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Optimizing A Method For Simultaneous Recovery of Proteins and DNA from Fingerprints

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This thesis has been presented to and accepted by the office of Graduate Studies, John Jay College of Criminal Justice in partial fulfillment of the requirements for the degree of Master of Science in Forensic Science.

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Abstract

DNA testing on touched objects is a valuable tool in forensic investigations, but DNA is usually present in low amounts, causing poor STR typing results. For touch DNA evidence, there is a clear need for additional individualization, especially for highly probative samples. This could be achieved by testing genetically variable proteins. The goal of this project was to develop a DNA/protein co-extraction method to facilitate DNA and protein testing on the same evidence item. Existing DNA extraction methods were carefully adjusted to allow for downstream mass spectrometry analysis. Initial experiments on saliva and fingerprints placed on glass suggested that trypsin and Millipore microcon MW100 units can be used to extract both DNA and protein from forensic samples, as well as produce interpretable DNA profiles and peptide sequences. The Microcon separation of both fractions was more suitable for both STR and mass spectrometry analysis than simply dividing the sample in half after trypsin digestion with no further purification. When tested in parallel to the standard Proteinase K method, the microcon co-extraction method had better DNA typing success rates. Mass spectrometry results for the microcon trypsin-based co-extraction method yielded expected amounts of identified proteins, including tissue specific proteins for both skin and saliva samples.

Introduction

In forensic DNA casework, short tandem repeats (STR) are routinely used as the primary genetic markers for human identification testing. In 1997, the FBI laboratory chose 13 mandatory STR loci to form the U.S. national DNA database. This panel was recently updated to 20 required STR to improve power of discrimination and ensure better international data compatibility (Hares, 2015). 99.7% of the human genome is the same from individual to individual. The 0.03% is what scientists use in order to tell people apart. STRs are Preferred Genetic Markers because: rapid processing is attainable, they are abundant throughout the genome, they are highly variable within various populations, they have a small size range allowing for multiplex development, discrete alleles allow for a digital record of data, allelic ladders simplify interpretation, PCR allows for small amounts of DNA material to be used, and a small product size is compatible with degraded DNA (Butler, 2012). In DNA analysis, the purpose of DNA extraction is to release the DNA molecules by lysing the cells, to remove inhibitors that could possibly reduce or affect PCR amplification, and to produce a stable solution consisting of high-quality DNA that will not degrade overtime. Two key components used for DNA extraction are Proteinase K and detergent. Proteinase K is an enzyme used in DNA extraction in order to break open cell membranes and break down proteins that protect DNA molecules, such as histones that are attached to chromosomes and block DNA sites. Detergent is used in DNA extraction in order to pull apart the lipids and proteins components of membranes that surrounding cell and nucleus. An example of a detergent that is commonly used is sodium dodecyl sulfate (SDS). Different types of biological evidence may require different reagents. When extracting semen, dithiothreitol

(DTT) is added to the Proteinase K/SDS mixture in order to break the strong disulfide bonds present in the cell wall. Another example is ethylene diamine triacetic acid (EDTA), which is a chelating agent that is added to samples when extracting blood in order to protect DNA molecules from nuclease enzymes (Butler, 2012).

For downstream testing, STRs are examined using multiplex polymerase chain reaction assays, with several generations of commercial kits available (Butler, 2012; Kline et al. 2011). Kit performance has been improving; validation studies of AmpF*l*STR® Identifiler® Plus PCR amplification kits, for example, have shown that this kit provides greater sensitivity, improved tolerance to PCR inhibitors, and more ease to decipher mixtures compared to older kits (Wang, et al., 2012). To compete with the ongoing improvements being made in STR assays and PCR kits, improvements have also been made in DNA quantitation. DNA quantitation is an important step in forensic casework because it determines the appropriate amount of DNA template to include in PCR amplification of short tandem repeat loci in order to avoid off-scale data and associate artifacts (Butler, 2012). If the PCR amplification contains too much DNA, the results will be overblown, making profile interpretation more challenging. If the PCR amplification contains too little DNA, this can result in loss of alleles due to stochastic amplification and failure to equally sample the STR alleles present in the sample. Validation studies on the Quantifiler Trio DNA Quantification Kit have established that this kit allows for greater detection sensitivity, and more robust performance with samples that contain PCR inhibitors or degraded DNA compared to older assays, such as the Quantifiler Duo (Holt et al. 2016). This is because the Quantifiler Trio DNA Quantification kit uses a multiplex TaqMan assay-based fluorescent probe to analyze

multiple-copy target loci, rather than just analyzing the single-copy loci used in the Quantifiler Duo (Holt et al. 2016). Improvements have also been made regarding instrumentation, such as design and analysis software. An internal validation study by NIST on the 3500 Genetic Analyzer found that full profiles were obtainable for DNA concentrations between 1.0ng to 0.1ng (Butts et al. 2011). The combination of improvements/advances in instrumentation, PCR kits, STR assays, and DNA Quantitation kits have, in turn, increased success rates and demand for the analysis of degraded/low template DNA evidence samples. One type of evidence that has recently become more popular, due to its frequent occurrence at crime scenes, is touch DNA evidence.

Touch DNA

Touch or transfer DNA analysis, "refers to the DNA that is left behind from skin cells when a person touches or comes into contact with an item" (Williamson, 2012). Such items can be the handle of a weapon, a doorknob, or even the bruised neck of a rape victim. Since 1997, full and partial profiles have been obtainable from DNA that was transferred to objects through touch (van Oorschot & Jones, 1997). Thanks to recent advances in DNA techniques, DNA testing on forensic evidence has evolved in such a way that it is now considered routine to collect DNA from touched objects (Meakin & Jamieson, 2013).

Factors affecting Touch DNA

Numerous studies have been conducted on all aspects on touch DNA, for efficient use in casework. Most current studies about touch DNA reflect on the analyses of the many factors that affect the success rate of touch DNA testing. Some of these factors

include the shedder status of an individual, an individual's age, how sweaty or dry a person's hands are, the time between deposition and recovery of DNA, and even the surface type, or object that is touched. How touch evidence is collected also introduces variability.

Touch DNA is based on the Locard Exchange Principle, which states that "every contact leaves a trace" (Hanson et al. 2012). When an individual touches an object their skin cells are transferred to that object either via direct (primary) or indirect (secondary) transfer. Direct (primary) transfer, as the name implies, refers to any form of an individual transferring their DNA to an item. For example, an individual could cough, speak in a room, or directly touch an item that is later recovered at a crime scene. Indirect (secondary) transfer, is when an individual's DNA is found on an object after some form of intermediary transfer. (Meakin & Jamieson, 2013). An example of this transfer is that individual A shakes the hand of individual B, individual B then later strangled a person (individual C). After swabbing the bruises on the victim's neck (individual C), individual A's profile is found. One very important factor that affects how much DNA gets transferred to an object is the shedder status of the individual. Shedder status refers to how much skin cells an individual sheds when coming into contact with an item. This classification of good/bad shedder was originally proposed in 1997 by van Oorschot & Jones, after observing how different individuals shed different amounts of DNA. Objects, which were handled by multiple individuals were typed and analyzed. After analyzing the results, it was concluded that the strongest profile from an object does not depend on the last person who came into contact with it, but instead the individual themselves. Another study was also able to observe a difference between individuals' ability to deposit skin

cells on a touched item (Lowe et al. 2002). One study however, was not able to distinguish individuals as good or bad shedders. Their results found no good shedders out of the 60 volunteers analyzed and, in turn, suggested no evidence of being able to distinguish individuals as good or bad shedders. Instead, their findings suggested that the amount of DNA an individual sheds is dependent on which hand they use and the time since they last washed their hands. (Phipps & Petricevic 2007).

Another factor that can affect the amount of DNA that gets transferred onto an object is how dry or sweaty an individual's hands are. There has been evidence showing that more individuals who are termed to be "good shedders," tend to have drier skin than bad shedders. One study found that individuals with skin diseases such as atopic dermatitis and psoriasis, which causes flaky skin, resulted in higher DNA deposition and better DNA profile quality (Kamphausen et al. 2012). Another study swabbed the hands and feet of volunteers, in order to test that DNA could be obtainable from items that have been worn or handled, due to the transfer of skin cells. Based on their findings, the authors hypothesized that individual donor variation could be the result of how dry or moist their hands are. The authors go on to explain that when an individual has dry skin, the skin begins to flake and chap, resulting in an increase in epithelial cells to be sloughed off when an item is touched. (Bright & Petricevic, 2004). Other studies have been conducted on the elapsed time since hand-washing, before an individual touches an item. One study analyzed touched items from both good and bad shedders who washed their hands at various intervals. After analyzing their results, the authors found that only good shedders can produce a full DNA profile immediately after hand-washing. The authors also stated that once the time interval since individuals washed their hands is

between 2 to 6 hours, the shedder type of an individual is no longer an issue. (Lowe et al. 2002).

In forensic casework, one of the biggest issues in determining whether or not an interpretable DNA profile will be obtained from a piece of evidence, is the time between deposition and recovery. As time elapses from deposition to recovery, factors such as temperature, dust, and sunlight can degrade the cells. One study analyzed 643 fingerprints on glass slides for the following time periods: 1, 3, 10, 20, and 40 days. Results showed significant decreases in profile fractions between to first day and 10 days or more (Ostojic & Wurmbach, 2017).

Another factor which can play a role into the amount of skin cells an individual may leave behind is the type of substrate that is touched. One study, conducted by Daly et al. 2012, examined the amount of DNA left behind based on the type of surface that was touched. In this study, men and women volunteered to hold one of the three objects (glass, fabric, or wood) for a minute, giving 100 samples of each substrate. DNA was then extracted from these touched objects using minitapes and amplified in a PCR to produce genetic profiles. Based on their findings, results showed a significant difference between the amount of DNA left behind depending on the object. Wood showed to be the most useful out of the three, followed by fabric, and glass was found to leave behind the least amount of DNA.

There are various types of collection methods used in forensic casework such as cotton or polyester swabs, flocked swabs, and tape-lifts. However, there is much debate on which type of swab/swabbing technique is the best for certain samples. For example, one study found that both the substrate and sample type (blood, saliva, touch DNA, etc.)

affect the results of swab efficiency. In regards to recovering touch DNA samples from smooth/nonporous substrates, polyester swabs were found to be most effective (Verdon et al. 2014 a). Tape-lifting is another type of collection method for touch DNA that has increased in use in forensic casework. The concept of the tape-lift collection method is that the adhesive on the tape will leave behind less shed skin cells, than swab collection methods. One study compared three different swabs (dry swab, DNA flocked swab, and self-saturating swab) and Scenesafe FAST minitape with trace DNA samples. Results showed that there was no difference between any of the three swabs for collecting trace DNA. However, their results do suggest the tape-lift collection method to be better suited than the three swabs. (Hansson et al. 2009). Another study evaluated a variety of tapes that are used in forensic casework for touch DNA samples on four different substrates. Based on their findings the authors stated that tape-lifting with Scenesafe FAST minitape obtained more DNA than any of the swabbing techniques that were tested. (Verdon et al. 2014 b). Flocked swabs have also proven to be a good collection method in forensic DNA collection. The concept behind these swabs is that the strands that make up the head of the swab are directed outward, increasing the surface area of the swab during collection (Plaza et al. 2016).

In summary, one can state that DNA testing on touched objects is a valuable tool for forensic investigations. Success rates vary widely, based on donor to donor variation and the type of substrate. Especially for smooth substrates, DNA that is collected from touched objects may be either too degraded or of insufficient quantity to result in a good quality DNA profile. One of the major challenges in examining touch DNA evidence, is that the results from STR typing often show partial profiles with allelic dropout, and/or

DNA mixtures. Carefully adjusting each step such as the laboratory's collection, extraction, and typing strategy can improve success rates. Another approach to supplement touch DNA evidence analysis is the addition of protein testing.

Protein and Touch DNA

An individual's hands act as a vector for transferring cells to the object that is touched, and these cells do not only contain DNA. One study was able to identify five highly specific and sensitive mRNA biomarkers for the identification of skin (LCE1C, LCE1D, LCE2D, CCL27, and IL1F7), that were analyzed through two different multiplex systems (Hanson et al. 2012). From their results, LCE1C was found to be the most sensitive marker out of the five tested. However, despite these findings, another study, which employed this multiplex of five mRNA biomarkers was only able to obtain a 50% detection rate (Ballantyne, 2014).

Morphologically, shed skin cells are most likely derived from the outer layer of the epidermis, known as the stratum corneum. The stratum corneum, in healthy individuals, consists of fully keratinized, cornified cells, which have gone through apoptosis and lost their nuclei (Alessandrini et al. 2001). Theoretically, because of this increase in keratinization and apoptosis of the stratum corneum, the reduction in cell metabolism should also result in loss of DNA and mRNA production, while the protein content still accumulates. This, in turn, makes epidermal proteins better candidates for tissue type identification on touch evidence. When an individual leaves a fingerprint behind, for example, shed skin cells are left behind that contain various keratins, calmodulin like 5 (CALML5), secretoglobulin 2A2 (SCGB2A2), chemokine 27 (CCL27), interleukin 37 (IL1F7), and late cornified envelope proteins (LCE1C, LCE1D,

LCE2D). Recent advances in mass spectrometry allow this technique to be used to detect protein polymorphisms in tissue specific proteins. Even though an additional mass spectrometry step may not prove to be practical when dealing with high volume crime, the application could focus on such objects that are known to be challenging when trying to obtain an interpretable DNA profile. Examples of such cases would be the DNA on fired/unfired cartridge casings, explosive devices, or even certain items found at a scene involving a missing person. Since the DNA from such samples often suffers from degradation, PCR inhibition, and low DNA yields, analyzing the proteins of such samples may prove to be more feasible.

Even though there are no current studies on the forensic application of mass spectrometry to touch DNA evidence, there are publications of proteomes on the components of interest such as skin (Parkinson et al. 2014), saliva (Amado et al. 2013), and sweat (Park et al. 2011) that could guide marker selection. A recent study analyzed genetically variant protein polymorphisms in hair shafts for human identification. Hair shaft proteins in this study were characterized through the use of mass spectrometry-based shotgun proteomics of 66 individuals. From all the hair shafts of individuals tested, 596 single nucleotide polymorphism alleles were correctly imputed in the subjects' DNA. Results also showed that most of the detected peptides were keratins or keratin associated proteins. This study demonstrated that genetically variant proteins could be obtained from the hair shaft of individuals and through mass spectrometry, could discriminate between different individuals. (Parker et al. 2016).

Goal of this Study

After assessing all the information explained above, the goal of this research is to optimize a method for a DNA/protein co-extraction that would result in the highest yield for both DNA and protein from fingerprints. Existing methods for low template DNA will be used and modified such that proteins can be extracted at the same time. If such a method can be developed, one could apply it towards obtaining enough protein from touch evidence and use mass spectrometry to analyze the protein component to search for protein polymorphisms.

Methods and Materials

Sample Collection and Substrate Preparation:

Glass slides were cleaned with 10% bleach, deionized water, and then 75% ethanol. Glass slides were then dried with kimwipes and placed in a glass slide holder tray. Volunteers were recruited using flyers and group emails. All samples were collected anonymously following approval by the CUNY University Integrated Institutional Review Board. Prior to sample donation, volunteers were asked to wash their hands with soap and water to remove extraneous DNA, then dry them with paper towels. After they washed and dried their hands, volunteers rubbed their face for 15 seconds and then rubbed their hands together for 15 seconds to produce sebaceous fingerprints. Volunteers then applied their fingerprints (thumb and three fingers, except pinky) on a clean/labeled glass slide by pressing down for 5 seconds. For all method comparisons, left and right hand prints for each donor were tested in parallel.

Proteinase K Based DNA Extraction

Standard:

Within 24 hours, the latent fingerprints on the glass slides were swabbed with a moistened swab using a standard swabbing method (10 times vertically followed by 10 times along the slide horizontally). Swabs were moistened with 5µl of 0.01% Sodium Dodecyl Sulfate (SDS) buffer. Opening one tube at a time, the tip of each swab was cut with a pair of sterile scissors into an irradiated and labeled 1.5mL tube. The cut swabs were incubated at 56^{o}C in 100µl of an SDS:Proteinase K:DTT solution for 60 minutes with shaking at 1400rpm. As a negative control, 100µl of this solution was transferred into an irradiated and labeled 1.5mL tube. The 100µl of incubation buffer added to each sample had the following concentrations: 0.01% SDS, 0.80mg/mL Proteinase K, and 35mM DTT. Samples were then placed on a heat block set at 99^{o}C for 10 minutes and then cooled for 10 minutes in an ice tray. Opening each 1.5mL tube at a time, each entire sample (including each swab substrate) was then transferred onto an irradiated spin basket in an irradiated and labeled 2mL dolphin tube. Swabs were transferred using a previously cleaned pair of tweezers and scissors. Between samples, tweezers and scissors were cleaned with 10% bleach, reverse osmosis water, followed by 75% ethanol, and then dried off with kimwipes. Once transferred, samples were balanced out in a microcentrifuge and spun for 5 minutes at 1500rcf. After centrifugation, the liquid DNA extracts were collected, transferred onto Microcon-100 fast flow membranes, and centrifuged for 20 minutes at 500rcf. This removed the SDS and salts in the flow through, leaving purified DNA on the top of the membranes. To recover the DNA fraction of each sample, 30µl of 0.1 x TE buffer was added on top of the Microcon-100 membrane, where

the microcons were inverted into a new irradiated/labeled microcon tube and centrifuged again for 3 minutes at 1000rcf. The recovered extract (consisting of purified DNA) was transferred into a labeled and irradiated 1.5mL tube. Prior to transferring the sample, the volume was estimated using the pipette. Volumes were then recorded on the extraction batch sheet. DNA fractions were stored at either +4^{o}C or -20^{o}C.

High Yield Method:

For the High Yield extraction method, the method consists of the same as above with the following adjustments. Swabs were moistened with 5µl of 0.05% Sodium Dodecyl Sulfate (SDS) buffer. Opening one tube at a time, the tip of each swab was cut with a pair of sterile scissors into an irradiated and labeled 1.5mL tube. The cut swabs were incubated at 56^{o}C in 200µl of an SDS:Proteinase K solution for 30 minutes with shaking at 1400rpm. The negative controls for this method consisted of 200µl of the prepared incubation buffer solution. The 200µl of incubation buffer added to each sample consisted of the following concentrations: 0.05% SDS, 0.80mg/mL Proteinase K. Before adding each sample onto its proper microcon membrane, a total of 1mg of fish sperm was used to pre-coat the microcon membranes after diluting the stock fish sperm DNA according to the following:

Reagent	**1 Sample (2 Microcons per sample)**
0.05% SDS	398µl
Fish Sperm DNA (1mg/mL)	2µl

Each sample extract was then added to its properly labeled/pre-coated membrane. Another adjustment with this method is that two elutions were used instead of one. The first elution consisted of the following. Samples added to their pre-coated membranes

were centrifuged at 500rcf for 20 minutes, 200µl of irradiated water was added onto the membranes, which were then inverted into a new labeled/irradiated collection tube, and centrifuged for 3 minutes at 1000rcf. Each samples eluate was then added to the second pre-coated microcon membrane, where the samples were centrifuged again at 500rcf for 20 minutes. To recover the DNA, 20µl of 0.1 x TE buffer was added onto the membranes, which were then inverted one at a time into a new irradiated/labeled collection tube. Tubes were then centrifuged at 1000rcf for 3 minutes. The recovered extract (consisting of purified DNA) was transferred into a labeled and irradiated 1.5mL tube. Prior to transferring the sample, the volume was estimated using the pipette. Volumes were then recorded on a batch sheet. DNA fractions were stored at either +4^{o}C or -20^{o}C.

Trypsin Based DNA/Protein Co-Extraction

Microcon Separation:

For the Trypsin Microcon Separation extraction method, the method consisted of similar steps as the standard Proteinase K method with the following adjustments. In order to make this extraction compatible with protein extraction as well as downstream DNA typing, Proteinase K was replaced with methylated/TPCK-treated trypsin. The SDS buffer was replaced with "fresh" 50mM Ammonium Bicarbonate, to account for downstream Mass Spectrometry. "Fresh" is referring to being made within the past three days. 1% Protease Max was used in this method in order to enhance protein digestion, and 0.5M DTT was used to help break down disulfide bonds. The reagents used in the incubation buffer consist of the following concentrations: 50mM Ammonium Bicarbonate (NH_4HCO_3), 0.01% Protease Max, and 5mM DTT. Swab tips were cut as

described above, covered with 100µl of trypsin incubation buffer and incubated at 56°C, while shaking at 1400rpm for 20 minutes. 1µl of a 0.1µg/µl trypsin solution was added to each sample after the initial incubation, followed by a 3-hour incubation period at 37°C with shaking at 1400rpm. Following the heating and cooling steps described above, opening each 1.5mL tube at a time, each entire sample (including the swab substrate) was transferred onto an irradiated spin basket in an irradiated and labeled 2mL dolphin tube. Swabs were transferred using a previously cleaned pair of tweezers and scissors. Once transferred, samples were spun for 5 minutes at 1500rcf. Liquid extracts were transferred onto Microcon-100 membranes and centrifuged for 20 minutes at 500rcf. The flow through contains digested peptides (protein fraction), while what remains on top of the Microcon-100 membrane contains the DNA (DNA fraction). The flow through was transferred to Protein Low Bind tubes and volumes were measured and recorded. To recover the DNA fractions, 30µl of dH_2O was added on top of each membrane, instead of 0.1 x TE buffer, and inverted into a new irradiated and labeled microcon tube. Tubes were centrifuged for 3 minutes at 1000rcf, where volumes of each DNA fraction were estimated as described above in the standard Proteinase K extraction method. DNA fractions were stored at either +4°C or -20°C and protein fractions were stored at -80°C. Table 1 lists the modifications of this initial trypsin DNA extraction protocol that were introduced to optimize DNA and peptide yields. All modifications were tested in comparison to the initial method described above.

Table 1. Trypsin Microcon DNA/Protein Co-extraction Method Modifications	
Modification	**Description**
Addition of Poly ARNA	10 ng/µl stock of PolyA-RNA was diluted to 0.05 ng/µl with dH_2O. 20µl of this diluted PolyA-RNA was then used to pre-coat each microcon membrane prior to sample addition and centrifugation at 500rcf for 20 minutes.
Use Microcon 30 instead of Microcon 100	Following trypsin digestion, samples were either transferred on to a microcon 30 membrane or a microcon 100 membrane.
Alkylation	After the initial incubation period, 3µl of iodoacetamide was added to each sample, followed by a 30 minute incubation period at room temperature in the dark.
Replace Protease Max with Sodium Laurate	The incubation buffer was made using 1% Sodium Laurate diluted to 0.01% in ammonium bicarbonate.
Additional Wash Step	After adding samples onto Microcon membranes, samples were centrifuged for 30 minutes at 500rcf. After this first concentration step, 40µl of dH_2O was added and samples were then spun for an additional 15 minutes at 500rcf before moving on to Protein and DNA fraction recovery steps.

Different Sample Collection Methods:

All tests of different collection methods involved the additional wash step modification for the trypsin based DNA/Protein Co-extraction: Microcon Separation method. After volunteers applied fingerprints on a glass slide, the slide was swabbed using either a 6-inch plastic shaft sterile polyester swab (Fisherbrand), a COPAN flocked swab (COPAN FLOQSwabs), or a tape-lift using a 1.2cm x 1.2cm piece of Sello Tape. For the flocked swabs, in order to have the entire swab submerged in incubation buffer, a 200µl volume was used. For the tape-lift collection method, a piece of irradiated tape, approximately

0.5cm x 0.5cm, was applied to the glass slide over the latent print ten times using sterile tweezers.

No Microcon "Split" Method

For the Split Method, samples were collected as described above. The concentrations of each of the reagents used to make the incubation remained the same as those used for the Microcon Separation extraction method: 50mM Ammonium Bicarbonate (NH_4HCO_3), 0.01% Protease Max, and 5mM DTT. But after incubation, the DNA fraction was not purified/concentrated, which meant the incubation volume needed to be lower in order to maintain DNA concentration levels. Therefore, 50µl of incubation buffer was added to each sample, instead of 100ul, before incubating at 56^{o}C with shaking at 1400rpm for 20 minutes. After samples were spun down in dolphin tubes using irradiated spin baskets, the volume was determined and half was placed into an irradiated and labeled 0.5mL tube, whereas half of the volume was then transferred into a 0.5mL Protein Low Bind tube. DNA fractions were stored at -20^{o}C and protein fractions were stored at -80^{o}C.

Reference Sample Extraction

Reference samples of volunteers were extracted from buccal swabs using the following Chelex extraction method. One at a time, each buccal swab was cut and transferred to an irradiated/labeled 1.5mL tube using a pair of cleaned scissors and tweezers. Opening one tube at a time using kimwipes, 1mL of irradiated dH_2O was transferred to each tube and then mixed by vortexing. 1mL of irradiated dH_2O was transferred to a labeled tube as an extraction negative control. The tubes were then incubated for 20 minutes at room temperature, occasionally inverting and vortexing the sample during this time. After this incubation period, tubes were spun in a balanced microcentrifuge for 3 minutes at

13,400rpm. After centrifugation, approximately 950µl of supernatant was carefully removed from each tube. 175µl of well-resuspended 5% Chelex solution was then transferred to each tube and briefly vortexed. Tubes were then incubated in a 56^{o}C water bath for 20 minutes. Once this time elapsed, tubes were vortexed at high speed for 10 seconds, and then incubated in a 100^{o}C water bath for 8 minutes. After this time, tubes were vortexed again at high speed for 10 seconds. Tubes were then balanced in a microcentrifuge and spun for 3 minutes at 13,400rpm. After centrifugation, approximately 100µl of supernatant was transferred to a clean/labeled 1.5mL microcentrifuge tube. Tubes containing the extracted DNA products were stored at -20^{o}C.

DNA Quantification

All the extracted DNA samples were quantitated using the Quantifiler® Trio DNA Quantification kit (Life Technologies, Thermo Fisher Scientific) on the Applied Biosystems®, Life Technologies 7500 Real-Time PCR system. Five standards were prepared through serial dilutions as per manufacturer guidelines. These standards consisted of the following concentrations:

Standard	Concentration
Standard 1	50ng/µl
Standard 2	5ng/µl
Standard 3	0.5ng/µl
Standard 4	0.05ng/µl
Standard 5	0.005ng/µl

Sufficient master mix consisting of 5µl of Quantifiler THP PCR reaction mix and 4µl of Quantifiler Trio Primer Mix was prepared for all standards, controls, and unknown samples, where 9µl of each were loaded into previously designated positions in a 96 well optical plate. Based on the plate map, designating which sample, negative control, and standard would be loaded into which well; 2µl of each sample, negative control, or

standard were used to determine the concentrations. Plates were sealed with optical foil. The 7500 system parameters were set to the following settings:

Stage 1: 50.0°C for 2min

Stage 2: 95.0°C for 10min

Stage 3: 95°C for 15sec then 60.00°C for 1min (40 x)

The data was analyzed by the HID Real-Time PCR Analysis Software v1.2, which plotted the standard concentration curve and used it to determine the concentration of each sample.

Peptide Quantification

All the extracted protein samples were quantitated using the Pierce Quantitative Fluorometric Peptide Assay Kit (Thermal Fischer Life Technologies). After each sample and standard was completely thawed from the -80^{o}C freezer, they were briefly vortexed and spun down. Five standards were prepared through serial dilutions as per manufacturer guidelines. These standards consisted of the following concentrations:

Standard	Concentration
Standard A	1000µg/mL
Standard B	500µg/mL
Standard C	250µg/mL
Standard D	125µg/mL
Standard E	62.5µg/mL
Standard F	31.3µg/mL
Standard G	15.6µg/mL
Standard H	7.8µg/mL
Blank	0µg/mL

After filling out a plate map, 10µl of each standard, control, and unknown samples were transferred into a well of a microplate according to the plate map. 70µl of Fluorometric Peptide Assay Buffer followed by 20µl of Fluorometric Peptide Assay Reagent was then

added to each well, and mixed by pipetting up and down. The microplate was then covered with sealing tape and incubated at room temperature for 5 minutes. The fluorescence of each sample and standard was then measured using an excitation of 390nm/emission of 475nm using a BioTek Synergy MX Microplate Reader.

Identifiler Plus Amplification

Polymerase chain reactions (PCR) were set up using the AmpF*l*STR® Identifiler Plus™ PCR Amplification Kit by Life Technologies, Thermo Fisher Scientific. Reduced volume reactions (12.5µl) were set up containing 5.0µl of Master Mix and 2.5µl Primer Mix. Each sample consisted of concentrations up to a maximum of 1ng. For samples with low quantitation results the maximum amount (5.0µl) of DNA extract was added. The positive control provided in the kit was diluted to 0.05ng/µl in 0.1 x TE buffer and 5µl of 0.1 x TE buffer was used as an amplification negative control. All samples and controls were placed in the thermal cycler set to the following cycling parameters:

Initial Incubation	**Denature**	**Anneal/Extend**	**Final Extension**	**Final Hold**
HOLD	29 CYCLES		HOLD	HOLD
95°C 11 min	94°C 20 sec	59°C 3 min	60°C 10 min	4°C ∞

3500 Electrophoresis:

All samples were analyzed on the 3500 genetic analyzer (Applied Biosystems®, Life Technologies). Sufficient master mix consisting of 0.36µl of 600 LIZ internal size standard v2.0 and 11µl of Hi-Di™ Formamide was prepared for all allelic ladders, controls, and unknown samples, where 11µl was loaded into each previously designated position in a 96 well optical plate. Based on the plate map, designating which sample, 9947A positive control, negative control and allelic ladder would be loaded into which

well, 1.2µl of each was transferred. An allelic ladder was loaded into the first well of each column used. Any empty wells were filled with 10ul of Hi-Di™ Formamide. Once all allelic ladders, 9947A positive controls, negative controls, samples, and empty wells were filled the plate was sealed with a septa, briefly centrifuged to get all contents at the bottom of the plate, and placed into the GeneAmp 9700 PCR system (Applied Biosystems®, Life Technologies) set to the denature/chill protocol (95°C for 5min followed by 4°C for 5min). The sample tray was then briefly centrifuged again and placed into the 3500 instrument. All runs were performed using a 36cm capillary with POP-4 polymer (Life Technologies) and injection settings of 1.2kV and 15 seconds that had been optimized in preliminary experiments. All raw sizing data from the 3500 Genetic Analyzer were converted into allele calls using GeneMapper ID-X v. 1.5 software (Life Technologies Thermo Fisher Scientific). The analytical detection threshold was set to 50 RFU; -4 basepair stutter filters were left at validated Life Technologies settings.

Mass Spectrometry Analysis:

Mass spectrometry analysis was run with 5uL of extract or flow-through and peptides were separated by reversed-phase liquid chromatography using an Easy-nanoLC 1000 HPLC (Thermo Scientific, Asheville, NC, USA), fitted with a Thermo Scientific Q Exactive Orbitrap mass spectrometer. All mass spectrometry analysis was performed by the Genetically Variable Protein (GVP) team at Lawrence Livermore National Laboratory in Livermore, CA.

Statistical Methods

To test for significance, Mann Whitney U tests were performed for tests that consisted of a sample size of $n \geq 5$. This included the Microcon versus No Microcon "Split" and the finalized method batch testing (standard Proteinase K versus Microcon Methods). Each test performed, used a significant P-value of 0.05.

Materials

Sample collection:

Skin: D-Square Standard sampling discs – CuDerm Corporation D100

Liquid Saliva: Pure Sal Saliva Collection device – Oasis PRSAL-401

Buccal cells: Puritan Capped Cotton Tipped Swabs – Puritan 25-806 1WC EC

Saliva trace, prints: Puritan Cotton Swabs – Puritan 806-WC

Prints: Fisherbrand Sterile Polyester Tipped Swabs – Fisher Scientific 23400122

COPAN FLOQSwabs 30mm Break Point – COPAN 520CS01

Reagent sources and order numbers:

Ammonium Biocarbonate – Fisher Chemical A643

Dithiothreitol – LifeTechnologies D1532

Gene Scan™ 600 LIZ™ Size Standard v2.0 – Thermo Fisher Scientific 4408399

Hi-Di Formamide – Thermo Fisher Scientific 4311320

Identifiler Plus STR Kit – LifeTechnologies 4427368

Iodoacetamide – Sigma Aldrich I1149

Microcon Fastflow – Millipore MCRFOR100 and MCRFOR030

Poly A RNA – Sigma Aldrich 10108626001

Protease Max - Promega V2071

Proteinase K (recombinant) – LifeTechnologies EO0492

Quantifiler Trio Kit – LifeTechnologies 4482919

Quantitative Fluorometric Peptide Assay (Pierce) – Thermo Fisher Scientific 23290

Sodium Dodecyl Sulfate – Ambion AM9820

Sodium Laurate – Sigma Aldrich TCI L0016

Trypsin (Sequencing Grade Modified) - Promega V5113

Results

Evaluation of DNA Extraction Methods and Optimized DNA/Protein Recovery

Comparison of Initial Extraction Methods

In order to make the standard Proteinase K method compatible with protein extraction, as well as downstream typing the following changes in reagents were made. Proteinase K was replaced with methylated/TPCK-treated trypsin, the ammonium bicarbonate was added to the buffer, and 0.01% SDS was replaced with Protease Max in order to enhance protein digestion to account for downstream mass spectrometry. Several experiments compared fingerprints (three fingers plus thumb, except pinky finger) and 5ul of spotted saliva samples. For the High Yield extraction method, no saliva samples were initially tested, only fingerprint samples. The average DNA and protein yields of each of the initial extraction methods tested (High Yield, standard Proteinase K, and Microcon Method) can be seen in the table below (Table 2).

Table 2. Average DNA Yields and Protein Results for Initial Extraction Methods Tested			
Test	**Average DNA Yield (Total ng)**	**Average Peptide Yield (Total ng)**	**Identified Protein Count (n=1)**
High Yield (ProK + carrier) Fingerprints (n=2)	5.4	n/a	n/a
Standard (ProK) Fingerprints (n=2)	4.8	n/a	n/a
Standard (ProK) Saliva (n=2)	104.8	n/a	n/a
Microcon Method (Trypsin) Fingerprints (n=2)	3.6	Inconclusive	54
Microcon Method (Trypsin) Saliva (n=2)	102.3	39229.3	204

These results suggest that trypsin can be used to extract both DNA and digested peptides, which was never thought to be possible. Samples did not have to be processed further prior to peptide sequencing on the QExactive. Samples from each extraction method were also typed in Identifiler Plus, giving interpretable DNA profiles (data shown in heatmap in Appendix, Figures 3 & 4).

Modifications and Variations Tested

The following modifications and variations were then tested with the Microcon extraction method: addition of polyA-RNA, Microcon 30 vs. Microcon 100 fast flow membranes, addition of a 30 minute alkylation step, the use of protease max vs. sodium laurate, the use of an additional wash step. The addition of polyA-RNA expected to improve DNA yields by blocking non-specific binding to the Microcon membrane (Schiffner et al. 2005). The different pore sizes of microcon fast flow membranes were tested with the idea that the microcon membranes with the smaller pore size (microcon 30) should

capture even degraded DNA and potentially increase DNA yields (Garvin & Fritsch, 2013). Normally with peptide extractions iodoacetamide, an alkylating agent, is used in an additional 30-minute alkylation step. During this step, the iodoacetamide prevents disulfide bond formation of cysteines. Finally, proteins were being detected in the DNA fraction, so an additional wash step was tested to see if it would help reduce the amount of proteins found on the top of the membrane. A summary of the results seen from each of the variations tested can be seen below (Table 3).

Table 3. Results of Modifications/Variations Testing with the Microcon Method			
Variation Tested	**Average DNA Yield (Total ng)**	**Average Peptide Yield (Total ng)**	**Identified Protein Count (n=1)**
PolyA-RNA (n=4)	6.7	6373.5	59
Fingerprints			
No PolyA-RNA (n=4)	18.9	4624.1	67
Microcon 30 (n=2)	3.5	Inconclusive	111
Fingerprints			
Microcon 100 (n=2)	2.1	623.6	128
Alkylation (n=2)	93.8	5700.1	224
Saliva			
No Alkylation (n=2)	239.0	8587.9	241
Protease Max (n=2)	7.5	4158.5	81
Fingerprints			
Sodium Laurate (n=2)	8.0	3850.7	74
Additional Wash (n=2)	1.5	7035.4	92
Fingerprints			
No Additional Wash (n=2)	3.0	6889.7	116

Results for the polyA-RNA modification showed higher protein yields with the addition of polyA-RNA, while the DNA yields were reduced when compared to samples where polyA-RNA was not added. From the mass spectrometry results, a higher amount of identified peptides was observed when polyA-RNA was not used. The sample with polyA-RNA resulted in 59 identified peptides, while the sample without polyA-RNA resulted in an identified peptide count of 67. For the different microcon membranes, fingerprint samples showed no difference in regards to DNA yields. In regards to the protein fractions, the microcon 30 membranes gave a result of either zero or in the

negative range, but considering the mass spectrometry results, this result must be interpreted with caution and was deemed inconclusive. For the microcon 100 membranes, only one out of the two samples tested, gave a protein yield that was greater than zero. After being analyzed through mass spectrometry at the Lawrence Livermore National Laboratory, the microcon 100 fast flow membrane sample resulted in 128 identified peptides, while the microcon 30 fast flow membrane sample resulted in 111 identified peptides. These mass spectrometry results suggested that the amount of identified peptides detected for the microcon 30 fast flow membranes, should have been similar to other samples. This suggested that the Thermal Fisher Life Technologies Pierce Quantitative Fluorometric Peptide Assay Kit failed, or was not sensitive enough. For the alkylation test, the modification was only tested on 1µl saliva samples and results showed that no peptides were detected with the addition of the 30-minute alkylation step. However, these samples had protein counts when later analyzed through mass spectrometry by the Lawrence Livermore National Laboratory, which further supports that the Thermal Fisher Life Technologies Pierce Quantitative Fluorometric Peptide Assay Kit was not the best assay to predict mass spectrometry results (data not shown). This test was then repeated using 5µl of saliva sample rather than 1µl. This time, results showed that both DNA and protein yields were higher without the alkylation step (Table 3). Mass spectrometry results suggested that the microcon co-extraction method is better suited for downstream mass spectrometry when no 30-minute alkylation step is used. Mass spectrometry results gave an identified peptide count of 241 when no alkylation step was used in the extraction method, while an identified peptide count of 224 was observed when an alkylation step was added. Results showed no difference in DNA

yields between using either Protease Max or sodium laurate. In regards to the protein fractions, the average protein yield for Protease Max was slightly improved compared to sodium laurate. Mass spectrometry results showed a slightly higher identified peptide count of 81 for the sample with Protease Max, while an identified protein count of 74 was observed for the sample with sodium laurate. For the additional wash test, results showed protein yields to be higher and DNA yields to be lower, when an additional wash step was used. Less proteins were also observed in the DNA fractions when an additional wash step was used. The mass spectrometry results for the additional wash test showed a count of 116 identified peptides when an additional wash was not used, while an identified peptide count of 92 was observed for the sample when an additional wash was used. This reduction would be consistent with the increase in volume and thus decreasing the peptide concentration for the flow through after the additional wash. All samples from each variation/modification tested with the microcon extraction method were also typed for Identifiler Plus STR markers. Out of all the samples, only five gave bad partial profiles, while the rest resulted in good partial to full DNA profiles (data shown in heatmap in Appendix, Figures 3 & 4). Typing results also showed signs of mixtures for some of the samples. However, the alleles of the actual donors were never the minor component in the resulting DNA profile.

Different Collection Methods

Since the additional wash step was successful with reducing the amount of peptides found in the DNA fraction, this method was chosen when testing the next type of modifications to the microcon method, different sample collection methods. The different types of collection methods that were tested were polyester swabs, flocked swabs, and

tape-lifts. The bristles on the flocked swabs were arranged similar to a brush, with the expectation that these swabs would efficiently pick up skin cells left behind on a fingerprint, but release material better than the standard coiled cotton polyester swab. The idea behind using the tape-lifts was that the adhesive would pick up skin cells left behind from the latent fingerprint. Each of these collection methods was tested in parallel to polyester swabs. A summary of these results can be seen in the table below (Table 4).

Table 4. Average DNA and Peptide Yields From Trypsin Extraction using Different Collection Methods

	Polyester Swab (n = 4)	**Flocked Swab (n = 2)**	**Tape-Lift (n = 2)**
Average DNA Yield (in ng)	2.9	0.7	2.1
Average Peptide Yield (in ng)	6988.0	3227.5	2373.0

Results from table 4 showed the performance of flocked swabs to be worse than expected. It was later noticed that the company of the flocked swabs (Copan Diagnostics) recommends to use a spin basket to separate substrate and liquid before the microcon purification step. Tests not included here, demonstrated DNA recovery improvement. However, compared to the other collection methods, the lysis buffer volume had to be doubled for flocked swabs in order for the swab to be completely submerged. In turn, this dilution makes the peptide fraction less concentrated, reducing the number of potential peptide identifications made when analyzed through mass spectrometry. Even if spin baskets had been used with flocked swabs, this disadvantage of diluting the peptide fraction would still remain. Table 4 suggests that polyester swabs are a suitable collection method, when looking at obtaining the most amount of both protein and DNA.

No Microcon (Split) Method

The concept of this method is basically the same trypsin digestion method as before, however there is no separation of DNA and protein components. Instead, after the trypsin digestion and the heating and cooling steps, the volume of each sample is simply divided (split) in half, where one half is stored at -20^{0}C to be used for DNA analysis and the other half meant for protein analysis is stored at -80^{0}C. There are some advantages and disadvantages in regards to the split method compared to the microcon method. One of the advantages of the split method is that it results in lower volumes and thus higher concentrations for peptides, than the microcon method. Another advantage is that the split method is cheaper to perform because no microcon units have to be purchased. One of the biggest advantages of this split method is that it requires less time and hands on work to perform, compared to the microcon method. This means that in routine casework, it will be easier to perform on a larger quantity of samples. One of the disadvantages of this method is that with a lower starting volume, one may run out of sample for either fraction if additional testing is needed. In turn, another disadvantage of the split method is that certain swabs cannot be used with this method. Since a lower volume also must be maintained for the DNA half, certain swabs will not be able to be completely submerged for the incubation and protein digestion steps. Another disadvantage was revealed after mass spectrometry testing. Without the filtration step, the digest contains polyester fibers and other particles that can clog the capillary. The split method was tested for feasibility using saliva and fingerprints. DNA was successfully typed in Identifiler Plus without purification. Both the split and microcon methods were ran in parallel to each other, in

order to compare both methods. The quantification results for both methods can be seen below (Table 5).

Table 5. Average DNA and Peptide Yields of Microcon and Split Extraction Methods

	Average DNA Yield For Samples(Total ng)	Average Peptide Yield For Samples (Total ng)	Average Identified Protein Count (n=2)
Microcon Method Fingerprints n=6	1.04 (± 0.580)	5900.2 (± 6374.07)	96
Split Method Fingerprints n=6	0.97 (± 0.397)	9881.4 (± 11359.40)	1 failed, 1 ≈ 10
Split Method Saliva n=1	22.14	1155	118

* Large autosomal target interpreted

Table 5 shows that DNA yields were similar for both the Microcon and split methods, while the split method had an improved average protein yield. Statistical analysis using a Mann Whitney ranking test detected no significant difference for either DNA (p-value = 0.93624) or peptide yields (p-value = 0.47152). Samples from each extraction method were typed in Identifiler Plus. Only two samples resulted with good partial profiles, while the rest of the samples results with full DNA profiles (data shown in heatmap in Appendix, Figure 4). The two samples that did not have full profiles, were samples that were extracted using the no microcon (split) method. Typing results for the samples that were extracted with the "split" method, also showed the ski slope pattern of larger STR loci having reduced peak heights, which is either a sign of PCR inhibition or DNA degradation. Two samples from both the microcon method, as well as the split method, were sent to the Livermore National Laboratory to compare mass spectrometry results. For the microcon method, both fingerprint samples were successfully analyzed through mass spectrometry, only differing by a count of 10 identified peptides. Out of the two

fingerprint samples that were sent from the split method, only one was successfully analyzed through mass spectrometry with an identified peptide count of 10, while the other sample failed. These results suggested that any sample extracted using the no microcon (split) method is not ready to be analyzed through mass spectrometry, and must first be filtered. Looking at the mass spectrometry workflow, results suggest that the microcon co-extraction method is more suitable for downstream mass spectrometry analysis.

Finalized Method Batch Testing

10 male volunteers applied a single thumbprint (one for each hand) on a clean/irradiated glass slide. Only males were chosen as volunteers for the final round of testing on account that 77.6% of crimes are committed by male offenders (Rand & Robinson 2011). A single thumbprint was used instead of pooling thumb and three fingerprints as before to test at a lower level closer to real touch DNA casework evidence. The microcon trypsin co-extraction method, with no additional wash step, was chosen for this final test. Even though the additional wash step was successful with reducing the amount of peptides found in the DNA fraction, it also diluted the peptide fraction and did not improve mass spectrometry results for identified protein counts. This microcon method was ran in parallel with the standard Proteinase K extraction method, in order to determine if this trypsin based extraction method can be safely used without compromising DNA results obtainable with current methods. A summary of the DNA and protein quantification results can be seen in the table below (Table 6).

Table 6. Average DNA and Peptide Yields for Finalized Method Testing for Fingerprint Samples		
Extraction Method	**Average DNA Yield (Total ng)**	**Average Peptide Yield (Total ng)**
Standard (ProK) n=8	0.67 (± 1.18)	n/a
Microcon Method (Trypsin) n=10	0.75 (± 0.78)	1032.32 (± 1116.47)

Out of the ten samples that were extracted with the standard Proteinase K method, two produced inconclusive DNA quantitation results and could not be included in the average and statistical test. Results from table 6 shows a higher average DNA yield for the trypsin-based microcon co-extraction method, compared to Proteinase K. The Mann Whitney test results showed no significance (p-value = 0.44726) between the average DNA yields of both methods. In addition, results from the microcon method showed less variation from sample to sample than the standard Proteinase K method and better Identifiler Plus STR results. A summary of the DNA typing results can be seen in the table below (Table 7), please refer to the heatmap in the appendix (Figure 5) for the full set.

Table 7. STR Results for Finalized Method Testing				
Extraction Method	**Percent of Full Profiles**	**Percent of Good Partial Profiles**	**Percent of Bad Partial Profiles**	**Percent of Amelogenin Only**
Standard Proteinase K n=9	22%	22%	44%	11%
Microcon Method n=10	70%	30%	0%	0%

Out of the ten samples that were extracted with the standard Proteinase K method, only nine samples were amplified and typed in Identifiler Plus. Typing results showed signs of mixtures in four out of nine samples (Proteinase K samples 1, 2, 8, and 10). Proteinase K

sample 10 gave a promising DNA yield of 2.7 total nanograms, but resulted in a bad partial profile with only 3 full genotypes called. A review of the real-time PCR amplification curve revealed an atypical shape, which was why this sample was omitted from the average DNA yield, shown in table 6. This indicates another problem with the Quantifiler Trio result for this sample set. Typing results also showed signs of mixtures for half of the trypsin-based co-extracted microcon samples (microcon samples 1, 3, 4, 9, and 10). However, none of the alleles pertaining to the actual donors were ever the minor component in the resulting DNA profile. Results from table 7 suggest that samples extracted using the microcon method produce more full/interpretable profiles than when extracted using the standard Proteinase K method.

Protein Results

After analyzing all the results from this study, both saliva and fingerprint samples were tested for a possible correlation between peptide and DNA yields. First, saliva samples from all tests conducted in this study were compiled, where the peptide yields were plotted against the DNA yields, as seen in the figure below (Figure 1).

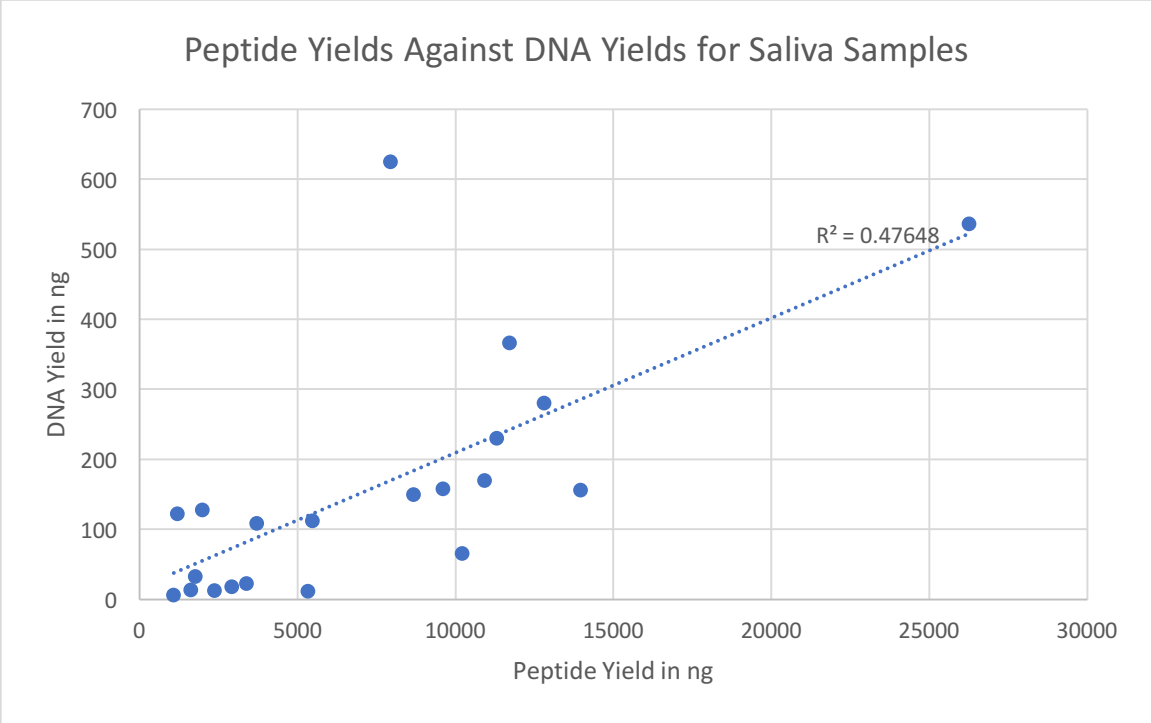

Figure 1. This figure represents a scatter plot consisting of 21 saliva samples from all tests conducted throughout this study. The peptide and DNA yields for each saliva sample are plotted in total nanograms. A linear regression line is also included, giving an R^2-value of 0.47648. Saliva samples with a DNA or peptide yield of zero were excluded.

As seen in Figure 1, the plot with an R^2-value of almost 0.5 suggests a moderate correlation between the DNA and peptide yields in saliva samples. The same test was performed for DNA and peptide yields for all the fingerprint samples tested in this study across all extraction methods. Peptide yields were plotted against DNA yields, which can be seen in the figure below (Figure 2).

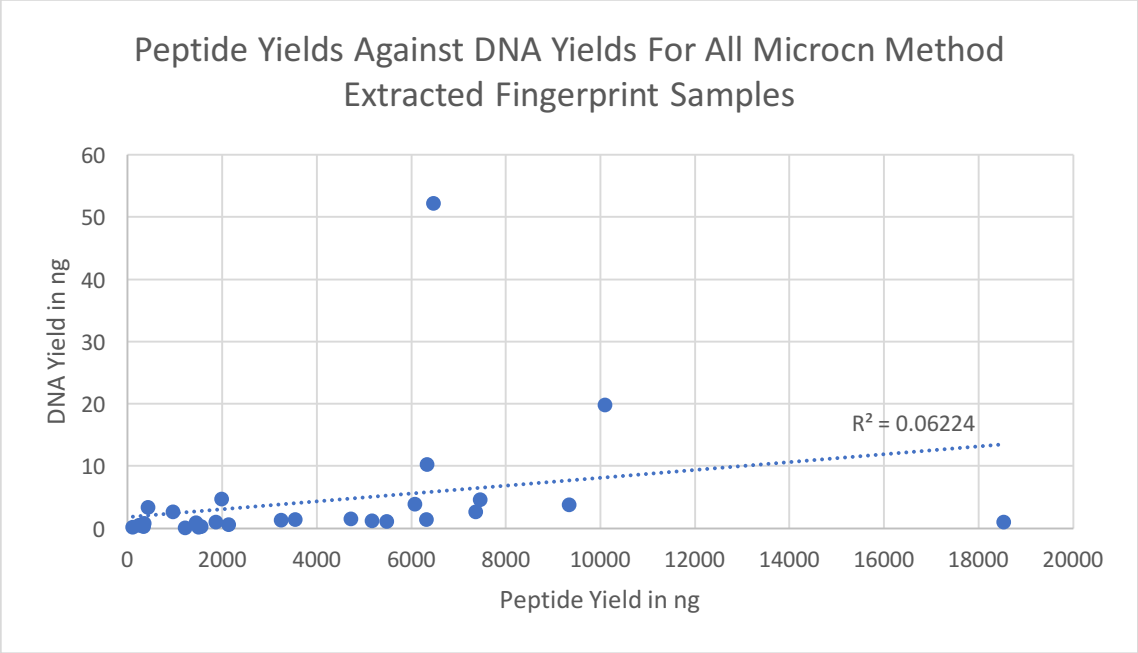

Figure 2. This figure represents a scatter plot consisting of 27 fingerprint samples from all tests conducted throughout this study. The peptide and DNA yields for each fingerprint sample are plotted in total nanograms. A linear regression line is also included, giving an R^2-value of 0.06224. Fingerprint samples with a DNA or peptide yield of zero were excluded.

Despite generating a low R^2-value of 0.06224, a slight trend is observed from figure 2, suggesting that there is some form of a relationship between the amount of DNA and peptide yield left behind by a fingerprint. Some correlation is to be expected. While there is variation based on tissue type, theoretically each cell should contain a constant amount of both DNA and protein, so if individuals leave behind more cells, then the potential amount of DNA, as well as the protein, should increase. That this relationship seems to be much stronger for saliva, is another indication that DNA from touched objects also has a cell free DNA component (Quinones and Daniel, 2012). It should be mentioned again that there were some technical problems with the Pierce Quantitative Fluorometric Peptide Assay Kit. As stated before, samples that gave a quantitation result of zero, still gave a result when analyzed through mass spectrometry. In order to compensate for background fluorescence, the extraction negative control had always been subtracted

from the samples that shared all buffer components, but not from the standards prepared in ammonium bicarbonate. This means some samples gave negative readings and it is not certain that the ng peptide yield is really accurate, but measurements definitely revealed relative amounts. As seen in figure 1 and 2, this relative measurement was sufficient to see a certain relationship between the amount of DNA and protein left behind by cells from either fingerprint or saliva samples.

Peptide sequencing via mass spectrometry on samples extracted with the co-extraction method was successful and identified many of the expected proteins. The results sent back from Lawrence Livermore Laboratory consisted of a sample view tab with a list of all the proteins that were identified in each sample. In terms of genetically variant proteins and amino acid variations, data analysis and discovery are still in progress. However, the proteins identified from the samples sent to the Lawrence Livermore National Laboratory so far, included several tissue specific protein for skin and saliva samples. Table 8 lists proteins specific to either skin or saliva that were identified through this proteomic peptide sequencing assay:

Table 8. Examples for Tissue Specific Proteins Detected in This Study

Saliva	Skin (candidates)
α-Amylase 1	Caspase 14
Cystatin-B	Cystatin-A
Cystatin-SA	Dermcidin
Histatin-1	Protein S100-A7
Submaxillary gland androgen-regulated protein	Keratin, type II cytoskeletal 1B
Statherin	Keratin, type I cytoskeletal 9

This list was compared to other studies that have identified tissue specific protein biomarkers through mass spectrometry (Yang et al. 2013 & Legg et al. 2017). There was no study, however, that identified specific protein biomarkers for skin samples. Cystatin

SA, histatin-1, α-amylase 1, statherin, and submaxillary gland androgen-regulated protein were found to be saliva specific and agreed with protein biomarkers found in previous studies (Yang et al. 2013 & Legg et al. 2017). Only Cystatin D, that had been mentioned as being a saliva marker by Legg et al. (2017) was not identified here. Various keratin proteins were detected in both saliva and skin samples. The mass spectrometry results also showed certain keratin types such as type I cytoskeletal 9, type II cytoskeletal 1, and type II cytoskeletal 2 to be more abundant in skin than saliva samples. The mass spectrometry results showed evidence for some skin specific proteins. Caspase 14, cystatin A, dermcidin, protein S100-A7, and keratin type II cytoskeletal 1B were only found in skin samples, suggesting that they may be skin specific.

Discussion

The purpose of this project was to develop a DNA/protein co-extraction. First, reagents in a standard DNA extraction method were replaced with protein and mass spectrometry compatible reagents. Results from the initial experiments (shown in Table 2) suggested that the microcon method, involving trypsin digestion, can extract both DNA and protein from forensic samples, as well as produce interpretable DNA profiles when typed using PCR-STR multiplex kits and capillary electrophoresis. Modifications to the microcon method were then tested to see if DNA and/or protein yields could be improved. Since it's a carrier, polyA-RNA was tested with the thought that it would help improve DNA yields. Different pore sizes were tested with the idea that a smaller pore size (microcon 30) should help improve DNA yields. Since the alkylating agent, iodoacetamide, is usually used in peptide mapping, an additional 30-minute alkylation step was tested. As a result of proteins being detected in the DNA fraction, an additional

wash step was tested in an attempt to reduce the amount of proteins found on the top of the membrane. After analyzing the results from all the modification experiments conducted, both the use of microcon 100 fast flow membranes and an additional wash step showed evidence of improvement to use in the microcon co-extraction method moving forward. However, no additional wash step was later used for the finalized method batch testing since mass spectrometry results didn't show improvement with protein identification counts. We could not confirm previously reported increases in DNA yields through "carrier" polyA-RNA (Schiffner et al. 2005), or the use of Microcon MW30 over MW100 units (Garvin and Fritsch, 2013). Once all protocol modification experiments were completed, various collection methods were then tested to determine which recovered the most amount of DNA and/or protein left behind from latent fingerprints. Results from these collection method experiments (shown in Table 4) suggested polyester swabs to be the optimal collection method, out of all the methods tested, after taking into account the amount of volume of reagents necessary to completely submerge flocked swab samples for incubation and protein digestion.

Inspired by the simple lysis approach published by Ostojic et al. 2014 & 2017, showing the extraction of single fingerprints and touched samples without using a purification step, a "no microcon method" (split method) was also tested. In order to test the feasibility of the "split" method, samples were processed in parallel to the microcon co-extraction method. Results from these experiments (shown in Table 5) seem to suggest that the split method would be better than the microcon method since the split method obtains a higher protein yield on average, as well as saves time and uses less reagents. However, when the split method samples were sent to the Lawrence Livermore

National Laboratory and analyzed through mass spectrometry, it was stated by their analysts that such samples had to first be filtered before analyzing, after having one sample clog the column of the mass spectrometer. As a result of first having to filter all of these "split" method digests, the samples were either consumed or diluted, which resulted in lower peptide concentrations. Additionally, since the samples require to be first be filtered before being analyzed through mass spectrometry, it counteracts the point of this extraction method not having a purification step. When these "split" method samples were typed by PCR, DNA results also showed signs of DNA degradation, even though the alleles were still being called. PCR inhibition causes a drop in peak heights for longer STR alleles similar to degradation and it seems logical that without purification, lysis buffer components interfered with Taq polymerase activity.

Looking at the results up to this point, the microcon co-extraction method seemed to be the most promising co-extraction method to obtain both DNA and protein from a single sample, where neither fraction compromises the analysis of the other. This co-extraction method was then tested in parallel to the standard Proteinase K Microcon extraction method on individual thumb prints from ten volunteers. After analyzing both DNA and protein yields from all ten volunteers for each extraction method, the results (shown in Table 6) suggests that the microcon co-extraction method obtains more consistent DNA yields across all donors. In addition, after analyzing all DNA typing results from both extraction methods, the results (shown in Table 7) shows evidence for the microcon co-extraction method to obtain more full/interpretable DNA profiles than Proteinase K. These results suggest that the microcon co-extraction method is better suited for low copy number touch DNA samples than the standard Proteinase K

extraction method employed here. This was noticeable when observing how certain samples extracted with the Proteinase K method either had similar, and in some cases, higher DNA concentrations compared to those extracted with the microcon co-extraction method, and produced a bad-partial profile, while the samples extracted with the microcon method produced a good/full profile. PCR may have been inhibited, for example by residual SDS or DTT in the DNA fraction. The Proteinase K method results may have been better with an additional purification step, or if we had used the high yield approach with carrier DNA for this experiment. This does not change the fact that trypsin extracted thumb prints had 100% full/interpretable profiles meaning this method can safely be used in casework without risking lower success rates for DNA typing.

In regards to the protein results, a slight correlation was observed for both saliva and fingerprint samples (Figures 1 & 2). Results from figures 1 and 2 also further supported that the mass spectrometer was more sensitive than the Thermal Fisher Life Technologies Pierce Quantitative Fluorometric Peptide Assay Kit and despite the issue of background fluorescence, a relationship between the amount of DNA and protein left behind by cells from either fingerprint or saliva samples was observed since the background fluorescence was accounted for. Additionally, from the data used to construct figure 2, a mean peptide yield of 4232.19 total ng and a mean DNA yield of 4.50 total ng was calculated for fingerprint samples. A standard deviation value of ± 4119.91 was calculated for the peptide yields of fingerprint samples and ± 10.35 for the DNA yields of fingerprint samples. The high standard deviation from this data set can be explained by one of the major challenges in touch DNA research, donor to donor variation. To control for this effect, all method or modification comparisons were performed on parallel sets of

left and right hand prints from the same donors. Furthermore, even though the discovery of genetically variant proteins is still in progress, protein samples analyzed by the Lawrence Livermore National Laboratory showed evidence of tissue specificity for both skin and saliva samples. For saliva samples, the protein biomarkers detected in this study were confirmed by previous studies (Yang et al. 2013 & Legg et al. 2017). For skin samples, the protein biomarkers caspase 14, cystatin-A, dermcidin, protein S100-A7, keratin type I cytoskeletal 9, and keratin type II cytoskeletal 1B suggested to be possible skin specific protein biomarkers. Further testing on these protein biomarkers for skin specificity is suggested.

Overall, the results of this study demonstrate that the microcon trypsin co-extraction method can extract and separate DNA and protein fractions from a single sample, and that either fraction can be successfully analyzed without compromising the analysis of the other. In regards to genetically variant proteins related to touch DNA samples, the discovery is still in progress. From the results sent back from the Lawrence Livermore National Laboratory in California, mass spectrometry showed that most proteins were not covered 100%. Despite these results, the peptide identifications that have been made, up to this point, demonstrated that peptides can be sequenced and the expected different types of keratins, skin and saliva specific proteins can be detected after microcon DNA co-extraction (Table 8). Skin and saliva samples sent to the Lawrence Livermore National Laboratory, could clearly be identified as such through their proteome (Tables 3, 5, & 8). The information obtained from this project has led to the development of a new type of co-extraction method that can be used to analyze touch DNA samples, with then having the option to also test proteins for genetically variable

markers and/or body fluid specific peptides. However, further investigation and research is needed on this method before it can be adopted into routine casework regarding touch DNA evidence. One of the biggest issues with touch DNA research is the variation of DNA obtained from individual to individual. In order to incorporate this variation, another study should be conducted consisting of a significantly larger sample size than just ten individuals. The low sample number may also have affected interpretation of some of the tested modifications, for example the use of carrier RNA, which should be repeated. Further investigation should also be conducted on testing this co-extraction method on various types of substrates that are known to degrade DNA and result in a low probability to produce an interpretable DNA profile. Persistence of DNA at the scene is an important concern, this variable is unknown for protein (Raymond et al. 2009). For the current study, all samples were collected within 24 hours of application. Therefore, additional testing should be conducted with this co-extraction method in regards to the amount of time that can elapse in order to still generate an interpretable DNA and/or peptide profile from a single latent fingerprint.

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Appendix

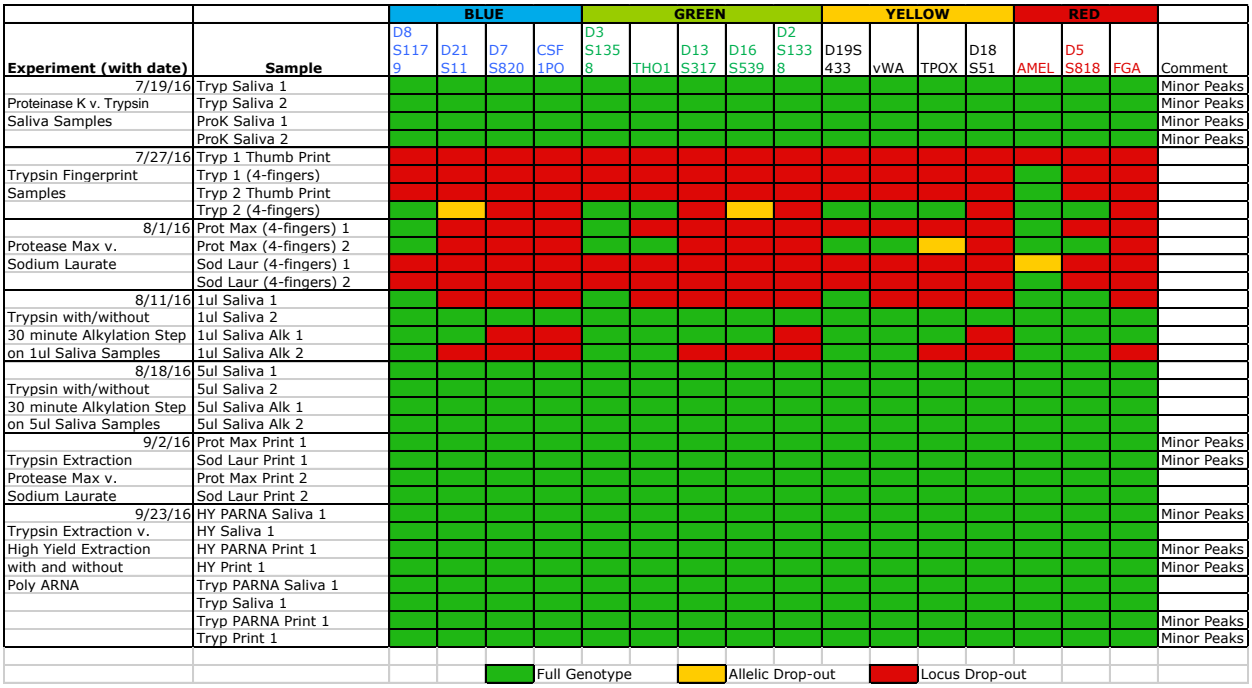

Figure 3. Heatmap of experiments from 7/19/16 to 9/23/16.

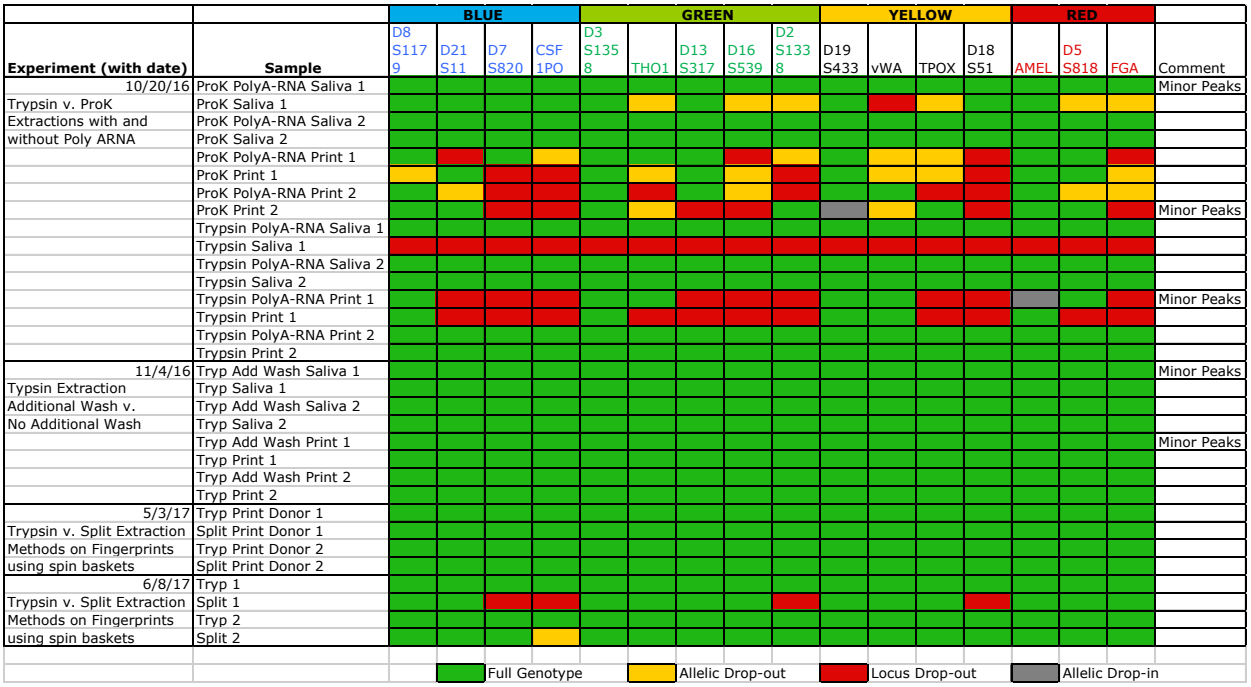

Figure 4. Heatmap of experiments from 10/20/16 to 6/8/17.

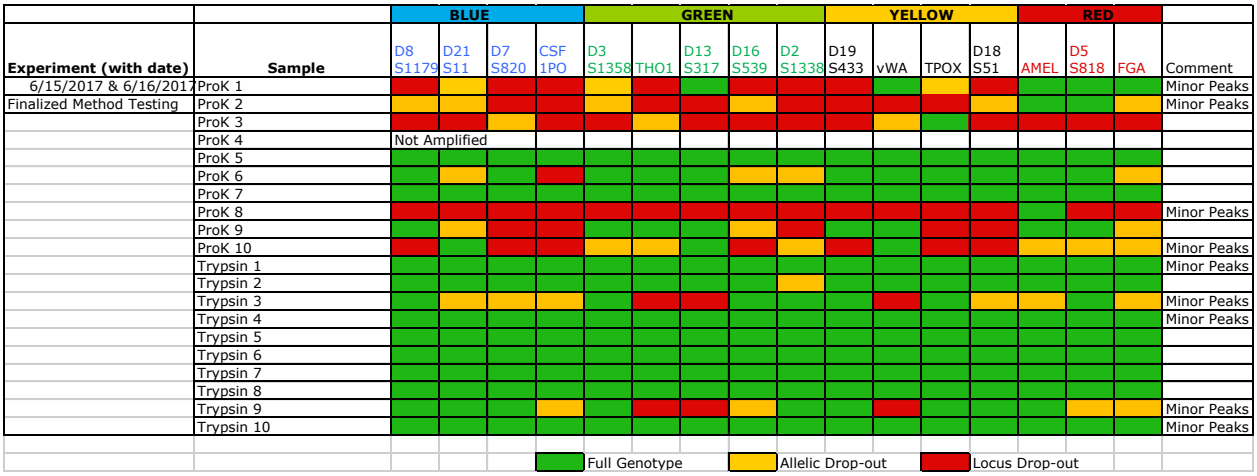

Figure 5. Heatmap from final method batch testing (6/15/17 to 6/16/17).