeared to be altered or destroyed from use of the Secondary Enhancement Spray (Appendix A: Data/Graph Sets 1-10). The Primary Fluorogenic Enhancement Sprays utilized in T5 (Spray #1) was effective for the enhancement of blood (Figures 25-26) impressions (Appendix A: Data/Graph Sets 1 and 6) and T6 (Spray #2) was effective for blood (Figures 27-28), saliva, and semen but neither spray exhibited fluorescence (Appendix A: Data/Graph Sets 1-3 and 6-8).

The spray utilized in T7 (Spray #3) was highly fluorescent and effectively enhanced blood and semen, and was partially effective for the enhancement of saliva, eccrine/sebaceous sweat and non-human oil impressions (Appendix A: Data/Graph Sets 1 -10). The pink coloration of the enhancement spray allowed for the visualization of impression details in blood (Figures 29, 31, 33, and 35), semen and saliva when visualized under normal lighting. When the sprayed impressions were visualized under alternate lighting blood (Figures 30, 32, 34, and 36), semen (Figures 37-39), saliva (Figure 40) eccrine/sebaceous sweat (Figure 41), and non-human oil (Figure 42) impression details were highly fluorescent making the impression details more readily visible for analysis.

Primary Fluorogenic Enhancement Spray #3 utilized in T7 effectively enhanced the majority of the five biofluids, but on fewer substrates than the Zar-Pro™ Lifters utilized in T2, T3, and T4. This was primarily due to limitations of spray methods as encountered with most spray-based enhancement. The spray methods enhanced the impression on the substrate itself, thus it must contend with the substrate variables, such as background colors, patterns, texture and porosity which often hinder visualization of impression details. The highly fluorescent nature of this spray helped mitigate the substrate characteristics, as the fluorescence of the biofluid allowed the examiner to better separate the ridge details from the background. Background staining was problematic and can be visualized on some of the semi-porous and porous substrates (Figures 29-42), but the spray rinse helped to minimize this occurrence.

Preliminary results of these experimental trials indicate that Zar-Pro™ Fluorescent Lifters can be utilized to lift, enhance, and preserve impressions deposited in blood, semen and sometimes saliva on a wide variety of substrates with varying degrees of porosity. The primary Fluorogenic Enhancement Spray #3, utilized in T7 can be used as a substrate enhancement method for blood, semen, eccrine/sebaceous sweat, and saliva. Spray enhancement, however is not as effective across a wide range of substrates due to background staining on some semi-porous and porous substrates. The ability to enhance impressions from a broad range of substrates with different biofluids is invaluable, as it is often difficult to identify the biological fluid prior to enhancement. Therefore, it is important to consider the substrate porosity when deciding whether to lift the impression from the substrate using Zar-Pro™ Lifters or to enhance it with a spray method.

Photography in the Substrate Trials were deemed less than optimal, as photography was standardized to make the collection of photographs from the complete sample set across the rating intervals more efficient. This practice, although efficient resulted in photographs that

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could be improved with optimal settings based on the biofluid and the substrate in which the impression was deposited. Also, there was contamination to the Zar-Pro ™ lifted semen impressions due to ink on the photography staging area, thus many of these photos contain the same shaped fluorescent stain (Figures 15, 17-21). This problem was rectified before additional photos were taken.

Figure 2: NL

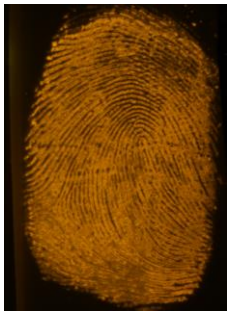

Figure 3: AL

T3 Blood
Lifted from Aluminum

Figure 4: NL

Figure 5: AL

T2 Blood
Lifted from Finished Wood

Figure 6: NL

Figure 7: AL

T3 Blood
Lifted from Painted Drywall

Figure 8: NL

Figure 9: AL

T4 Blood
Lifted from Unfinished Wood

Figure 10: NL

Figure 11: AL

T4 Blood
Lifted from Poster Board

Figure 12: NL

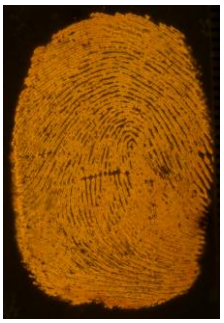

Figure 13: AL

T4 Blood
Lifted from Wall Paper

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Figure 14: NL

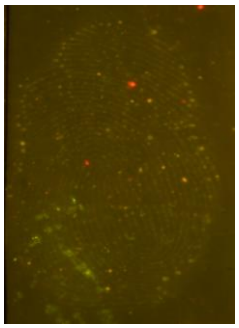

Figure 15: AL
T4 Semen
Lifted from Aluminum

Figure 16: NL

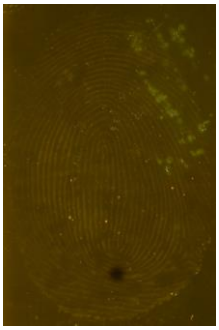

Figure 17: AL
T3 Semen
Lifted from Glossy Paper

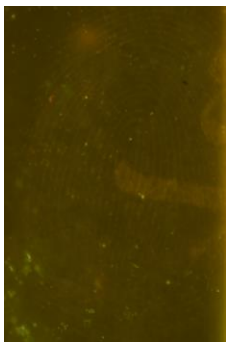

Figure 18: AL
T2 Semen
Lifted from Plastic

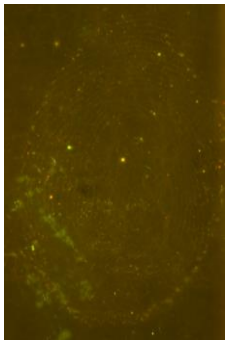

Figure 19: AL
T3 Semen
Lifted from Vinyl Tile

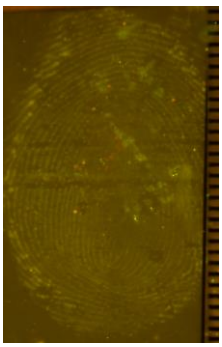

Figure 20: AL
T3 Semen
Lifted from Finished Wood

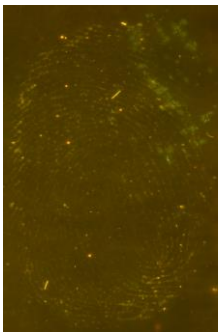

Figure 21: AL
T2 Semen
Lifted from Leather

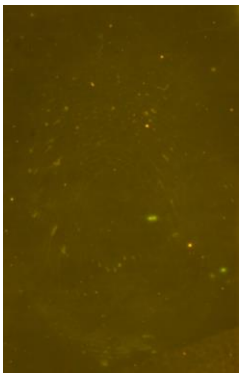

Figure 22: AL
T3 Saliva
Lifted from Plastic

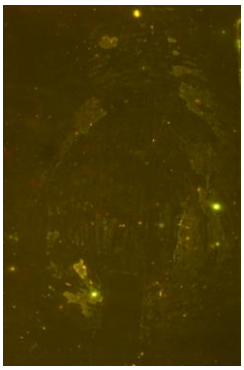

Figure 23: AL
T2 Saliva
Lifted from Finished Wood

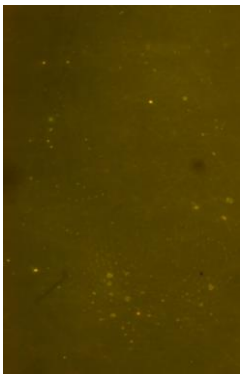

Figure 24: AL
T2 Saliva
Lifted from Steel

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Figure 25: NL T5 Blood on Vinyl Tile

Figure 26: NL T5 Blood on Wall Paper

Figure 27: NL T6 Blood on Unfinished Wood

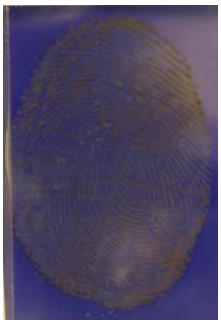

Figure 28: NL T6 Blood on Glossy Paper

Figure 29: NL T7 Blood on Aluminum

Figure 30: AL T7 Blood on Aluminum

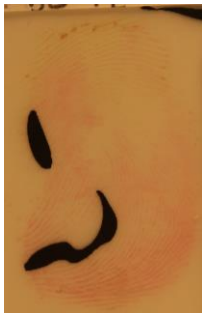

Figure 31: NL T7 Blood on Plastic

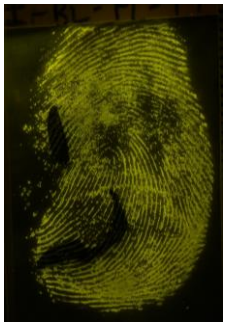

Figure 32: AL T7 Blood on Plastic

Figure 33: NL T7 Blood on Leather

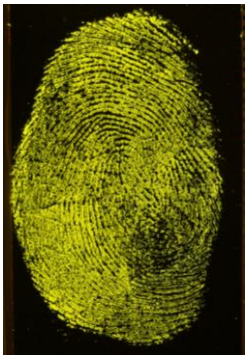

Figure 34: AL T7 Blood on Leather

Figure 35: NL T7 Blood on Wallpaper

Figure 36: AL T7 Blood on Wallpaper

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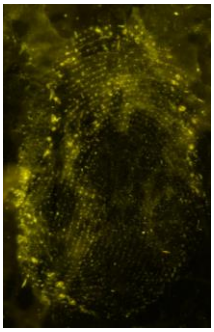

Figure 37: AL T7 Semen on Ceramic Tile

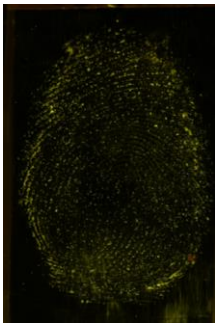

Figure 38: AL T7 Semen on Aluminum

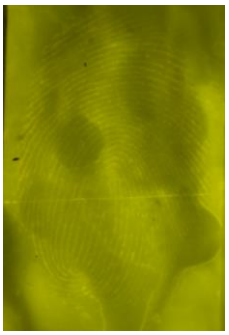

Figure 39: AL T7 Semen on Glossy Paper

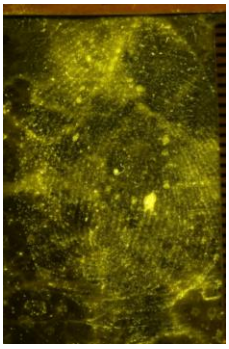

Figure 40: AL T7 Saliva on Vinyl Tile

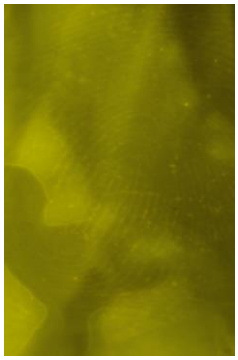

Figure 41: AL T7 Sweat on Glossy Paper

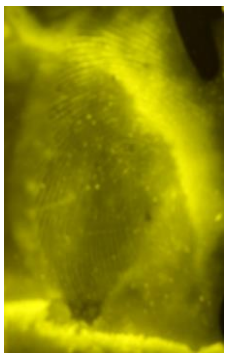

Figure 42: AL T7 Oil on Plastic

7. Aged Trials

The premise of the Aged Trials was to determine the effectiveness of Zar-Pro™ Lifters and the three novel primary Enhancement Sprays (Dye Stain Spray #1, Fluorogenic Dye Stain Sprays #2, and #3) to enhance impression deposited in biofluids (blood, semen, saliva, eccrine/sebaceous, and non-human oil) across three substrates (ceramic tile, glossy paper, and cotton) over a series of aged intervals (1 month, 3 months, 6 months, and 1 year). Zar-Pro™ Lifters are utilized in forensic science for the detection, enhancement, and preservation of blood impressions, but attempts had not been made to expand this methodology to other biofluids. The possibility of using a Secondary Enhancement Spray to increase fluorescence on Zar-Pro™ lifted impressions to determine if visualization of impression details could be improved for analysis was also explored. The second aim of the project was to determine the effectiveness of three novel Primary Fluorogenic Enhancement Sprays (#1, #2, and #3) developed in the course of this project for impressions aged across a one year interval. The final aim was to determine whether the DNA contained within the biofluid of the enhanced impressions would still be viable for

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analysis. These trials also set-up the framework to explore touch DNA from the impression depositor contained within the impression details made with biofluids and aged across a one year interval.

Impressions were deposited using the optimal deposition parameters determined for blood, semen, saliva, eccrine/sebaceous sweat, and non-human oil on tile, glossy paper, and cotton (Tables 1-5). The Aged Trials were conducted in triplicates and the protocols are defined as follows:

Trial 1 (T1) *- DNA Substrate Control*
Trial 2 (T2) *- First Touch Impressions for DNA*
Trial 3 (T3) *- Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) after 1 day*
Trial 4 (T4) *- Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) after 1 day and treated with a secondary fluorescent enhancement spray after 2 days*
Trial 5 (T5) *- Novel Dye Stain Spray #1 (blue color)*
Trial 6 (T6) *- Novel Fluorogenic Spray #2 (blue color)*
Trial 7 (T7) *- Novel Fluorogenic Spray #3 (pink color)*

DNA Substrate Controls (T1)

An impression free substrate sample was packaged separately to provide a substrate control to eliminate the possibility of DNA contamination from the substrate itself.

First Touch Impressions for DNA (T2)

For each biofluid on each substrate, the first impression laid was selected to determine the possibility of obtaining the touch DNA of the depositor. Due to the nature of deposition during the commission of crimes, it is highly likely that many samples subjected to fluorescent enhancement methods, such as Zar-Pro™ Lifters or Fluorogenic Enhancement Sprays will generate mixed profiles during DNA analyses. Therefore, a set of samples was generated on a variety of substrates that involve a "perpetrator" depositing blood, semen, saliva, eccrine/sebaceous sweat or non-human oil from a potential "victim" will be analyzed for the presence of a mix of DNA profiles.

Application of Zar-Pro™ Fluorescent Lifters (T3 and T4)

The Zar-Pro™ Fluorescent Lifters and Zar-Pro™ Activator Solution were purchased from a forensic science supplier (Tri-Tech Forensics, Inc.). The Lifters were cut into 1"x3" pieces and a small diagonal cut was made in the upper left corner of the non-activation side to ensure the activation side would be applied to the impression area at the time of use. The Lifters were then re-sealed in their original packaging. The Zar-Pro ™ Activator Solution was stored in the research laboratory.

The Zar-Pro™ Fluorescent Lifters were activated using the Activator Solution which comes in a pump spray bottle (SKS Bottles). The Activator Solution was applied to the Lifters until completely saturated (approximately five pumps). After spray activation, the lifter was air dried for 30-45 seconds to allow for the evaporation of any pooled alcohol; excessive alcohol left

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on the Lifters could alter the impression. Evaporation happens quickly and can be visualized as it is occurring. After the lifter was activated without any observable alcohol pools, it was ready to be applied to the impression area. The lifter was placed onto the substrate with the activation side on lifter contacting the impression (the diagonal cut corner of the lifter on the left). The user then applied 8-10lbs of pressure measured using a digital scale (Taylor) with their hand for 30 seconds to the backside (non-activation side) to completely affix the blood impression to the lifter. The lifters were then carefully removed from the substrate for analysis.

Application of Fluorogenic Dye Stain Sprays (T5, T6, and T7)

The Enhancement Dye Stain Sprays (#1, #2, and #3) were freshly made (250mls at a time) and stored in an atomizing spray bottle. A single full pump of spray (1.2ml of the formulation) was applied to the impression areas at a distance of 12cm while the substrates were placed in a horizontal position. After being sprayed the substrates were then moved to a vertical position and allowed to incubate for one minute before being rinsed. The rinse solution consisted of a 50% methanol water mixture pre-made and stored in an aerosol spray bottle. The rinse was sprayed twice from 15 cm distance and allowed to dry in vertical position for two minutes. The dried impressions were placed on trays for analysis.

Application of Secondary Enhancement Spray (T4)

The enhancement spray used after 2 days in T4 of the trials to test post lift enhancement consisted of 25% ethanol, 25 % n-butanol, and 2% -sulfosalicyclic acid. The spray was applied from an atomizing spray bottle 15 cm from the lifted impression until visible wetness was observed.

Rating of Impression Detail and Fluorescence Intensity:

Impression details and fluorescent intensity were rated prior to enhancement (T3-T7), after one day (T3-T7), two day (T3-T7), and one week (T3-T7) intervals under normal (NL) and alternate lighting (AL) conditions using the following guidelines:

<u>Impression Details (ID)</u>

0 = no visible proteinaceous material, no visible ridge detail
1 = visible proteinaceous material, no visible ridge detail
2 = visible proteinaceous material, visible ridge detail; such as a partial impression pattern with minimal ridge details
3 = visible proteinaceous material, visible ridge detail: overall impression pattern, ridge paths and deviations, such as enclosures, dots, ridge endings, bifurcations, etc.

<u>Fluorescence Intensity (FI)</u>

0 = no visible fluorescence
3 = moderate fluorescence
6 = intense fluorescence

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One examiner rated the impression details of the five biofluids on the three selected substrates (ceramic tile, glossy paper, and cotton) over aged intervals at 1 month, 3 months, 6 months and 1 year prior to enhancement under both normal and alternate lighting. Two examiners then independently rated the impression details and fluorescent intensity after one day (T3-T7), two day (T2-T7), and one week (T2-T7) intervals under normal and alternate lighting for the 1 week, 1 month, 3 months, 6 months, and 1 year intervals.

Visualization with alternate lighting

The alternate lighting units (ALS) used in the scope of this project were the handheld Rofin Polilight Flare Plus and Flare Plus 2 fitted with 505nm Cyan heads, both of which provide high-intensity light in the range of 460-530 nm and have the ability to generate fluorescence for the visualization of impressions using orange barrier filter goggles. A solid phase fluorescence scale was made prior to each research trial to provide a standard of measurement for fluorescent intensity (Figure 1).

Biofluid impressions deposited on the fourteen substrates were visualized with alternate lighting prior to enhancement. This allowed for the marking of the impression area with a sterile pin to better direct the enhancement method to the impression area, which was especially important for Aged Trials as even blood impressions become latent due to the effects of age. The alternate light source and orange barrier filter goggles were also used to rate the fluorescence of the enhanced impressions after one day, two days and one week throughout the 1 year aged intervals (1 month, 3 month, 6 months and 1 year).

Photography

All samples within the Aged Trials were photographed using a Canon EOS T5i digital SLR fitted with an EF 100mm Macro lens 1:2.8 ratio at 12.5 inches away from the stage. An Orange barrier light filtering was done with a 0.125 inch thickness orange acrylic plexiglass #2422 amber sheet. The 505nm ALS was mounted nine inches away from the stage at an oblique angle of 30 degrees. The sensitivity was set to ISO 100 with F stop set to an aperture of 5.6. The shutter speed was set to open for 6 seconds for weak to moderate fluorescent samples and 1 second, or $1/25^{th}$ second for more strongly emitting samples. Standardization of photography was conducted in order to facilitate the photography of all samples at the above mentioned intervals in an efficient manner, however, this practice resulted in less than optimal photographs. In the future, optimal photography parameters will be set per substrate and biofluid to improve the quality of the resulting sample set.

Subsets of impressions deposited on ceramic tile, glossy paper, and cotton were photographed under normal and the alternate lighting prior to enhancement. A full set of enhanced impressions were photographed at 1 week, 1 month, 3 month, 6 months, and 1 year intervals under normal lighting conditions, at the two day interval under alternate lighting condition, and a subset of enhanced impressions were again photographed under alternate lighting at the one week interval.

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The photographs were labeled and stored on an external hard drive in the research lab and have been archived as part of the raw data from the research trials in accordance with institutional guidelines.

Storage

All impressions in the Aged Trials were stored in paper envelopes labelled and affixed to a file folder which was then placed in a plastic sheath and stored in a separate three ring binders organized by substrate and biofluid. The binders were then placed in a secured cabinet in the research laboratory.

Significant Results

The Aged Trial ratings have been graphed for two examiners (Appendix A: Data/Graph Sets 11-20). Based on the preliminary review of this data, the Zar-Pro™ Fluorescent Lifters (activated with Zar-Pro™ Activator Solution) utilized in T3 and T4 were very effective for lifting blood (Figures 46-51 and 67-72) and semen (Figures 52-55 and 73-76) from one month through the one year interval (Appendix A: Data/Graph Sets 11-12 and 16-17). The use of the secondary enhancement spray after 2 days in T4 was determined not to be effective for use with the Zar-Pro™ Lifters (Appendix A: Data/Graph Sets 11-20). The Lifter produced overall better results when left untreated, even though the secondary fluorescent enhancement spray did produce a slight increase in fluorescence. This was primarily because the use of the spray often resulted in the destruction of visible impression details. It also created a color loss in the heme of blood impressions under normal lighting conditions.

Blood is generally visible under normal lighting conditions on non-porous light colored substrates, and is therefore easier to detect than semen impressions which are generally colorless. However, when blood is aged color loss occurs, resulting in a more latent impression (Figures 43-45 and 64-66). This occurrence is of importance in the Aged Trials, specifically at and following the six month interval in which coloration in blood is noticeably diminished, coinciding with substrate porosity. For example, blood impressions deposited on glossy paper were still partially visible at the one year interval (Figures 44 and 65). The visualization of impressions on the substrate and even post lift on the Zar-Pro™ Lifters is hampered by lack of contrast between the biofluid and the background. The fluorescent capabilities of the Zar-Pro™ Lifters and Fluorogenic Enhancement Sprays (#1, #2, and #3) are crucial as they allow for visualization of impression details through fluorescence when visualized with alternate lighting Appendix A: Data/Graph Sets 11-20).

Zar-Pro™ Lifters utilized in T3 and T4 were found to be effective for the enhancement of blood and semen impressions, but inconsistent in the ability to enhance saliva and non-human oil even when excited and visualized with alternate lighting. (Appendix A: Data/Graph Sets 13, 15, 18, and 20). Lifted blood impressions could often be visualized under normal lighting conditions, although this contrast was noticeably reduced as the impression aged on the substrate prior to enhancement (Figures 46, 48, 50, 67, 69, and 71). However, this was not a limiting factor for the visualization of lifted blood impressions as they fluoresce when visualized under alternate

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lighting (Figures 47, 49, 51, 68, 70, and 72). Blood impressions were effectively lifted and enhanced with Zar-Pro™ Lifters from all three substrates throughout the one year aged interval (Appendix A: Graph/Data Sets 11 and 16).

Latent impressions such as semen (Figures 52, 54, 73, and 75), saliva, eccrine-sebaceous sweat, and non-human oil could not be visualized on the Lifters without the use of alternate lighting. However, semen impressions could be lifted from ceramic tile and glossy paper and fluoresced throughout the one year interval (Figures 52-55 and 73-76). Semen impression were not effectively enhanced from cotton, regardless of the age interval prior to enhancement (Appendix A: Graph/Data Sets 12 and 17). Saliva impressions had some partially visible ridge details on ceramic tile and glossy paper through the one year interval (Figure 85) (Appendix A: Graph/Data Sets 13 and 18). Vegetable oil had some ridge details visible on ceramic tile through the one year interval (Figure 87) (Appendix A: Graph/Data Sets 15 and 20). Whereas, eccrine/sebaceous impressions and were not overly effective again this was regardless of the aged interval prior to enhancement (Appendix A: Graph/Data Sets 14 and 19).

Fluorogenic Enhancement Spray #1 utilized in T5 and Spray #2 utilized in T6 effectively enhanced blood (Figures 90-93) and semen impressions, but not the other biofluids (Appendix A: Data/Graph Sets 13, 15, 18, and 20). Sprays #1 and Spray #2 had fluorescent capabilities allowing for enhanced impression details to be visible under normal and alternate lighting conditions.

The most effective of the Fluorogenic Enhancement Sprays was Spray #3 utilized in T7, which enhanced impressions deposited in all biofluids (Figures 56-63, 77-84, 86, 88, and 89). The enhanced impressions were partially visible in normal lighting due to the presence of the pink coloration produced by the spray binding to the biofluid (Figures 56, 58, 60, 62, 77, 79, 81, and 83). Yet the most beneficial property of Spray #3 was the intensity of fluorescence produced when enhanced impressions were visualized under alternate lighting, improving the visualization of impression details for analysis while minimizing the background variables that hinder visualization (Figures 57, 59, 61, 63, 78, 80, 82, 84, 86, 88, and 89). Impressions were effectively enhanced with Spray #3 throughout the one year interval across a variety of biofluids and substrates (Appendix A: Data/Graph Sets 11-20).

Figure 43: Ceramic Tile

Figure 44: Glossy Paper

Figure 45: Cotton

Blood Aged 1 Month Impressions prior Enhancement

Figure 46: NL

Figure 47: AL

1 Month T3 Blood Lifted from Ceramic Tile

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Figure 48: NL **Figure 49: AL**
1 Month T4 Blood
Lifted from Glossy Paper

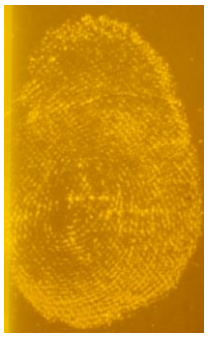

Figure 50: NL **Figure 51: AL**
1 Month T3 Blood
Lifted from Cotton

Figure 52: NL **Figure 53: AL**
1 Month T4 Semen
Lifted from Ceramic Tile

Figure 54: NL **Figure 55: AL**
1 Month T4 Semen
Lifted from Glossy Paper

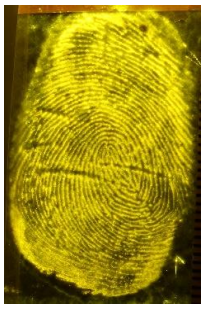

Figure 56: NL **Figure 57: AL**
1 Month T7 Blood
on Ceramic Tile

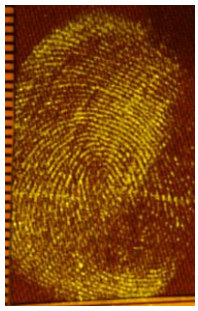

Figure 58: NL **Figure 59: AL**
1 Month T7 Blood
on Cotton

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Figure 60: NL

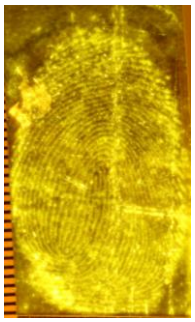

Figure 61: AL

1 Month T7 Semen
on Ceramic Tile

Figure 62: NL

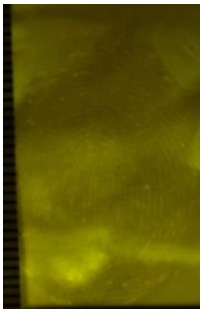

Figure 63: AL

1 Month T7 Semen
on Glossy Paper

Figure 64:
Ceramic Tile

Figure 65:
Glossy Paper

Figure 66:
Cotton

Blood Aged 1 Year
Impressions prior Enhancement

Figure 67: NL

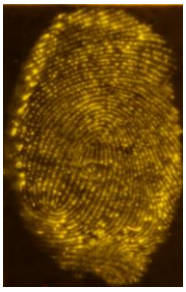

Figure 68: AL

1 Year T4 Blood
Lifted from Ceramic Tile

Figure 69: NL

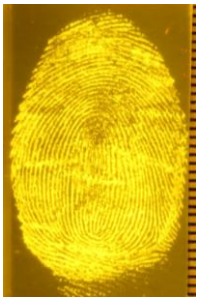

Figure 70: AL

1 Year T3 Blood
Lifted from Glossy Paper

Figure 71: NL

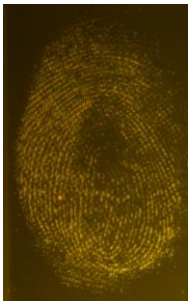

Figure 72: AL

1 Year T4 Blood
Lifted from Cotton

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Figure 73: NL

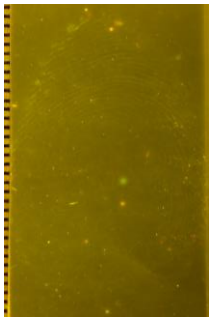

Figure 74: AL

1 Year T3 Semen
Lifted from Ceramic Tile

Figure 75: NL

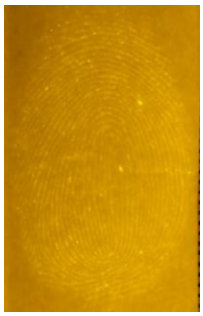

Figure 76: AL

1 Year T4 Semen
Lifted from Glossy Paper

Figure 77: NL

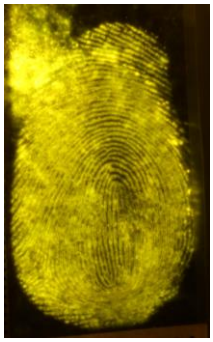

Figure 78: AL

1 Year T7 Blood
on Ceramic Tile

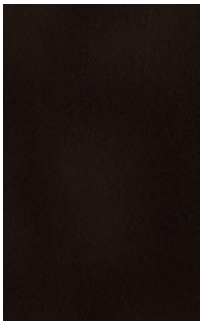

Figure 79: NL

Figure 80: AL

1 Year T7 Blood
on Cotton

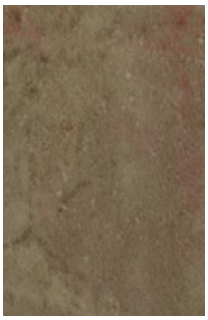

Figure 81: NL

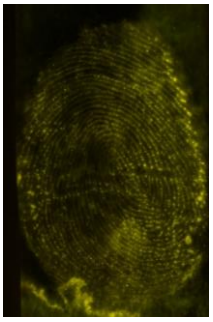

Figure 82: AL

1 Year T7 Semen
on Ceramic Tile

Figure 83: NL

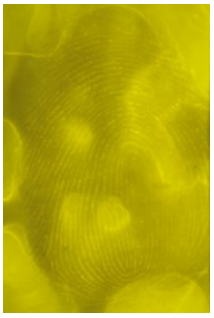

Figure 84: AL

1 Year T7 Semen
on Glossy Paper

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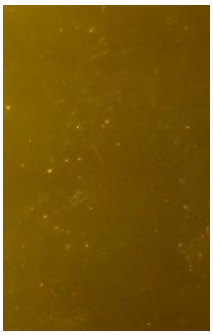

Figure 85: AL 1 Year T4 Saliva Lifted from Ceramic Tile

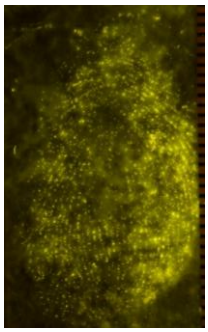

Figure 86: AL 1 Year T7 Saliva on Ceramic Tile

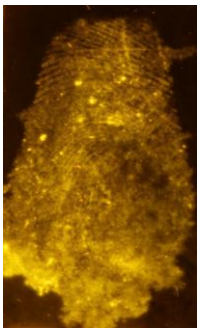

Figure 87: AL 1 Year T3 Oil Lifted from Ceramic Tile

Figure 88: AL 1 Year T7 Oil on Ceramic Tile

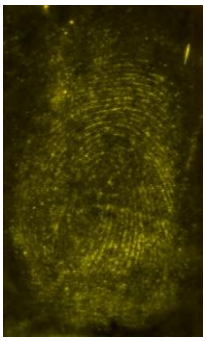

Figure 89: AL 1 Year T7 Sweat on Ceramic Tile

Figure 90: NL 1 Year T5 Blood on Ceramic Tile

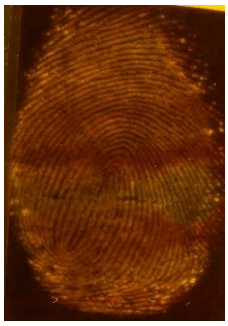

Figure 91: AL

Figure 92: NL 1 Year T6 Blood on Glossy Paper

Figure 93: AL

8. Substrate and Aged Trials Data Analysis

The inter-observer reliability or the ability of independent examiners to observe the same impression and come to the same conclusion [60] is an important aspect of this research project to gauge the overall effectiveness of the Zar-Pro™ Lifters and the novel Fluorogenic Enhancement Sprays to enhance impressions deposited in various biofluids over a one year interval. In addition, the intra-observer reliability was also assessed, which is the ability of a single examiner to make the same conclusion about the same ratings; but on a separate occasion [60]. This allowed for further objectivity in the verification of impression ratings and were conducted by each examiner after their initial determination to assess consistency and reliability within their individual ratings.

Cohen's Kappa statistical analysis of inter- and intra- observer ratings were chosen for this study as ratings are subjective in nature. Impression analysis by itself is considered highly subjective relying heavily on examiner training and experience to make reliable determinations. Therefore, three examiners of various education and experience were selected to conduct ratings in attempt understand the variability in impression based research and to verify conclusions. Three independent examiners rated the quality of ridge detail and the intensity of fluorescence

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under normal and alternate lighting conditions. Numerical designators were used to quantify these results; impression details were rated on a four point scale (0 indicating no visible proteinaceous material, no ridge detail; 3 indicating visible proteinaceous material, visible ridge detail: Overall impression pattern, ridge paths and deviations, such as enclosures, dots, ridge ending, and bifurcations, etc.) and fluorescence intensity was rated on a six point scale (0 indicating no visible fluorescence, 3 indicating moderate fluorescence and 6 indicating intense fluorescence).

To date, the data from the Aged Trial 1 year, 1 week and 1 year, 1 week, and 1 day intervals for all five biofluids (blood, semen, saliva, eccrine/sebaceous sweat, and non-human oil) have been input into an excel document and graphed (Appendix B: Graph/Data Set 1-5) and Appendix C: Graph/Data Set 1-5), but only blood trials (T3-T7) at the above mentioned interval have been assessed using the Kappa model (Appendix B: Graph/Data Set 1 and Appendix C: Graph/Data Set 1). Cohen's Kappa statistical analysis was used to verify the significance of the results obtained from inter-observers and intra-observers [62]. The observed agreement between examiners and the expected agreement between examiners was assessed utilizing the Kappa equation (Figure 94) [38]. In the case of this research, with a third examiner, Cohen's Kappa was used by comparing Examiner A to Examiner B, Examiner B to Examiner C, and Examiner A to Examiner C. Once the Kappa value was obtained it can be interpreted using an agreement chart (Figure 95) [62]. A Kappa value of 1 indicates a perfect agreement among examiners, a Kappa value of 0 indicates the agreement was due to chance alone. This statistical model was applied to the ratings of ridge detail and fluorescent intensity, and the rating validity was assessed.

$$\text{kappa} = \frac{(p_o - p_c)}{(1 - p_c)}$$

where p_o is the observed proportion of agreement and p_c is the proportion expected by chance.

Figure 94: Cohen's Kappa statistical formula

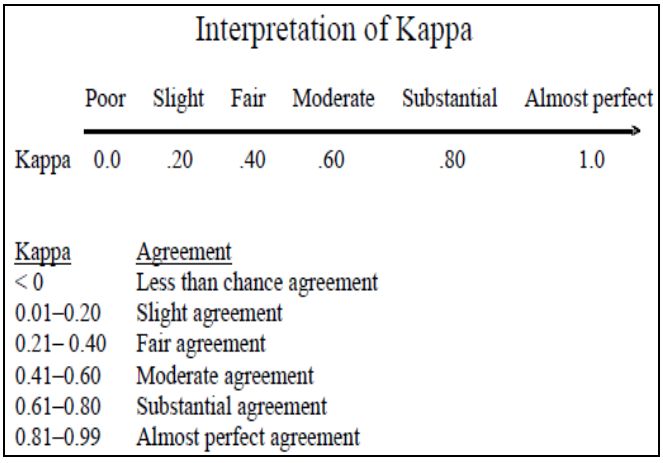

Figure 95: Interpretation of Kappa Value

Independent Examiners

Three examiners rated the ridge detail and fluorescent intensity of the enhanced impressions. All ratings were conducted independently of each other, but within a 12 hour time interval. The examiners utilized oblique lighting to rate each impression under normal and alternate lighting. When viewing an impression the use of oblique angles allows light to bounce off of the impression, allowing for better visualization of impression details within the pattern

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area, this technique can be used under both alternate and normal lighting conditions to better visualize impression details for analysis.

Examiner background, education and experience were considered when selecting examiners to rate impressions for this study. Examiner A, 35 year old female that does not wear glasses; however, she did have LASEK eye surgery. She has a Master's Degree with 11 years of experience in impression analysis and has received advanced training in this area. Examiner B, 50 year old male that wears prescribed glasses (-6.75); however, he is myopic and therefore did not wear glasses when rating and has never had any type of laser eye surgery to correct his vision. He has a Doctorate degree with less than one year experience in impression analysis and has received no specialized training in this area. Examiner C, 29 year old female that does not wear contacts or glasses; however, she had LASIK eye surgery. She has a Bachelor's degree with less than one year of experience in impression analysis but has received specialized training in this area.

Inter-observer Variability

Inter-observer reliability was assessed between three examiners that rated ridge quality and fluorescence intensity under normal and alternate lighting conditions for blood impression at the one year, one week interval (T3-T7). The data sets from each examiner were graphed (Appendix B: Data/Graph Set 1) and the results were analyzed as follows to determine agreement level; Examiner A was compared to Examiner B, Examiner B to C, and Examiner A to C. Agreement ratings were also assessed for ridge detail ratings, fluorescence intensity ratings, and ratings conducted when visualization under normal and alternate lighting (Appendix D: Table 1). Inter-observer variability between examiners, as well as variability in the above mentioned categories were calculated using the Kappa model and their level of agreement was determined (Table 6).

Examiner A and Examiner B had a Kappa value of 0.42 which indicated a moderate level of agreement, Examiner A and Examiner C had a Kappa value of 0.66 a substantial level of agreement, and Examiner B and Examiner C had a Kappa value of 0.40 a fair level of agreement in ratings of ridge detail and fluorescence intensity under normal (NL) and alternate lighting (AL). Examiner A and Examiner C showed the highest level of agreement amongst the three examiners (Table 6), this could be in part to both examiners had reported training in impression analysis.

In order to further examine the possible sources of variance in inter-observer ratings, additional statistical calculations were conducted for the specific enhancement trials (T3-T7). The agreement between examiners for the rating of fluorescence was compared to the agreement between examiners for the rating of ridge detail for each of the research trials (Table 6). For T3 (Zar-Pro™ Lifters), the Kappa value obtained for agreement on fluorescence was 0.42, while the Kappa value obtained for agreement on ridge detail was 0.54. Although there is a 0.12 difference in the Kappa values, both provided a moderate level of agreement. T4 (Zar-Pro™ Lifters with Secondary Enhancement Spray), the Kappa value obtained for agreement on fluorescence

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Table 6: Kappa statistical calculations made for inter-observers

Inter-Observer Variability		
Comparisons	**Kappa Value**	**Agreement Level**
Examiner A/ Examiner B	0.42	Moderate
Examiner A/ Examiner C	0.66	Substantial
Examiner B/ Examiner C	0.40	Fair
T3 Fluorescence/ Ridge Detail	0.42 / 0.54	Moderate/ Moderate
T4 Fluorescence/ Ridge Detail	0.40 / 0.50	Fair/ Moderate
T5 Fluorescence/ Ridge Detail	0.26 / 0.56	Fair/ Moderate
T6 Fluorescence/ Ridge Detail	0.78 / 0.56	Substantial/ Moderate
T7 Fluorescence/ Ridge Detail	0.34 / 0.56	Fair/ Moderate
Overall Fluorescence/ Ridge Detail	0.44 / 0.54	Moderate/ Moderate
T3 Normal/ Alternate Lighting	0.72 / 0.28	Substantial/ Fair
T4 Normal/ Alternate Lighting	0.54 / 0.34	Moderate/ Fair
T5 Normal/ Alternate Lighting	0.78 / 0.04	Substantial/ Slight
T6 Normal/ Alternate Lighting	1.00 / 0.34	Perfect/ Fair
T7 Normal/ Alternate Lighting	0.62 / 0.26	Substantial/ Fair
Overall Normal/ Alternate Lighting	0.73 / 0.25	Substantial/ Fair
T3 Overall	0.50	Moderate
T4 Overall	0.46	Moderate
T5 Overall	0.40	Fair
T6 Overall	0.66	Substantial
T7 Overall	0.44	Moderate
T3-T7 Overall	0.48	Moderate

indicated a fair level of agreement (0.40), while the value on ridge detail rating indicated a moderate level of agreement (0.50). T5 (Spray #1) fluorescence rating indicated a fair level of agreement (0.26), while agreement on ridge detail rating was moderate (0.56) and T6 (Spray #2) fluorescence ratings indicated a substantial level of agreement (0.78), while the agreement on ridge detail was moderate (0.56). T7 (Spray #3) fluorescence ratings indicated a fair level of agreement (0.34) and ridge detail rating was moderate (0.56).

Kappa values alone do not indicate the intensity of the fluorescence rating or quality of ridge detail in enhanced impressions but some deductions can be made from reviewing the examiners agreement levels. T6 (Fluorogenic Enhancement Spray #2) had the highest level of agreement between examiners for both fluorescence and ridge detail ratings, but was not an effective enhancement spray. Thus, the examiners tended to agree that the enhancement method produced poor results which was indicated in their scoring and level of agreement. T5 (Spray #1) had the lowest Kappa values combined for fluorescence and ridge detail. Yet, T5 was also a poor performing enhancement spray making the agreement of effectiveness higher amongst

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examiners. It was also found that a higher Kappa value was obtained for ridge detail ratings than ratings on fluorescent intensity ratings (Table 6).

Agreement between normal lighting and alternate lighting ratings for all examiners were also compared (Table 6). For T3 (Zar-Pro™ Lifters), the Kappa value for normal lighting provided a substantial level of agreement (0.72), while the value obtained for alternate lighting resulted in a fair level of agreement (0.28). T4 (Zar-Pro™ Lifters with Secondary Enhancement Spray), the value obtained for normal lighting rating was moderate (0.54), while the alternate lighting rating was fair (0.34). T5 (Spray #1), the agreement for normal lighting was substantial (0.78), while alternate lighting indicated a slight level of agreement (0.04). T6 (Spray #2) normal lighting rating was perfect (1.00), yet the alternate lighting rating was only fair (0.34). T7 (Spray #3) normal lighting rating was substantial (0.62) and the alternate lighting rating was fair (0.26).

The Kappa values obtained in the comparison of ratings conducted under normal and alternate lighting provided the largest difference in value, with normal lighting providing a higher Kappa value in each trial (T3-T7). This result was expected since there are fewer variables in the observation of ridge details under normal lighting. The rating variation could be due to the added variables associated with visualization of enhanced impressions under alternate lighting, such as the darkness of the space used to view the impression, the type and battery power of the ALS, and eye strain as visualization under alternate lighting is not "normal".

Rating conducted for T5 (Spray #1) under alternate lighting provided the lowest Kappa value (0.04) out of all calculations conducted (Table 6). Thus verifying the hypothesis that it is easiest for examiners to agree on ratings when no ridge detail or fluorescence can be observed, or if the ridge detail and fluorescent intensity are very clear. However, when the quality of the enhanced impression lies in the middle, as seen with T5 more variance should be expected.

Additional to the abovementioned categories, an overall agreement calculation was conducted for each trial. For T3, the Kappa value obtained was 0.50, which provides a moderate level of agreement; T4, had a Kappa value of 0.46, a moderate level of agreement; T5 had a Kappa value of 0.40, a fair level of agreement; T6 had a Kappa value of 0.66, a substantial level of agreement; and T7 had a Kappa value of 0.44, a moderate level of agreement. Again, T6 had the highest Kappa value, which is most likely due to the results of the trial being of extreme low quality, or extreme high quality.

Intra-observer reliability was also examined, wherein all three independent examiners conducted a secondary rating of the same set of enhanced impressions at one year, one week (T3-T7) and at one year, one week, and one day (T3-T7). The data sets from each examiner at the one year, one week interval were graphed (Appendix B: Data/Graph Set 1) and compared to the data sets from the one year, one week, one day interval (Appendix C: Data/Graph Set 1). This time interval is important, as it was deemed the fluorescent intensity of the enhanced impression would peak and be fixed at the one week interval post-enhancement and would thus be comparable a day later at this interval. Examiners rated ridge quality and fluorescence intensity under normal and alternate lighting conditions at both time intervals. The results were analyzed to examiner variability in the agreement with their prior rating and the amount of times each

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examiner agreed or disagreed with their ratings were recorded for each trial (Appendix D: Table 2). Intra-observer variability was calculated using the Kappa model for each Examiner to indicate the level of agreement with their previous ratings of the same sample set. (Table 7).

Table 7: Kappa statistical calculations made for intra-observers

Intra-Observer Variability		
Comparison	**Kappa Value**	**Agreement Level**
Examiner A/ Examiner A	0.92	Almost perfect
Examiner B/ Examiner B	0.99	Almost perfect
Examiner C/ Examiner C	0.88	Almost perfect
T3 Fluorescence/ Ridge Detail	0.88 / 0.90	Almost perfect/ Almost perfect
T4 Fluorescence/ Ridge Detail	1.00 / 0.90	Perfect/ Almost perfect
T5 Fluorescence/ Ridge Detail	0.82 / 0.96	Almost perfect/ Almost perfect
T6 Fluorescence/ Ridge Detail	1.00 / 1.00	Perfect/ Perfect
T7 Fluorescence/ Ridge Detail	0.82 / 0.96	Almost perfect/ Almost perfect
Overall Fluorescence/ Ridge Detail	0.92 / 0.94	Almost perfect/ Almost perfect
T3 Normal/ Alternate Lighting	0.98 / 0.80	Almost perfect/ Substantial
T4 Normal/ Alternate Lighting	0.90 / 1.00	Almost perfect/ Perfect
T5 Normal/ Alternate Lighting	0.96 / 0.82	Almost perfect/ Almost perfect
T6 Normal/ Alternate Lighting	1.00 / 1.00	Perfect/ Perfect
T7 Normal/ Alternate Lighting	1.00 / 0.82	Perfect/ Almost perfect
Overall Normal/ Alternate Lighting	0.97 / 0.89	Almost perfect/ Almost perfect
T3 Overall	0.88	Almost perfect
T4 Overall	0.96	Almost perfect
T5 Overall	0.88	Almost perfect
T6 Overall	1.00	Perfect
T7 Overall	0.88	Almost perfect
T3-7 Overall	0.92	Almost perfect

When the agreement was compared between Examiner A and Examiner A at 1 day, a Kappa value of 0.92 was obtained which provides an almost perfect level of agreement (Table 7). When the level of agreement was compared between Examiner B and Examiner B at 1 day, a Kappa value of 0.99 was obtained, which provides an almost perfect level of agreement. When the level of agreement was compared between Examiner C and Examiner C at 1 day, a Kappa value of 0.94 was obtained, which correlates to an almost perfect level of agreement between Examiners. Although all Examiners showed an almost perfect level of agreement, Examiner B had the highest Kappa value.

To further examine the possible sources of variance in intra-observer ratings, additional statistical calculations were conducted for specific categories, such as rating of fluorescence intensity, quality of ridge detail, ratings conducted under normal and alternate lighting, and the

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enhancement method (T3-T7). For T3-T7, the agreement between the three examiners of the same sample set conducted at one year, one week and one year, one week, and one day. T3 the Kappa value obtained for agreement on fluorescence rating was 0.88, while the Kappa value obtained for agreement on ridge detail rating was 0.90. Although there is a 0.02 difference in the Kappa value, both provide an almost perfect level of agreement. For T4, the Kappa value obtained for agreement on fluorescence rating was 1.00, which provides a perfect level of agreement, while the Kappa value obtained for agreement on ridge detail rating was 0.90, which provides an almost perfect level of agreement. For T5, the Kappa value obtained for agreement on fluorescence rating was 0.82, which provides an almost perfect level of agreement, while the Kappa value obtained for agreement on ridge detail rating was 0.96, which provides an almost perfect level of agreement as well. For T6, the Kappa value obtained for agreement on fluorescence rating and ridge detail rating were both 1.00, which provides a perfect level of agreement. For T7, the Kappa value obtained for agreement on fluorescence rating was 0.82, which provides an almost perfect level of agreement, while the Kappa value obtained for agreement on ridge detail rating was 0.96, which provides an almost perfect level of agreement.

In the intra-observer comparison of ridge detail and fluorescent intensity, the Kappa values are much closer than in the inter-observer comparison; therefore, less information can be deduced. However, some items of interest include T6, where all three examiners provided ratings 1 day apart that were in perfect agreement for ridge detail and fluorescent intensity. This could be due to the lack of impression details or fluorescent intensity at all, or the presence of very high quality impression details and fluorescent intensity, which are both observed in the Examiner results of T6. Three out of four of the other trials had a Kappa value that was slightly higher for ridge detail than fluorescent intensity, which is most likely due to the abovementioned variance when analyzing fluorescence.

A comparison of the agreement between normal lighting and alternate lighting ratings for all intra-observers was also calculated. For T3, the Kappa value obtained for agreement on the normal lighting rating was 0.98, which provides an almost perfect level of agreement, while the Kappa value obtained for agreement on the alternate lighting rating was 0.80, which provides a substantial level of agreement. For T4, the Kappa value obtained for agreement on the normal lighting rating was 0.90, which provides an almost perfect level of agreement, while the Kappa value obtained for agreement on the alternate lighting rating was 1.00, which provides a perfect level of agreement. For T5, the Kappa value obtained for agreement on the normal lighting rating was 0.96, which provides an almost perfect level of agreement, while the Kappa value obtained for agreement on the alternate lighting rating was 0.82, which provides an almost perfect level of agreement. For T6, the Kappa value obtained for agreement on the normal lighting rating and alternate lighting rating was 1.00, which provides a perfect level of agreement. For T7, the Kappa value obtained for agreement on the normal lighting rating was 1.00, which provides a perfect level of agreement, while the Kappa value obtained for agreement on the alternate lighting rating was 0.82, which provides an almost perfect level of agreement.

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The Kappa values obtained in the comparison of normal lighting and alternate lighting ratings provided a variety of agreement levels from substantial to perfect. Just like the inter-Examiner comparison of normal light and alternate lighting, the Kappa values were generally higher for normal lighting ratings than alternate lighting ratings. Again, intra-observer comparison for T6 showed there was perfect agreement for normal and alternate lighting ratings, which adds to the previous statement that it is easier for Examiners to provide consistent ratings when there is either a lack of ridge detail and fluorescent intensity to rate, or there is high quality ridge detail and fluorescent intensity.

Lastly, additional to the abovementioned categories, an overall intra-observer agreement calculation was conducted for each trial. For T3, the Kappa value obtained was 0.88, which provides an almost perfect level of agreement; T4, had a Kappa value of 0.96, an almost perfect level of agreement; T5 had a Kappa value of 0.88, an almost perfect level of agreement; T6 had a Kappa value of 1.00, a perfect level of agreement; and T7 had a Kappa value of 0.88, an almost perfect level of agreement. Again, T6 had the highest Kappa value, which is most likely due to the results of the Trial being of extreme low quality, or extreme high quality.

Significant Results

The Kappa values that were calculated for all three inter-observer comparisons provided at least fair agreement, and are therefore the impression ratings are deemed to be valid. The Kappa values calculated for intra-observer variance showed that all three examiners had an almost perfect agreement, which shows that their blind ratings of the same sample set were also consistent and valid.

9. DNA Protocols and Analysis

A subset of impressions enhanced using both the lifter and spray methods were assessed to determine viability of the captured DNA and the attainment of optimal yield necessary for subsequent STR amplification.

Optimization of DNA extraction

The purpose of this phase of the study was to test the viability of DNA in impression evidence lifted with Zar-Pro™ Fluorescent Blood Lifters, as well as that which was enhanced and preserved with the Fluorogenic Enhancement Sprays. For evidence lifted with Zar-Pro™ Fluorescent Blood Lifters, the samples were obtained by taking a cutting of the fixed impression from a section of the lift where removal was least likely to influence the impression quality ratings. This ensures the long-term maintenance of the impression details and prevents unnecessary destruction of evidence. In the case of impression evidence fixed by Fluorogenic Enhancement Sprays, the sample was either cut from the substrate, if possible, or scraped from the surface of the substrate if necessary. All cuts and scrapings were performed using disposable, sterile scalpel blades to prevent contamination of samples. Subsequent extraction and

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analysis of these samples indicated that no quantifiable DNA was present in cuttings or scrapings from spray treated impressions.

Four commonly used DNA extraction methods were examined by using quantitative PCR (qPCR) to quantify and compare the efficacy of each method. This was necessary because CODIS profiler kits such as the PowerPlex 16® System [29] and the AmpF*l*STR® Identifiler® kit [16] recommend using 0.5-1.0 ng and 0.5-1.25 ng of template DNA, respectively. Achieving this concentration for a whole genomic extraction may prove difficult when starting from a small sample cut with the intention of keeping impression evidence as intact as possible. Efforts were made to determine how much of the physical sample on average is required in order to reach optimal extract concentration.

In addition, the measures of the *amount* of total DNA are used as proxies for how much DNA on average should represent any given marker. However, when the concentration of DNA is determined by spectrophotometry (e.g. NanoDrop), total DNA measures include the remaining intact genomic DNA endogenous to the sample, as well as full genomic DNA from any exogenous organisms, typically viral and bacterial species [45]. Therefore, it is essential to understand that when one targets CODIS markers (or any set of markers) by traditional PCR, non-target genomic DNA is prevalent in the PCR reaction. As a result, amplification efficiency of targeted DNA is sub-optimal as the non-target DNA interferes between polymerase and targeted DNA molecules and/or competes as binding sites for primers [43, 66]. Proper optimization of DNA concentration has been shown to be less problematic when a real-time qPCR assay is used to specifically quantify target human DNA [24, 56], as is the case with this project.

DNA extraction

Protocols and commercial kits that are commonly used in forensic and low DNA copy number laboratories were directly compared to determine the most effective DNA extraction method to retain the maximum number of target copies and thus reduce the problems that are induced by low copy numbers (allelic dropout, PCR failure) in downstream applications. The goal was to best optimize total genomic DNA extraction to reduce or eliminate the low copy number effect that hampers amplification protocols regardless of the target regions of interest, such as the CODIS markers, while recognizing that the amount of DNA available for extraction in these samples may be minimal.

To explore the feasibility of DNA extraction from enhanced impressions, four different DNA extraction methods were evaluated; organic extraction method using phenol:chloroform was compared to a loose silica extraction (homemade resin, Promega Wizard® Genomic DNA Purification Kit), a magnetic bead protocol (Promega DNA IQ™ System), and a magnetic bead protocol specific for bones, teeth, and adhesives (Applied Biosystems PrepFiler® BTA Forensic DNA Extraction Kit). Extracts from three of the extraction methods were subsequently tested using known concentrations of whole genomic human control DNA added directly to the reaction, placed onto a piece of Zar-Pro™ Lifter included in the reaction, or fixed to cotton with fluorogenic enhancement spray and extracted.

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Quantitative PCR (Qiagen Investigator Quantiplex Kit) was used to determine the viability and yield of DNA from organic extraction, the Promega DNA IQ™ Extraction System, and the Applied Biosystems PrepFiler® BTA Forensic DNA Extraction Kit in order to choose a method that provided consistent yields at the lowest cost.

Preliminary DNA extraction trials resulted in the adoption of the Promega DNA IQ™ System as the protocol of choice due to its consistent yield and cost efficiency for the large number of samples to be processed in this study. The Applied Biosystems PrepFiler® BTA Forensic DNA Extraction Kit was concurrently optimized for use because this protocol has been chosen for validation by the newly established DNA Unit at the Oakland County Sheriff's Office, Forensic Science Laboratory, which is acting as professional collaborator for this project.

Determining viability of DNA in enhanced impressions

Early experiments clearly indicated that DNA extractions from impressions enhanced using the Fluorogenic Enhancement Sprays in Trial T5, T6, and T7 did not produce any amplifiable DNA. DNA experiments for these trials were discontinued in order to further explore DNA preservation in the presence of the sprays and to devote resources to developing protocols that can be used to successfully amplify DNA from impressions deposited in biofluids and lifted onto Zar-Pro™ Fluorescent Blood Lifters.

DNA extracted from biofluid samples laid on glass and lifted using Zar-Pro™ Lifters were tested using the PowerPlex ESX 16® Fast System (Promega) to ensure the viability of generating reliable STR profiles suitable for forensic casework. Profile analysis was successful for impressions in both blood and semen lifted from glass and extracted after 24 hours post enhancement at levels as low as 27 pg of quantifiable DNA. The optimized protocol was subsequently applied to a subset of the impressions lifted from various substrates over the Aged Trials time intervals to assess the effectiveness of the protocol for detection, lifting, extraction, quantification, STR amplification, and profile analysis (see Tables 9 and 10).

Sample size optimization

A series of DNA extraction and quantification experiments designed using known concentrations of whole genomic human control DNA to measure inhibition caused by components of the Zar-Pro™ Lifters indicated that the maximum cutting size utilized should not exceed 16 mm^2 in order to prevent inhibition of subsequent downstream applications. A dramatic drop in DNA return was noted as sample cuttings exceeded this size limit regardless of the starting DNA concentration added to the extraction reaction (Table 8). This suggests that the inhibition is caused directly by the inclusion of a larger piece of the lifter and not as a result of reduced DNA concentration causing stochastic return.

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Table 8: Summary of average DNA return when known DNA concentrations are extracted from Zar-Pro™ Lifters.

Zar-Pro (mm^2)	DNA IN (pg)	DNA OUT (pg)	% Return (avg)	Variance	DNA IN (pg)	DNA OUT (pg)	% Return (avg)	Variance
1	133	124	93.23	0.04905	66.7	65.62	98.38	4.91E-05
4	133	117.4	88.27	0.2118	66.7	66.66	99.94	0.1836
9	133	106.3	79.92	0.1116	66.7	63.03	94.49	1.12E-04
16	133	93.3	70.15	0.2088	66.7	55.43	83.1	2.09E-04
25	133	62.95	47.33	0.1488	66.7	32.85	49.25	1.83E-05
64	133	42.36	31.85	0.02476	66.7	29.28	43.90	2.90E-06
121	133	6.23	4.69	1.102E-06	66.7	3.59	5.39	6.68E-07
196	133	3.33	2.50	1.588E-05	66.7	5.13	7.70	8.92E-06

Figure 96: NL DNA Cutting Size Zar-Pro™ Lifted Impression

Figure 97: AL DNA Cutting Size Zar-Pro™ Lifted Impression

Given that the sample itself is an impression deposited in biofluid, the amount of nucleated cells captured within the ridge detail in each cutting, and therefore, the amount of DNA preserved, can be variable (Figures 96-99). It was determined experimentally that only impressions deposited in blood and semen contained enough DNA for effective analysis and that samples in the range of 9 to 16 mm^2 provided the most likely chance of containing an amplifiable quantity of DNA.

DNA Analysis of Zar-Pro™ Lifted Blood and Semen Impressions (Aged Trials T3)

Enhanced impressions from the blood and semen Aged Trials on ceramic tile, glossy paper, and cotton, were moved forward to DNA analysis in order to replicate the time frames that would be most in line with an average forensic laboratory work flow. This included blood and semen samples from T1 and T3. As an indicator of preservation, the first DNA extraction attempts were made from Zar-Pro™

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Lifters used to enhance blood and semen impressions one month and one year post deposition. This means that the DNA was analyzed at either twelve months or one month post enhancement. The initial DNA extractions and quantification reactions indicated that no, or very scant, amplifiable DNA was present in any of the extracts from these samples (Tables 9 and 10).

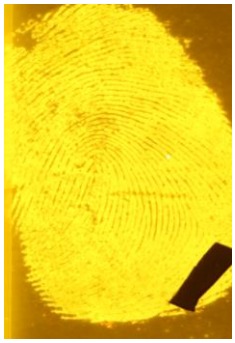

Figure 98: T3 Blood DNA Cutting

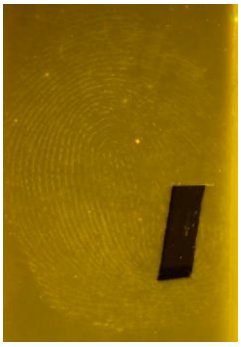

Figure 99: T3 Semen DNA Cutting

Table 9: Quantification of DNA Extracts from semen and blood impressions lifted after 1 month on the substrate (= 12 months on Lifter).

Sample	DNA OUT pg	Variance
se-tl-s3-t1	0	0.00E+00
se-gp-s3-t1	0	0.00E+00
se-ct-s1-t1	0	0.00E+00
bl-tl-s3-t1	0	0.00E+00
bl-gp-s2-t1	0.2524	1.12E-04
bl-ct-s2-t1	0	0.00E+00
se-tl-s3-t3	0	0.00E+00
se-gp-s3-t3	0	0.00E+00
se-ct-s1-t3	0	0.00E+00
bl-tl-s3-t3	0	0.00E+00
bl-gp-s2-t3	0	0.00E+00
bl-ct-s2-t3	0	0.00E+00

se:semen; bl: blood; tl: tile; gp: glossy paper; ct: cotton

Table 10: Quantification of DNA Extracts from semen and blood impressions lifted after 1 year on the substrate (= 1 month on Lifter).

Sample	DNA OUT pg	Variance
se-tl-s2-t1	0.002242	3.55E-09
se-gp-s2-t1	0.0072	3.29E-08
se-ct-s2-t1	0.8275	2.72E-04
bl-tl-s2-t1	0	0.00E+00
bl-gp-s2-t1	0.01315	1.47E-07
bl-ct-s2-t1	0	0.00E+00
se-tl-s2-t3	0	0.00E+00
se-gp-s2-t3	0.09132	9.81E-06
se-ct-s2-t3	0.002134	9.11E-09
bl-tl-s2-t3	0.0164	5.38E-07
bl-gp-s2-t3	0	0.00E+00
bl-ct-s2-t3	0.0009193	1.69E-09

se:semen; bl: blood; tl: tile; gp: glossy paper; ct: cotton

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Tables 9 and 10 show the DNA quantification results for the blood and semen samples tested from lifts at one month and one year. These were tested concurrently meaning that samples labeled as one month, had been lifted and stored on the Zar-Pro™ Lifter one year prior to DNA extraction, and those labeled as one year had been lifted and stored on the Lifter one month prior. The result clearly showed that although the quantity was marginal, it was much more common to have DNA present in the one year samples whose impressions had been lifted onto the Lifter in the previous month compared to those impressions that had been stored on the Lifters for one year and effectively yielded no DNA (Tables 9 and 10). This suggests that the question of DNA preservation is not as closely tied to the length of time on the substrate as it is to how long the impression was on the Zar-Pro™ Lifter.

In order to better understand the preservation of DNA on the Zar-Pro™ Lifters, a series of experiments were conducted using impressions deposited on glass (Table 3 Blood, Table 4 Semen) to determine the time interval within which DNA is preserved and how to better optimize the yield of DNA from the lifted impression. The time intervals chosen for DNA recovery were between 24 and 60 hours post enhancement to determine if an obvious degradation pattern could be observed.

Table 11. Quantification and STR profile analysis of DNA extracts from lifted semen and blood impressions over various time intervals.

Blood		**Avg DNA Concentration (pg)**				**STRs in Profile**	
Time (hrs)	**Size (mm^2)**	**Cut 1**	**Cut 2**	**Cut 3**	**Total**	**≥ 16**	**≥ 10**
24	9	18.0^	53.3	n.a.	35.7	2 of 2	2 of 2
36	9	19.9	7.7*	n.a.	13.8	1 of 2	1 of 2
48	9	32.6*	30.9*	n.a.	27.0	0 of 2	0 of 2
60	9	88.8	32.4^	n.a.	60.6	2 of 2	2 of 2
24	16	96.7	40.0	96.7	77.8	3 of 3	3 of 3
36	16	25.3^	15.6"	23.6^	21.5	2 of 3	3 of 3
48	16	34.7^	117.0	178.9	110.2	3 of 3	3 of 3
60	16	58.2	17.1"	90.1^	55.1	2 of 3	3 of 3
Semen		**Avg DNA Concentration (pg)**				**STRs in Profile**	
Time (hrs)	**Size (mm^2)**	**Cut 1**	**Cut 2**	**Cut 3**	**Total**	**≥ 16**	**≥ 10**
24	9	169.4	4.7*	297.6	157.2	2 of 3	2 of 3
36	9	120.0	56.1	347.6	174.6	3 of 3	3 of 3
48	9	0.6*	12.7"	8.5*	7.3	0 of 3	1 of 3
60	9	0.1*	150.6	0.9*	50.5	1 of 3	1 of 3
24	16	241.4	1.9*	105.0	116.1	2 of 3	2 of 3
36	16	0.9*	0.5*	4.9*	2.1	0 of 3	0 of 3
48	16	0.7*	9.7*	1.8*	4.1	0 of 3	0 of 3
60	16	0.9*	1.2*	0.8*	1.0	0 of 3	0 of 3

no symbol = complete GlobalFiler™ profile; ^ = partial profile (≥ 16 STRs); " = minimal profile (≥ 10 STRs); * = no profile (< 10 STRs); based on 70 rfu cut-off

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DNA was extracted using the Applied Biosystems PrepFiler® BTA Forensic DNA Extraction Kit, STR amplification was performed using the GlobalFiler™ PCR Amplification Kit, and profiles were analyzed using Applied Biosystems GeneMapper® ID-X 1.5 software. Profiles were categorized based on the number of amplified STRs (minimum 70 rfu) that could be accurately identified for each sample as follows (Table 11; Appendix E):

Complete profile:	all STRs in the GlobalFiler™ kit amplified and identified
Partial profile:	≥ 16 STRs amplified and identified
Minimal profile:	≥ 10 STRs amplified and identified
No profile:	< 10 STRs amplified and identified

For some of the samples, as post-lift intervals increased, the DNA appeared to degrade after it was bound onto the lifter (Table 11; Figure 100). This may be the result of acidic conditions in the chemical composition of the Zar-Pro™ Lifters and may be improved through buffering the system to prevent DNA breakdown.

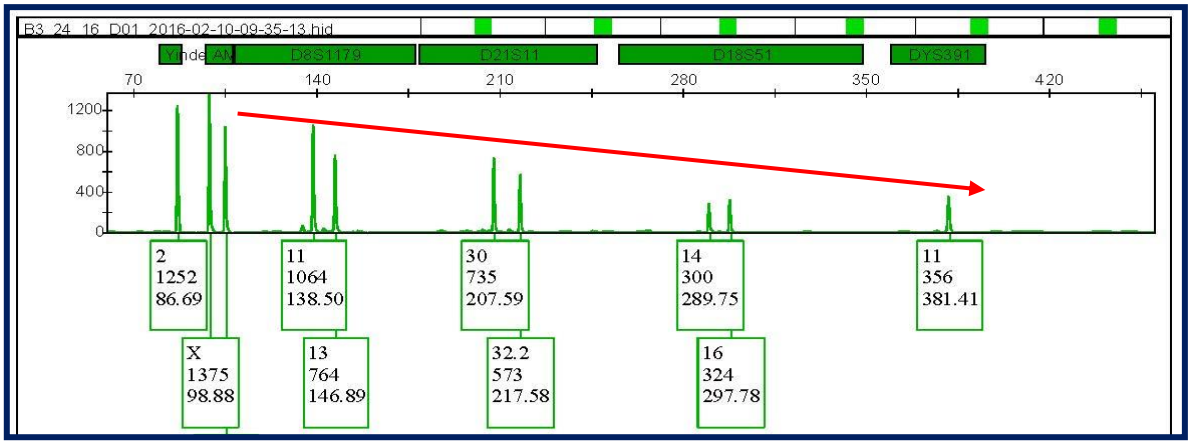

Figure 100. An example of decreasing STR peak height vs amplicon length suggests degradation in the DNA extracted from a 16mm^2 sample of a Zar-Pro™ lifted blood impression, 24 hours after lifting.

As expected, the DNA yields are highly stochastic even between same sized samples within the same time interval (Table 11). Despite trying to control for many variables, it is neither possible to quantify the number of cells present in the ridges lifted onto the Zar-Pro™ Lifter, nor to ensure that the same number of ridges are included in each sample despite trying to control the cutting size (Figures 96-99). Also interesting is the fact that quantity of DNA at these low concentrations is not as tightly correlated with the success of amplification as expected. A 9 mm^2 sample with a DNA concentration at 12.7 pg/µl produced a minimal profile, one at 18.0 pg/µl produced a partial profile, while samples at 30.9 pg/µl and 32.6 pg/µl produced no usable profiles (Table 11). This may be within the stochasticity parameters expected however, replicating these experiments may help by increasing sample numbers in order to clarify this trend.

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These results also suggest that increasing concentrations of components in the Zar-Pro™ formulation may be detrimental to the extraction of DNA from semen lifts as evidenced by the fact that there were no useable STR profiles recovered from the 16 mm^2 samples after the initial 24 hour interval (ie. 36, 48, and 60 hours) (Table 11). Despite having some relatively high DNA yields over the course of the time intervals using 9 mm^2 samples, and in the early stage of the 16 mm^2 sample extraction, there is a steep decline in DNA recovery at and following the 36 hour interval for the 16 mm^2 samples of the lifted semen impressions (Table 11). While the 9 mm^2 samples from the semen lifts did show a noticeable decrease in amplification success over time, one third still provided a profile after 60 hours post lift (Table 11). An in depth examination of factors that may contribute to this phenomenon is necessary in order to optimize the preservation of DNA in this particular biofluid once it is exposed to the components in the Zar-Pro™ formulation. This knowledge is also essential in order to continue work on developing a DNA friendly Fluorogenic Enhancement Spray that can be used to detect, enhance, and preserve evidence in biofluids.

A sample cut size effect was not observed in the amplification of extracts from the lifted blood impressions. In contrast, these samples showed an increase in DNA yield that correlated with the increase in the size of the cutting. This increase was maintained across the time course and contributed to the retrieval of more complete and partial STR profiles from the 16 mm^2 samples than were obtained from the 9 mm^2 samples from lifts of blood impressions (Table 11). More work is needed to determine the outer time limits of DNA preservation in blood impressions lifted with Zar-Pro™ Lifters. The understanding gained during the course of this project that DNA preservation is affected not only by time interval on the substrate but also by time interval on the Lifter offers a two pronged approach to optimizing DNA preservation and subsequent extraction methodologies going forward.

Research is ongoing to develop a method for long-term preservation of the DNA once it has been lifted from the substrate onto the lifter and to continue developing methodologies to utilize single physical evidence items for more than one outcome. During current field trials in the DNA Unit of the Oakland County Sheriff's Office, Forensic Science Laboratory, optimized protocols are being applied to a subset of the impressions lifted from glass over optimal time intervals to assess the effectiveness of the protocol for detection, lifting, extraction, quantification, STR amplification, and profile analysis.

Aged Trials DNA Control T1 and Touch Impression T2

Due to the nature of deposition during the commission of crimes, it is highly likely that many samples subjected to fluorescent enhancement methods, such as Zar-Pro™ Lifters or Fluorogenic Enhancement Spray, will generate mixed profiles during DNA analyses. Therefore, a set of samples was generated on a variety of substrates that involve a "perpetrator" depositing blood from a "victim" that will subsequently be analyzed for the presence of a mix of DNA profiles. Further, given that it is not always possible to analyze all evidence in a timely manner, the samples containing touch DNA were aged with the rest of the Aged Trials and are available for study as protocols become optimized.

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Significant results

It was determined that the DNA IQ system (Promega), provided the most affordable and consistent results for DNA extraction. It was also decided to concurrently optimize the Applied Biosystems PrepFiler® BTA Forensic DNA Extraction Kit because the DNA Unit of the Oakland County Sheriff's Office, Forensic Science Laboratory had chosen to validate this protocol in their newly established division.

Systematic extraction and quantification analysis of all biofluids from a subset of substrates clearly indicated that the fluorogenic enhancement sprays developed did not produce amplifiable DNA within the protocols tested. Ongoing research will focus on determining the mechanism behind compromised DNA preservation.

Extraction and amplification analysis did indicate that DNA was preserved and amplifiable from blood and semen impressions lifted using Zar-Pro™ Lifters. Experimental optimization for balance between inhibitors and DNA yield indicated that the size of the sample should be within an optimal range of 9 – 16 mm^2. Further exploration through quantification of DNA yield from lifted blood and semen impressions across time intervals revealed that DNA preservation in semen impressions is contingent on the time on the Zar-Pro™ Lifter between lift and extraction and that increasing the size of the sample cut from the lifter greatly diminishes yield. DNA in lifted blood impressions appeared to be more stable and could be more readily extracted and amplified to produce STR profiles.

10. Impression/DNA Preservation

Preservation of impression evidence

The degree of stability afforded to impressions enhanced with the novel dye stain sprays has yet to be determined, however, the Zar-Pro™ Lifters are durable with previous studies demonstrating they have the ability to preserve lifted impressions in a manner similar to a photograph with long-lasting fluorogenic properties. Future research projects will evaluate the quality of the ridge detail and fluorescence intensity of the preserved sample sets to better address the issues pertaining to preservation.

In general, there are no specific storage requirements recommended for impression samples on the Lifters or impressions enhanced on the substrates using the novel dye stain sprays other than the packaging being paper-based, such as an envelope. The research samples have been stored in paper envelopes secured inside manila envelopes placed in plastic sheaths within three ring binders. The binders are secured in a locked storage cabinet within the Madonna University Forensic Science Research Facility laboratory.

11. Field Testing

Initial data sharing and field test design has begun in conjunction with the appropriate personnel at the Oakland County Sheriff's Office**.** Optimization of DNA extraction and amplification of STR profiles has been conducted to support the conclusions obtained in the research laboratory and test the applicability of the methods within a typical forensic science laboratory work flow. Subsequently, a subset of blood and semen impressions will be provided

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to personnel in the latent print unit for blind testing in order to replicate previous results and to provide feedback regarding the feasibility of adapting these protocols for use by practitioners in forensic science.

III. Conclusions

Discussion of Findings

In summary, three raters were used across the various experimental designs. Inter- and intra-observer reliability was assessed in the Aged Trials with ratings conducted by 1 examiner prior to enhancement, by two examiners at the 1 day, 2 days, and 1 week intervals, and by three examiners for the 1 year Aged Trials at the 1 week and 1 week, 1 day intervals. The data collected was amalgamated and graphed to visually represent results from each substrate and biological fluid within the Substrate and Aged Trials.

The data trends demonstrate that Zar-Pro™ Lifters as utilized in the Substrate Trials T2, T3, and T4 and Aged Trials T3 and T4 were highly effective in their ability to lift, enhance, and preserve blood and semen impressions in the Substrate and Aged Trials through the one year interval. In assessing the quality of the Zar-Pro™ Lifters (T3) with the combined use of a secondary fluorescent enhancement spray after the 48 hour rating interval (T4) it was determined that the Zar-Pro™ Lifters produced overall better results when left untreated. The secondary fluorescent enhancement spray did produce a slight increase in fluorescence, however it was not deemed a considerable improvement and the use of the secondary enhancement spray often resulted in a destruction of ridge detail for analysis, which was not favorable.

Blood is generally visible under normal lighting conditions on non-porous light colored substrates, and is therefore easier to detect than semen impressions which are colorless. However, as blood ages it may lose color and become more latent, which was seen in the Aged Trials, specifically at and following the six month interval. This change was substrate dependent showing variation across the substrates tested. The increasingly latent properties of blood impressions and the inherent tendency for semen impressions to be latent often resulted in decreased visualization of impression details under normal lighting. The fluorescent capabilities of the Zar-Pro™ Lifters allowed for visualization of impression details under alternate lighting across all trials, often producing superior quality impressions for analysis. The ability to effectively enhance semen impressions correlated to the porosity of the substrate on which the impression was deposited, this was observed with porous substrates in the Substrate Trials and with the porous substrate, cotton in the Aged Trials.

Zar-Pro™ Lifters utilized in the Substrate Trials T2, T3, and T4 and Aged Trials T3 and T4 performed inconsistently with saliva and vegetable oil impressions and was deemed ineffective for eccrine/sebaceous impressions across both the Substrate and Aged Trials. Saliva and vegetable oil impressions showed sporadic effectiveness with the Zar-Pro™ Lifters even when fluoresced and visualized under alternate lighting. Saliva impressions had some partially visible ridge details on ceramic tile and glossy paper through the one year interval, whereas

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vegetable oil had some ridge details visible on tile through the six month interval. The visualization of lifted vegetable oil and saliva impressions was substrate dependent across the Substrate Trials and often improved under alternate lighting. It is believed that the preservation of eccrine/sebaceous and non-human oil impressions on the substrates prior to enhancement may have resulted in the smudging or smearing of impression details, as these type of impressions are never truly "dry" which makes their preservation more difficult.

Both the DNA IQ system (Promega) and the Biosystems PrepFiler® BTA Forensic DNA Extraction Kit were optimized for use extracting DNA from blood and semen impressions lifted with Zar-Pro™ Lifters. A sample 9 – 16 mm^2 in size is optimal for DNA extraction and can easily be removed from the lift without causing large scale damage to the impression evidence. Full STR profiles could be generated from lifted blood and semen impressions using the PowerPlex ESX 16® Fast System and the GlobalFiler™ PCR Amplification Kit meaning that the methods are applicable across both Promega and Applied Biosystems kits, which are commonly used in forensic science laboratories.

Implications for Policy and Practice

Given the problems associated with methods currently used for the enhancement and preservation of blood and proteinaceous impression evidence, the potential benefits of this research carry broad implications for criminal justice at all levels, from local to international. This project produced novel Fluorogenic Enhancement Sprays that provide simple, time and cost effective, non-toxic methods for detecting, enhancing, and preserving proteinaceous impression evidence. A review of the effectiveness of Zar-Pro™ Lifters for non-blood impressions in biofluids commonly encountered at crime scenes was also conducted on a variety of substrates over a one year interval. Impression evidence in the majority of cases is fixed and preserved on the Zar-Pro™ Lifter allowing for repeat visualization over long time intervals. Experiments conducted were also able to identify that DNA could be recovered from blood and semen impressions lifted using Zar-Pro™ Lifters and field trials are currently being conducted at the Oakland County Sheriff's Office Forensic Science Laboratory to test the viability of implementing these protocols into the work flow of an active forensic science laboratory. Thus, this project expanded the methods available for the recovery of impression evidence that are safe for use at crimes scenes and provide opportunities for subsequent DNA recovery at the laboratory.

This research project also identified further questions that need to be explored regarding DNA preservation and timing of recovery from evidence fixed with a Fluorogenic Enhancement Spray or lifted with Zar-Pro™ Lifters. These questions will need to be explored in order to reach the maximum benefit that this methodology has to offer. A considerable amount of experimental materials were generated and are now stored and available for testing. This will allow for continued research with reduced resource investment.

Implications for Further Research

This research has led to a greater understanding of the fluorescent properties of blood and other biological fluids, which allows for better targeting of these fluids for enhancement. This is important in

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the development of novel applications in the field of forensic science. A continued focus on understanding inherent blood fluorescence specifically, and biofluorescence in general, will contribute much needed practical knowledge that will facilitate improved product design and implementation within the field of forensic science. This should include the development of an eosin fluorescence scale, and continued research to produce a fluorogenic enhancement spray that is suitable for use in DNA extraction and amplification, while also being capable of preserving fluorescence and ridge detail in enhanced impressions. In addition, efforts to improve photographic records of experimental evidence by developing methods to deal with variations in fluorescence should continue to ensure optimal quality photographs representative of both fluorescent intensity and ridge detail of impressions.

Efforts to recover DNA from lifted blood and semen impressions over longer time intervals should continue. A number of variables that may affect DNA preservation while on the Zar-Pro™ Lifters have been identified during this research and should be explored to examine if changes in chemical formulation might contribute to increased stability of DNA after lifting. These lessons could then be incorporated into the formulation of a Fluorogenic Enhancement Spray capable of fixing and preserving DNA for later extraction. Future attention should also focus on whether mixed DNA profiles may be commonly generated in lifted impressions and if the DNA, after extraction, is recovered in the concentrations expected given a known concentration of DNA from each contributor. This would assist in providing answers to questions that could arise during practical application of this methodology.

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V. Dissemination of Research Findings

Scientific meeting presentations:

An abstract was submitted for presentation at the 2015 and 2016 NIJ Forensic Science R&D Symposia. The following presentations were given during the course of the grant and the NIJ was acknowledged for full support. Abstracts were published in meeting proceedings for select presentations.

Alexander Hulscher and Jodi Lynn Barta (2014) Recovery of DNA Evidence from Zar-Pro™ Blood Lifters. Poster presented at the 99th Annual **International Association for Identification Educational Conference**, August 12th (awarded First Place in the Student Division).

Christine Siress and Wilson Muse (2015) Generating fluorescence in blood. Oral Presentation at the **Michigan Academy of Science, Arts, and Letters Annual Conference**, March 13th.

Christine Siress and Wilson Muse (2015) Fluorogenic Enhancement: Making Blood Fluorescent. Poster presented at **Madonna University Symposium of Research, Scholarship, and Creativity**, April 8th.

Jodi Campo, Jessika Williams, Andrea Plewa and Jessica Zarate (2015) The compilation and interpretation of proteinaceous impression evidence and enhancement methods. Poster presented at **Madonna University Symposium of Research, Scholarship, and Creativity**, April 8th.

Alexander Hulscher and Jodi Lynn Barta (2015) Recovery of DNA Evidence from Zar-Pro™ Blood Lifters. Poster presented at **Madonna University Symposium of Research, Scholarship, and Creativity**, April 8th.

Jessika Williams (2015) The significance of inter-observer and intra-observer variability observed when rating the quality of enhanced impressions, and the magnitude of their fluorescence. Oral presentation at **Madonna University Senior Seminar**, July 17th.

Jessica Zarate and Jodi Lynn Barta (2015) No more either or: Working together to solve compatibility issues between impression enhancement and DNA analysis. Oral presentation at the 100th Annual **International Association for Identification Educational Conference**, August 6th.

Christine Siress and Wilson Muse (2015) Fluorogenic Enhancement of Latent Blood Impressions. Poster presented at the 44th Annual Meeting of the **Midwestern Association of Forensic Scientists,** September 23rd.

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Jessica Zarate and Jodi Lynn Barta (2015) No more either or: Working together to solve compatibility issues between impression enhancement and DNA analysis. Oral presentation at the 44th Annual Meeting of the **Midwestern Association of Forensic Scientists**, September 24th.

Jessica Zarate and Jodi Lynn Barta (2016) Working to Solve Compatibility Issues Between Impression Enhancement and DNA Analysis. Poster presented at the 68th Annual **American Academy of Forensic Sciences Scientific Meetings**, February 26th.

Jessica Zarate and Jodi Lynn Barta (2016) Working to Solve Compatibility Issues Between Impression Enhancement and DNA Analysis. Poster presented at the **Madonna University Symposium of Research, Scholarship, and Creativity**, April 13th.

Manuscripts submitted for publication:

Jessica Zarate, Nadeane Nasser-Beydoun, Alexander Hulscher, Nathan Jones, and Jodi Lynn Barta (2016) Defining Methods to Create Consistent and Reproducible Fingerprint Impressions Deposited in Biological Fluids on a Variety of Substrates. **Jacob's Journal of Forensic Science** – Accepted.

Manuscripts in preparation for publication:

Tentative Titles:

- Lifting, enhancing, and preserving blood and other proteinaceous impressions across a broad range of substrates using Zar-Pro™ Fluorescent Lifters
- Lifting, enhancing, and preserving aged blood and proteinaceous impressions using Zar-Pro™ Fluorescent Lifters
- Assessing variability in impression based research studies
- Developing novel fluorogenic enhancement sprays for impression enhancement
- Understanding mechanisms of fluorescence in blood
- The recovery of DNA from Zar-Pro™ lifted blood and semen impressions
- Utilizing a fluorescent standard in forensic science research

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Appendix D: Inter- and Intra-Examiner Agreement Tables

Inter-Examiner Variability

Table 1: Agreement Tables T3-T7

Trial #3

			Agree	Disagree
Observer A/Observer B	Fluoresence	NL	18	0
		AL	5	13
	Total:		**23**	**13**
	Ridge Detail	NL	11	7
		AL	14	4
	Total:		**25**	**11**
	OVERALL		**48**	**24**
Observer B/Observer C	Fluoresence	NL	18	0
		AL	7	11
	Total:		**25**	**11**
	Ridge Detail	NL	11	7
		AL	17	1
	Total:		**28**	**8**
	OVERALL		**53**	**19**
Observer A/Observer C	Fluoresence	NL	18	0
		AL	11	7
	Total:		**29**	**7**
	Ridge Detail	NL	17	1
		AL	15	3
	Total:		**32**	**4**
	OVERALL		**61**	**11**

Trial #4

			Agree	Disagree
Observer A/Observer B	Fluoresence	NL	18	0
		AL	7	11
	Total:		**25**	**11**
	Ridge Detail	NL	12	6
		AL	16	2
	Total:		**28**	**8**
	OVERALL		**53**	**19**
Observer B/Observer C	Fluoresence	NL	18	0
		AL	8	10
	Total:		**26**	**10**
	Ridge Detail	NL	9	9
		AL	16	2
	Total:		**25**	**11**
	OVERALL		**51**	**21**
Observer A/Observer C	Fluoresence	NL	18	0
		AL	7	11
	Total:		**25**	**11**
	Ridge Detail	NL	10	8
		AL	18	0
	Total:		**28**	**8**
	OVERALL		**53**	**19**

Trial #5

			Agree	Disagree
Observer A/Observer B	Fluoresence	NL	9	0
		AL	1	8
	Total:		**10**	**8**
	Ridge Detail	NL	6	3
		AL	6	3
	Total:		**12**	**6**
	OVERALL		**22**	**14**
Observer B/Observer C	Fluoresence	NL	9	0
		AL	0	9
	Total:		**9**	**9**
	Ridge Detail	NL	6	3
		AL	6	3
	Total:		**12**	**6**
	OVERALL		**21**	**15**
Observer A/Observer C	Fluoresence	NL	9	0
		AL	6	3
	Total:		**15**	**3**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**33**	**3**

Trial #6

			Agree	Disagree
Observer A/Observer B	Fluoresence	NL	9	0
		AL	6	3
	Total:		**15**	**3**
	Ridge Detail	NL	9	0
		AL	3	6
	Total:		**12**	**6**
	OVERALL		**27**	**9**
Observer B/Observer C	Fluoresence	NL	9	0
		AL	6	3
	Total:		**15**	**3**
	Ridge Detail	NL	9	0
		AL	3	6
	Total:		**12**	**6**
	OVERALL		**27**	**9**
Observer A/Observer C	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**36**	**0**

Trial #7

			Agree	Disagree
Observer A/Observer B	Fluoresence	NL	9	0
		AL	4	5
	Total:		**13**	**5**
	Ridge Detail	NL	8	1
		AL	8	1
	Total:		**16**	**2**
	OVERALL		**29**	**7**
Observer B/Observer C	Fluoresence	NL	9	0
		AL	2	7
	Total:		**11**	**7**
	Ridge Detail	NL	5	4
		AL	8	1
	Total:		**13**	**5**
	OVERALL		**24**	**12**
Observer A/Observer C	Fluoresence	NL	9	0
		AL	3	6
	Total:		**12**	**6**
	Ridge Detail	NL	4	5
		AL	9	0
	Total:		**13**	**5**
	OVERALL		**25**	**11**

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Intra-Examiner Variability

Table 2: Agreement Tables T3-T7

Trial #3				
			Agree	Disagree
Observer A	Fluoresence	NL	18	0
		AL	15	3
	Total:		**33**	**3**
	Ridge Detail	NL	17	1
		AL	16	2
	Total:		**33**	**3**
	OVERALL		**66**	**6**
Observer B	Fluoresence	NL	18	0
		AL	17	1
	Total:		**35**	**1**
	Ridge Detail	NL	18	0
		AL	18	0
	Total:		**36**	**0**
	OVERALL		**71**	**1**
Observer C	Fluoresence	NL	18	0
		AL	15	3
	Total:		**33**	**3**
	Ridge Detail	NL	18	0
		AL	16	2
	Total:		**34**	**2**
	OVERALL		**67**	**5**

Trial #4				
			Agree	Disagree
Observer A	Fluoresence	NL	18	0
		AL	18	0
	Total:		**36**	**0**
	Ridge Detail	NL	17	1
		AL	18	0
	Total:		**35**	**1**
	OVERALL		**71**	**1**
Observer B	Fluoresence	NL	18	0
		AL	18	0
	Total:		**36**	**0**
	Ridge Detail	NL	18	0
		AL	18	0
	Total:		**36**	**0**
	OVERALL		**72**	**0**
Observer C	Fluoresence	NL	18	0
		AL	18	0
	Total:		**36**	**0**
	Ridge Detail	NL	14	4
		AL	18	0
	Total:		**32**	**4**
	OVERALL		**68**	**4**

Trial #5				
			Agree	Disagree
Observer A	Fluoresence	NL	9	0
		AL	6	3
	Total:		**15**	**3**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**33**	**3**
Observer B	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	8	1
		AL	9	0
	Total:		**17**	**1**
	OVERALL		**35**	**1**
Observer C	Fluoresence	NL	9	0
		AL	7	2
	Total:		**16**	**2**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**34**	**2**

Trial #6				
			Agree	Disagree
Observer A	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**36**	**0**
Observer B	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**36**	**0**
Observer C	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**36**	**0**

Trial #7				
			Agree	Disagree
Observer A	Fluoresence	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**36**	**0**
Observer B	Fluoresence	NL	9	0
		AL	8	1
	Total:		**17**	**0**
	Ridge Detail	NL	9	0
		AL	9	0
	Total:		**18**	**0**
	OVERALL		**35**	**1**
Observer C	Fluoresence	NL	9	0
		AL	5	4
	Total:		**14**	**4**
	Ridge Detail	NL	9	0
		AL	8	1
	Total:		**17**	**1**
	OVERALL		**31**	**5**

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Appendix E: Examples of STR Profiles from Lifted Impressions

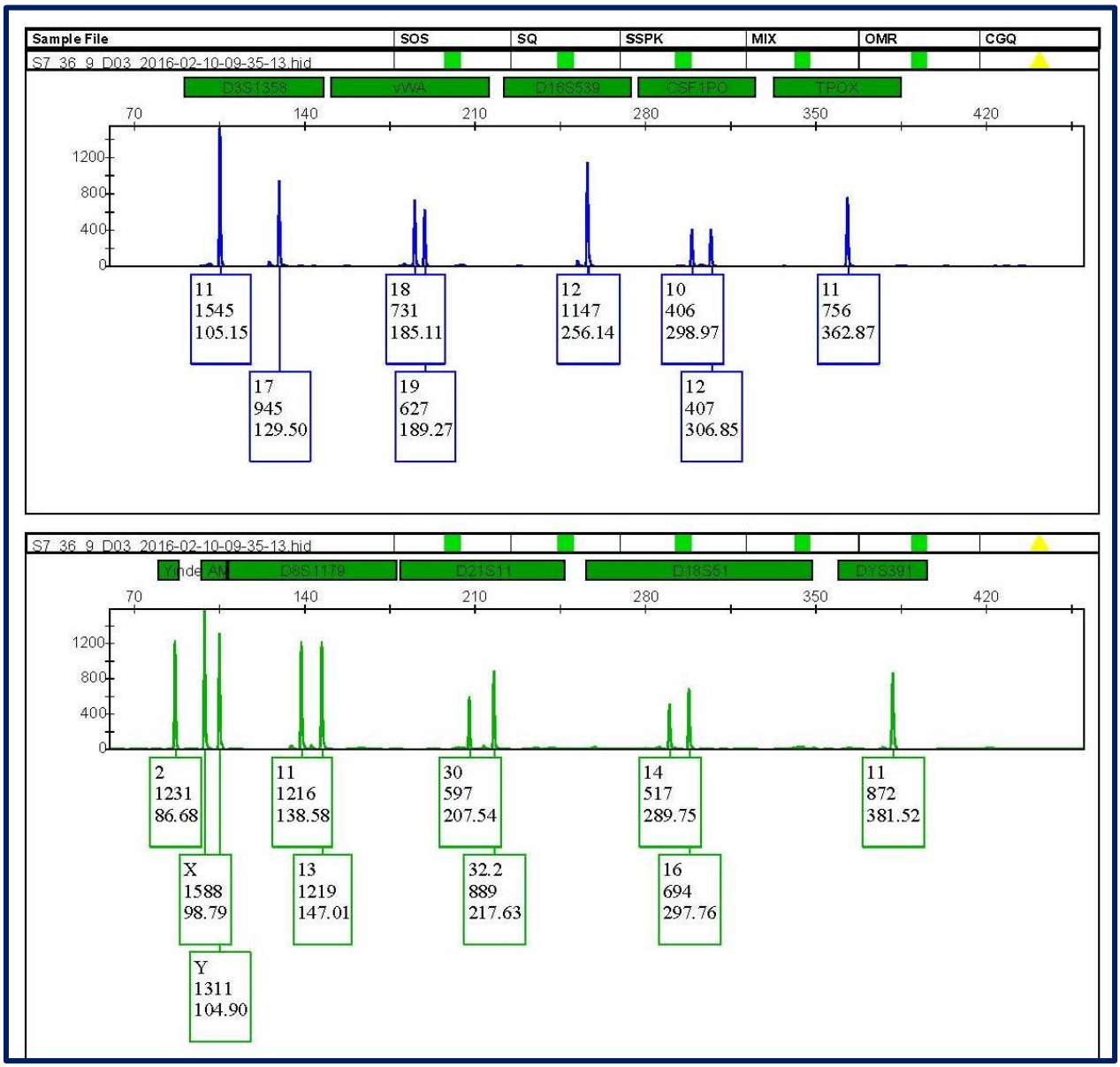

Figure 1. STR profile representing the quality of a full profile obtained for DNA extracted from a 9mm^2 sample of a Zar-Pro™ lifted semen impression, 36 hours after lifting.

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Figure 2. STR profile representing the quality of a partial profile obtained for DNA extracted from a 16mm^2 sample of a Zar-Pro™ lifted blood impression, 36 hours after lifting.

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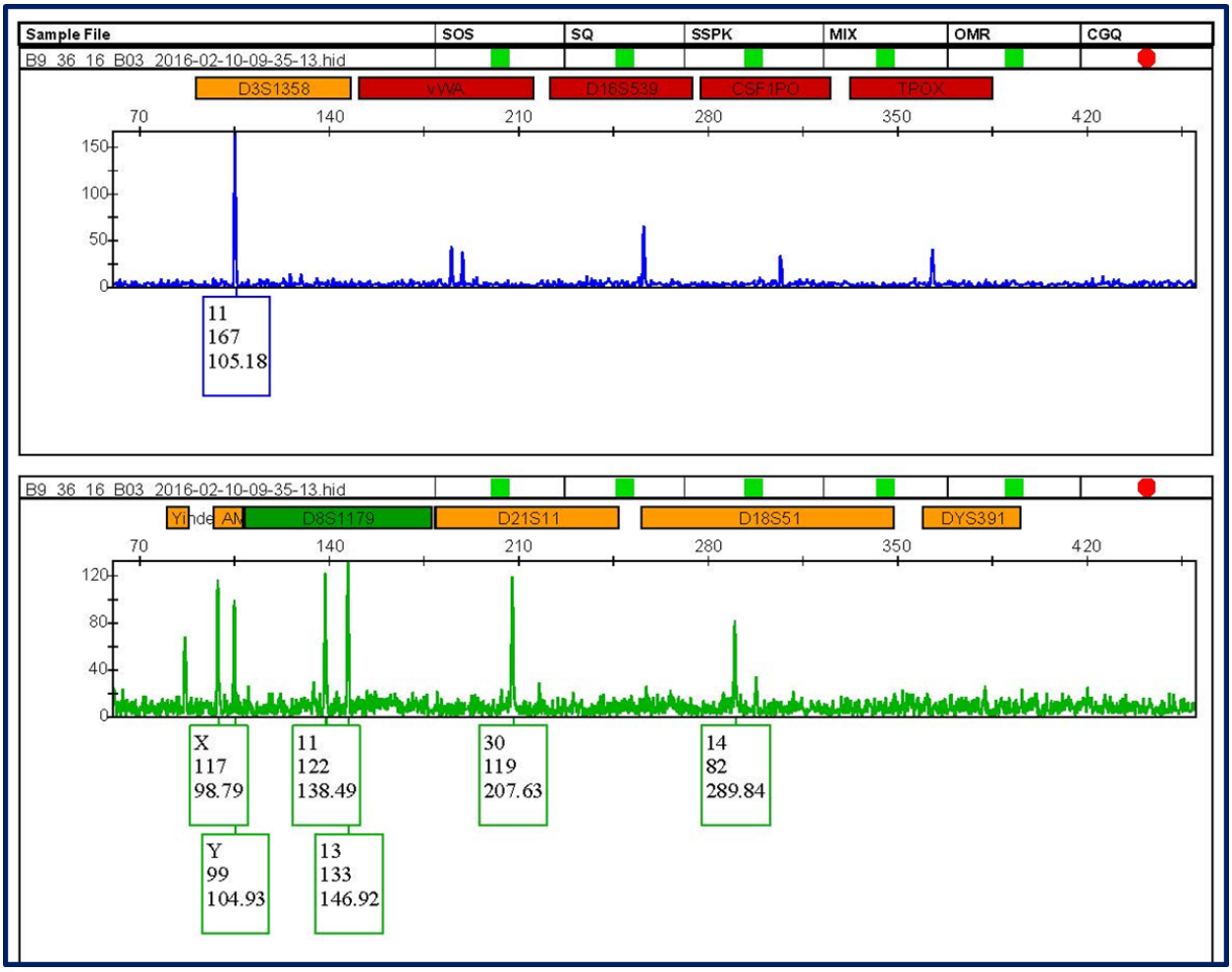

Figure 3. STR profile representing the quality of the no profile designation obtained for DNA extracted from a 16mm^2 sample of a Zar-Pro™ lifted blood impression, 36 hours after lifting.

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