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Strategies for Enhanced Genetic Analysis of Trace DNA from Touch DNA Evidence and Household Dust

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STRATEGIES FOR ENHANCED GENETIC ANALYSIS OF TRACE DNA FROM TOUCH DNA EVIDENCE AND HOUSEHOLD DUST

by

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the Department of Chemistry in the College of Sciences at the University of Central Florida Orlando, Florida

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ABSTRACT

In forensic casework it is often necessary to obtain genetic profiles from crime scene samples that contain increasingly smaller amounts of genetic material, called Low Template DNA (LTDNA). Two examples of LTDNA sources are touch DNA evidence and dust bunnies. Touch DNA refers to DNA that is left behind through casual contact of a donor with an object or another person. Touch DNA can be used to prove a suspect was present at a crime scene. Dust bunnies, or dust conglomerates, typically contain trapped shed skin cells of anyone in the vicinity along with fibers, dirt, hair, and other trace materials. Dust specimens are a potential source of forensic evidence that has been widely underutilized in the forensic community. This is unfortunate because a dust bunny could not only be used to associate a person or crime scene – through trace materials such as fibers – but also to positively identify – through a DNA profile. For example, if a dust specimen is found on a piece of evidence suspected of being moved from its original location, for instance as a body that is too heavy to carry and therefore collects dust while being dragged, then it could be used to link a suspect to a crime scene.

Standard methods for obtaining and analyzing touch DNA have been established, but the techniques are not ideal. First, by nature, the ‘blind-swabbing’ technique, which involves cotton swabs or adhesive tape being applied to an area of interest, can artificially create mixtures of biological material that was originally spatially separated. Second, because the amount of DNA present is typically very low, standard analysis methods may not be sensitive enough to produce probative profiles. In the case of mixtures, the minor component’s DNA may go undetected. Dust specimens contain degraded genetic material that has been accumulating for an unknown
amount of time. Additionally, dust is usually a conglomeration of genetic material from multiple donors so a mixed profile, if any, is likely to be recovered if standard analysis methods are used.

In order to overcome these obstacles presented by LTDNA, a micro-manipulation and combined cell lysis/direct PCR amplification technique has been developed that is sensitive enough to obtain full or probative STR profiles from single or clumped bio-particles collected from touch DNA and dust evidence. Sources of touch DNA evidence such as worn clothing items, touched objects, and skin/skin mixtures are easily sampled using an adhesive material on a microscope slide. Dust specimens can be dispersed onto an adhesive material as well. Targeted bio-particles are then “picked” with a water-soluble adhesive on a tungsten needle and deposited into a micro-volume STR amplification mix. Individual selection and analysis of isolated bio-particles reduces the chance of mixed profile recovery. To aid in the release of genetic material present in the bio-particles, a lysis mix containing a thermostable proteinase is then added to the sample. Samples are then analyzed using standard capillary electrophoresis (CE) methods.

In addition to identifying the donor source of these LTDNA sources, it would be beneficial to a criminal investigation to identify the tissue source of the biological material as well. While it is widely speculated that the material originates from shed skin cells, there is little confirmatory evidence proving this assertion. Knowledge of the nature of the evidence could be vital to prevent its misinterpretation during the investigation and prosecution of a crime. Here tissue specific mRNA biomarkers have been evaluated for their use in tissue source determination using a highly sensitive High Resolution Melt (HRM) temperature assay that detects the selectively amplified targets based on their melt temperatures.
Using the enhanced genetic analysis technique described above, DNA profile recovery has been markedly enhanced in sources of Touch DNA evidence and dust specimens compared to standard methods. Additionally, the molecular-based characterization method could potentially provide a better understanding of the meaningfulness of the recovered DNA profiles. This optimized strategy provides a method for recovering highly probative data from biological material in low template samples in an efficient and cost effect manner.
To my mother and father. I am forever grateful for your unwavering love and support.
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CHAPTER ONE: INTRODUCTION

“Every contact leaves a trace” is a principle that is well known among forensic scientists. Dr. Edmond Locard, a pioneer in the field, explained how a perpetrator unconsciously deposits clues for investigators to find, and how careful analysis of these clues could identify guilty parties [1]. Unknowingly, criminals deposit fibers from clothing, dirt from their shoes, hair and other biological material containing the often considered “Holy Grail” of forensic evidence; their DNA. While DNA is deposited in the form of biological stains visible to the naked eye or through alternative light sources, it is also left behind through much more discrete means. DNA is also present on surfaces and objects the perpetrator touched (touch DNA), and even potentially in household dust. As forensic scientist Paul Kirk explained, “Only human failure to find it, study and understand it, can diminish its value” [2].

The ability to detect DNA on touched objects and surfaces has been routinely demonstrated [3-5]. However, current methods can result in DNA profiles lacking in probative value due to insufficient biological material or the presence of multiple donors [6]. DNA profiles are obtained by amplifying certain regions, known as short tandem repeats (STRs), which are highly polymorphic within a population. Known frequencies of alleles at each STR loci in a given population are then used to determine the likelihood ratio (LR), which is the likelihood of some random person having the same combination of STR alleles. When a sample contains very little DNA, some STRs might be missing due to degradation and only partial profiles are obtained [7]. The LR might have limited probative value if only a few alleles are detected. Additionally, in many cases multiple donors are present in the sample and the resulting complex DNA profile could be difficult to interpret. Currently, touch DNA is recovered using a
‘blind-swabbing,’ which involves a sterile cotton swab or adhesive tape applied to the area of interest and extraction of the recovered DNA [8-14]. This technique co-samples cellular material that was originally spatially separated on the touched item. If the cellular material originated from different donor sources, for example a touch DNA sample taken from the neck of a strangulation victim, then a mixture could be artificially created. Extraction of an entire dust bunny sample would also likely result in complex admixed DNA profiles (Figures 62, 65 and 66). In order to overcome these obstacles a simple and sensitive technique for analyzing LTDNA in putative cells, termed ‘bio-particles,’ has been developed. This research describes how the protocol was initially developed as an enhanced analysis method for touch DNA evidence and subsequently applied to biological material found in household dust, particularly ‘dust bunnies’.

Touch DNA is easily recovered from objects and surfaces using a low retention adhesive material adhered to a glass microscope slide support. The adhesive material containing the recovered potential biological material can then be stained for visualization and examined under a microscope to ensure proper transfer of sample. Once the sample has been collected, bio-particle isolation is conducted using a water soluble adhesive on a tungsten needle. The isolated bio-particle is deposited into a micro-volume reaction mixture containing a combination of cell lysis and STR amplification reagents, allowing for a one-step lysis/amplification protocol followed by STR profile detection using capillary electrophoresis (CE).

The final micro-volume reaction mixture was developed using isolated buccal cells and validated with a variety of touch DNA sources. The final protocol was the result of an evaluation of a variety of strategies that have been employed for LTDNA analysis. These
strategies include an optimized cell lysis protocol, micro-volume reaction mixture and increased PCR cycle number. The cell lysis step was performed using the ForensicGEM™ saliva kit lysis buffer solution (ZyGEM, VWR Suwanne, GA) which contains a thermostable proteinase capable of lysing cell membranes at its activation temperature of 75°C [15]. The proteinase’s activation temperature allows for its convenient incorporation into an amplification program. The cells are lysed by the proteinase for 15 minutes at 75°C then the 95°C hot start step simultaneously inactivates the proteinase while activating the polymerase enzyme for PCR. The AmpFISTR® Identifiler® Plus kit (Life Technologies, Grand Island, NY) was selected for a variety of reasons; the small amplicon size range (<360 base pairs) enables amplification of degraded samples, the addition of AmpliTaq Gold® (Life Technologies) provides a more streamlined reaction setup and the combination of the annealing and extension steps reduces the amplification time. A study examining the effect of increased PCR cycle number and a reduced PCR reaction volume with the Identifiler® Plus kit demonstrated that it maintained its sensitivity and had only minor increases in stochastic effects [16]. Additionally, a comparative analysis study of the effectiveness of AmpFISTR® kits in profiling degraded DNA isolated from used cigarette butts demonstrated that Identifiler® Plus had improved profile recovery over Identifiler®, supposedly due to its improved ability to overcome PCR inhibitors [17]. Identifiler® Plus contains primers for the 13 CODIS loci plus 2 additional STR loci (D19S433 and D2S1338) a sex determinant (Amelogenin) for a total of 30 possible alleles. Full or probative (>20 alleles) were recovered from objects where casual contact occurred (Figure 12). This procedure offers the benefits of reduced analysis time (no DNA extraction or quantitation required), per-sample cost reduction (5µl reaction volume compared to standard 25µl amplification mixture) and increased sensitivity.
Once it was proven that this technique could be used to successfully detect DNA in isolated bio-particles, mixture scenarios resembling those commonly encountered in forensic casework were tested. It is often the case that forensic DNA samples associated with violent crimes are mixtures of the “victim” and the “assailant,” with the “assailant” often only contributing a minor portion of the DNA. Repeated analysis of individual bio-particles isolated from a mixed DNA source increases the chances of detecting the minor contributor’s DNA over standard methods. Here a male donor’s DNA (the “assailant”) was observed in a variety of simulated assault scenarios where DNA from both a female donor (the “victim”) and the “assailant” were present (Table 2). A comparison analysis using a standard ‘blind-swabbing’ technique resulted in no recovery of probative, single-source profiles matching the “assailant.” The ability to continuously test the same touch DNA sample is a key benefit of this technique; collection and analysis of bio-particles can be inexpensively repeated until a probative profile is obtained or until the biological material has been exhausted from the sample.

In addition to determining the donor source of touch DNA evidence, it would be beneficial to identify the tissue source of the biological material. Much speculation revolves around the nature of touch DNA. While there is evidence suggesting that DNA on touched objects could originate from epidermal cell in the cornified layer of skin, the possibility of other epithelial cell sources, such as saliva, cannot be disproved [18, 19]. The murky nature of the biological source of touch DNA allows the questioning of its significance to be raised. For example, in physical assault cases a defense attorney could claim the defendant’s DNA resulted from saliva transferred during conversation, not physical contact. RNA profiling using tissue specific biomarkers could provide insight into the nature of the crime. While saliva might negate
the assertion of physical contact, the presence of vaginal secretions could validate a sexual
assault claim, which would result in different criminal charges. Here a highly sensitive protocol
was developed to detect tissue specific messenger RNA (mRNA) biomarkers in single or few
isolated cells/bio-particles based on the known melt temperatures of the mRNA targets using
High Resolution Melt (HRM) temperature analysis. Based on the success of the micro-volume
one-step lysis/STR amplification protocol, a micro-volume one-step lysis/cDNA synthesis assay
was developed for use with single or few cells/bio-particles from touch DNA, saliva and vaginal
secretions. While further assay development and validation is still needed, the ability to detect
mRNA biomarkers for skin and vaginal secretions was demonstrated (Figures 47 and 48). The
ultimate goal would be to identify donor and tissue source of an isolated bio-particle
simultaneously. Initial evaluation of a DNA/RNA co-isolation assay was also conducted by
simply dividing a lysate for DNA and RNA analysis pipelines using the STR amplification and
HRM analysis protocols, respectively.

Dust bunnies are a source of LTDNA that has been under-utilized by forensic examiners. Dust bunnies are conglomerates of inorganic and biological material, accumulated together by
static forces over time, and are a “representation of our environment in miniature,” as speculated
by magistrate Hans Gross a century ago [20]. The ability to identify the donor source of DNA
found within a dust bunny associated with some crime or crime scene could be greatly beneficial
to forensic investigators. Here the micro-manipulation technique developed for touch DNA
evidence was applied to isolated bio-particles found in dust. Dust bunnies were broken up over
wax paper and the particulate material was dispersed onto the low retention adhesive adhered to
a glass microscope slide support. Sample collection and analysis was performed using the
micro-manipulation technique and one-step lysis/STR amplification protocol. Using the one-
step lysis/amplification protocol, probative, single source DNA profiles were observed in 70% of the dust bunnies tested. Additionally, 25% of the dust bunnies produced 2 distinct probative profiles matching 2 different donor sources.

The results of these studies demonstrate the highly probative value of biological material in touch DNA and household dust sources. Touch DNA and household dust could provide investigators with knowledge that previously could not be ascertained using standard methods of DNA and tissue source analysis. This ‘smart’ (i.e., specific, measurable, attainable, realistic and timely) is a comprehensive method for obtaining identifying information at the single cell level.
CHAPTER TWO: METHODOLOGY

Touch DNA Evidence – DNA Analysis

Sample Collection

All samples were collected from volunteers using procedures approved by the University of Central Florida’s (UCF) Institutional Review Board. Informed written consent was obtained from each donor. All samples were de-identified to maintain the donor’s anonymity.

Touch DNA Samples – Single Sources

Biological material was recovered from touched objects, surfaces, worn clothing items and direct skin areas using WF Gel-film® x8 retention level (Gel-Pak®, Hayward, CA) that was pre-cut to a desired size and adhered to a glass microscope slide support (3” x 1”mm, ThermoFisher Scientific, Suwanee, GA). Gel-film® size of 2” x 0.75” was typically used. To adhere gel-film to slide support, the white backing was removed and gel-film was pressed firmly down. Immediately prior to applying sample to the gel-film, the clear top protective layer was removed using sterilized tweezers. Using gentle pressure, the exposed Gel-film® surface was touched to the sample area several times for sufficient transfer of biological material. Table 1 lists the various locations and objects “single source” touch DNA was collected from. Samples were assumed to be single source because it was unlikely for DNA from a person other than the donor, owner or wearer of the item would be present. All samples were stained with Trypan blue (0.4%) (Sigma-Aldrich, St. Luis, MO) for 1-2 minutes then rinsed briefly with a gentle flooding of sterile Millipore water. The samples were air-dried at room temperature and stored at room temperature in microscope slide boxes protected from light.
Touch DNA Samples – Mixed Sources

Mixture scenarios typically encountered in forensic casework were simulated. The mixture scenarios included: (1) skin-clothing – male donor gripped the outside of a female donor’s shirt sleeve, (2) skin-skin – male donor held a female donor’s wrist, (3) skin-skin (“choking”) – male donor touched a female donor’s neck, and (4) bed sheets (simulated sexual assault) – a female donor slept on her personal sheets for 1 night, then the male donor made brief (a few minutes) direct skin contact with the sheets. For each scenario tested, 3 donor sets were used (3 males, 3 females), for a total of 6 different donors. Biological material was recovered using Gel-film® (see Gel-film® slide preparation described above). To collect bio-particles, the area of interest was touched several times with the Gel-film® surface. For skin-skin contact scenarios (“choking” and “wrist grab”) the area of contact on the female donor was touched with Gel-film®. For the bed sexual assault scenario, the area on the sheets where mutual contact occurred was touched several times with the Gel-film® surface.

A fifth assault scenario was simulated with a small sample set (1 male, 1 female) to represent another mixture type encountered in forensic casework; scratching. For the scratching scenario, a female donor scratched the inside of a male donor’s arms with enough force to leave a mark. The underside of the female donor’s nails were then scraped using a scrape stick to collect any biological material. For the simulated assault “scratching” mixture the scrape stick was rolled over the Gel-film® surface to deposit the material collected from underneath the female donor’s fingernails. All samples were stained with Trypan blue (0.4%) for 1-2 minutes then rinsed briefly with a gentle flooding of sterile Millipore water. The samples were air-dried.
at room temperature and stored at room temperature in microscope slide boxes protected from light.

**Buccal Epithelial Cells**

Saliva samples were obtained by swabbing the inside of the cheek with a sterile IntegriSwabs™ (Lynn Peavey Company, Lenexa, KS). Buccal swabs were rolled onto Gel-film® (see Gel-film® slide preparation described above). All samples were stained with Trypan blue (0.4%) for 1-2 minutes, and then rinsed briefly with a gentle flooding with sterile Millipore water. The samples were air-dried at room temperature and stored at room temperature in microscope slide boxes protected from light.

**Reference and Comparison Samples**

When possible, saliva samples were obtained from donors of touch DNA and dust bunny samples to obtain reference profiles for comparison with any profiles detected in the analysis of individual bio-particles. Saliva samples were obtained by swabbing the inside of the cheek with a sterile IntegriSwabs™. ‘Blind swab’ comparison samples were collected for 4 simulated physical assault mixture scenarios described above.

For scenarios 1-3, the area of contact on the female’s shirt/skin was swabbed with a pre-moistened (sterile Millipore water) IntegriSwab™. For scenario 4, the area of mutual contact on the sheets was swabbed with a pre-moistened (sterile Millipore water) sterile IntegriSwab™. The swabs were dried at room temperature prior to storage at -20°C until needed. A comparison sample was collected for the “scratching” mixture scenario, where a female donor used both hands to scratch the inside of a male donor’s arm with enough force to leave a temporary mark. A scrape stick was used to scrape the underside of the female donor’s nails (1 stick/hand) and the
end of 1 stick (see micro-manipulation section below for preparation of 2\textsuperscript{nd} stick) was cut off into a 1.5 mL Safe Lock tube extraction tube (Eppendorf, Westbury, NY).

**Isolated Buccal Epithelial Cells – Method Development**

*Buccal Epithelial Cell Collection*

Buccal cell collection was conducted using a Leica 205C Stereomicroscope (Micro Optics of FL, Inc, Davie, FL). For all samples a “0” cell sample was collected from an apparent blank area on the Gel-film\textsuperscript{®} to test for the presence of “cell-free” DNA. The sample was viewed under the stereomicroscope and a 3M\textsuperscript{™} water-soluble wave solder tape (5414 transparent) (3M, Saint Paul, MN) on the end of a tungsten needle was used to collect the saliva cells. The 3M\textsuperscript{™} water-soluble adhesive was adhered to a clean glass microscope slide using double sided tape (Fisher Scientific) and collected on the end of a tungsten needle while viewing the slide under the stereomicroscope. The collected saliva cell and water-soluble adhesive was then dissolved into AmpFISTR\textsuperscript{®} Identifiler\textsuperscript{®} Plus Master Mix (Life Technologies, Grand Island, NY) in the bottom of a sterile 0.2ml PCR flat-cap tube (Phenix Research, Candler, NC).

*Direct PCR– Increased PCR Cycle Number*

Two sets of buccal cells (1, 2, 5, 10 and 0) were collected from a female donor using the micro-manipulation technique into 10µl sterile Millipore water in the bottom of a sterile 0.2ml PCR flat-cap tube. Following sample collection 15µl of the Identifiler\textsuperscript{®} Plus Master Mix which consisted of 10µl PCR mix and 5µl primer mix was added to the sample. Samples were amplified using a modified Identifiler\textsuperscript{®} Plus protocol: 95°C 11min (hot start and lysis enzyme inactivation); 29 or 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a 9700 GeneAmp\textsuperscript{®} PCR system thermal cycler (Applied
Biosystems by Life Technologies). A positive control (9947a) and negative control (sterile Millipore water) was performed with each amplification.

**One-Step Lysis/STR Amplification - Standard Reaction Volume**

Buccal cells (1, 2, 5, 10 and 0) were collected from a female donor (same donor used above) using the micro-manipulation technique into 10µl lysis buffer solution (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) (ZyGEM forensicGEM™ saliva kit VWR, Suwanne, GA) in the bottom of a sterile 0.2ml PCR flat-cap tube (Phenix Research). Following sample collection 15µl of the Identifiler® Plus Master Mix which consisted of 10µl PCR mix and 5µl primer mix was added to the sample. Samples were amplified using a modified Identifiler® Plus protocol: 75°C 15 min (lysis step) 95°C 11min (hot start and lysis enzyme inactivation); 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a 9700 GeneAmp® PCR system thermal cycler. A positive control (9947a) and negative control (sterile Millipore water) was performed with each amplification.

**One-Step Lysis/STR Amplification - Micro-Volume Reaction**

Buccal cells (1, 2, 5, 10 and 0) were collected from 5 donors (4 female, 1 male) using the micro-manipulation technique into an Identifiler® Plus Master Mix which consisted of 1.4µl PCR mix and 0.7µl primer mix. Following sample collection 1.4µl lysis buffer solution (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) was added to the sample. Samples were amplified using a modified Identifiler® Plus protocol: 75°C (lysis step) 95°C 11min (hot start and lysis enzyme inactivation); 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a 9700 GeneAmp® PCR system thermal cycler.
A positive control (9947a) and negative control (sterile Millipore water) was performed with each amplification.

**Isolated Touch Bio-Particles**

*Touch Bio-Particle Collection*

Bio-particle collection was conducted using a Leica 205C Stereomicroscope. Images of the collected individual bio-particles were taken at a 250X magnification with trans-illumination. The bio-particles were measured using Leica Application Suite LAS v4.3 software. For each sample, 20 single and 20 ‘clumped’ or ‘fluid-like’ bio-particles were collected. Samples were considered to be ‘clumped’ if the material appeared to be an aggregate of multiple cells condensed into one solid structure. Single, nucleated cells were initially the primary target of the bio-particle collections. The ‘fluid-like’ descriptor refers to any bio-particle that’s shape is somewhat undefined and appears to be in a hydrated state (Figure 30). For all samples a “0” cell sample was collected from an apparent blank area on the Gel-film® to test for the presence of ‘cell-free’ DNA. As the sample was viewed under the stereomicroscope a 3M™ water-soluble wave solder tape (5414 transparent) on the end of a tungsten needle was used to collect the bio-particles. The 3M™ water-soluble adhesive was adhered to a clean glass microscope slide using double sided tape and collected on the end of a tungsten needle while viewing the slide under the stereomicroscope. The collected bio-particle and water-soluble adhesive were then dissolved into AmpFISTR® Identifiler® Plus Master Mix in the bottom of a sterile 0.2ml PCR flat-cap tube.

*One-Step Lysis/STR Amplification of Isolated Touch Bio-Particles*

In order to validate the combined cell lysis/STR amplification protocol for use with isolated bio-particles, 3 micro-volume reaction mixtures were compared with 20 single and 20
‘clumped’ bio-particles collected from a shirt collar sample from a single male donor. Three micro-volume reaction mixtures were tested using the Identifiler® Plus kit. Reaction mixture 1: ‘3.5µl’ consisted of 1.4µl PCR mix, 0.7µl Primer mix. Reaction mixture 2, ‘3.5µl+’ consisted of 1.4µl PCR mix, 0.7µl Primer mix and 0.2µl (1U) AmpliTaq® Gold polymerase (Life Technologies). Reaction mixture 3, ‘5µl+’ consisted of 2.2µl PCR mix, 1.1µl Primer mix and 0.2µl (1U) AmpliTaq® Gold polymerase. Following sample collection, a lysis buffer solution was prepared (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) and an aliquot was added to the sample: ‘3.5µl’ – 1.4µl aliquot; ‘3.5µl+’ – 1.2µl aliquot; ‘5µl+’ – 1.5µl aliquot. Samples were amplified using a modified Identifiler® Plus protocol: 75°C 15 min (lysis step) 95°C 11min (hot start and lysis enzyme inactivation); 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a 9700 GeneAmp® PCR system thermal cycler. A positive control (9947a) and negative control (sterile Millipore water) was performed with each amplification.

**DNA Isolation and Quantitation – Reference and Comparison Samples**

DNA was manually extracted from swabs using QIAamp® DNA Investigator kit (QIAGEN, Germantown MD) according to the manufacturer’s recommended conditions. The samples were incubated in 400µl Buffer ATL and 20µl proteinase K at 56°C for 1 hour in a 1.5 mL Safe Lock tube extraction tube. The samples were vortexed and spun down every 10 minutes to improve lysis during the 1 hour incubation. 400µl of Buffer AL was then added and the samples were mixed by pulse-vortexing for 15 seconds. The samples were then incubated for 10 minutes at 70°C, with a brief vortex every 3 minutes for complete lysis. The swabs were then transferred to a DNA IQ™ spin basket (Promega, Madison, WI) in the same 1.5 mL Safe Lock
tube extraction tube and spun down at 14,000 rpm (16,000 × g) for 5 minutes to remove all liquid from the swab. The swab and spin basket were then discarded and the entire lysate was transferred to a QIAamp® MinElute® spin column (QIAGEN) which selectively binds DNA. The sample was then washed to remove contaminants with 500µl Buffer AW1, 700µl Buffer AW2 and 700µl 100% ethanol. The membrane of the column was dried by centrifuging the sample at 14,000 rpm for 3 minutes then incubation at room temperature for 10 minutes. References samples were eluted into 60µl of buffer EB and the ‘blind swab’ comparison samples were eluted into 20µl of buffer EB for a more highly concentrated eluate (for increased DNA concentration). An extraction blank was included with each extraction as a negative control. DNA was quantified with the Quantifiler® Human DNA Quantification kit (Life Technologies) according to the manufacturer’s conditions. All quantitations were performed on an ABI Prism 7000 real time PCR instrument.

Autosomal STR Amplification – Reference and Comparison Samples

Reference and comparison samples were amplified with the Identifiler® Plus kit according to the manufacturer’s recommended conditions (25µl reaction volume). The samples were amplified using the Identifiler® Plus protocol: 95°C 11 min (hot start and lysis enzyme inactivation); 28 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. A DNA input concentration of 1ng or a maximum of 10µl sample extract (for low or undetected DNA concentration) was used. Amplifications were performed on a 9700 GeneAmp® PCR system thermal cycler. A positive control (9947a) and negative control (sterile Millipore water) were included with each amplification.
**Detection of PCR Products**

One microliter of amplified DNA product was added to 9.7µl Hi-Di™ formamide (Life Technologies) and 0.3µl of GeneScan™ 500 LIZ® size standard (Life Technologies). Samples were analyzed using capillary electrophoresis (CE) with the ABI Prism 3130 Genetic Analyzer using Module G5 (16 second injection, 15kV, 60°C) and analyzed with GeneMapper® analysis software v4.0. A peak height detection threshold of 50 Relative Fluorescent Units (RFUs) was used for allele detection.

**Touch DNA Evidence – Tissue Source Analysis**

**Body Fluid Samples**

Body fluids were collected from volunteers using procedures approved by the University’s Institutional Review Board. Informed written consent was obtained from each donor. Saliva and vaginal secretions were extracted for RNA for use as control samples in the RNA profiling assay. Human total skin RNA was obtained through a commercial source (Stratagene/Agilent Technologies Santa Clara, CA). Buccal samples (saliva) were collected from donors using sterile IntegriSwabs™ by swabbing the inside of the donor’s mouth. Semen-free vaginal secretion swabs were collected by swabbing the vaginal cavity with a sterile IntegriSwab™. All samples were stored at -20°C until needed.

**RNA Isolation and Quantitation – Control Samples**

Total RNA was extracted from saliva and vaginal secretions using a manual organic RNA extraction (guanidine isothiocyanate-phenol-chloroform mixture) [21]. A mixture of 500 µl of denaturing solution (4 M guanidine isothiocyanate, 0.02 M sodium citrate, 0.5% sarkosyl) and 3.6µl β-mercaptoethanol (pre-heated at 56°C for 10 minutes) was added to a 1.5 mL Safe Lock
tube extraction tube (Eppendorf) containing the swab and incubated at 56°C for 30 minutes. The swab was then transferred into a DNA IQ™ spin basket, re-inserted back into the original extraction tube, and centrifuged at 14,000 rpm for 5 minutes. After centrifugation, the basket with the swab was discarded. A mixture of 50 μl 2 M sodium acetate and 600 μl acid phenol: chloroform (5:1), pH 4.5 (Ambion by Life Technologies) was then added to the sample. The sample was then incubated at 4°C for 30 minutes to separate the organic and aqueous layers and then centrifuged at 14,000 rpm. The aqueous phase (top layer) was transferred to a new 1.5 ml microcentrifuge tube and incubated for 1 hour at -20°C with 2 μl of GlycoBlue™ glycogen carrier (Ambion by Life Technologies) and 500 μl of isopropanol to precipitate the RNA. The extracts were then centrifuged at 14,000 rpm for 10 minutes. The supernatant was removed and the pellet was washed with 900 μl of 75% ethanol/25% DEPC-treated water. The pellet was dried in a vacuum centrifuge for 3 minutes and incubated for 10 minutes in 20μl of 60°C nuclease free water (Ambion by Life Technologies). Samples were used immediately or stored at -20°C until needed. All extracts were DNase treated to remove any contaminating DNA using the Turbo DNA-free™ kit (Applied Biosystems by Life Technologies) according to the manufacturer’s protocol. RNA extracts were quantitated with Quant-It™ RiboGreen® RNA Kit (Invitrogen by Life Technologies) as previously described [21]. Fluorescence was determined using a Synergy™ 2 Multi-Mode microplate reader (BioTek® Instruments, Inc., Winooski, VT).

**Cell/Bio-Particle Isolation and Lysis**

Touch bio-particles, vaginal secretion cells and saliva cells were collected from the Gel-film® surface while viewing under the stereomicroscope using the micro-manipulation technique described in the touch DNA section. Touch bio-particles (10, 25 and 50 single and 1, 5, 10 and
20 ‘clumped’ bio-particles) were collected from a touch DNA sample from a male donor’s shirt collar. Collection of touch bio-particles with Gel-film® is described in the touch DNA section. Vaginal secretion cells (1, 2, 5, 10, 25, 50 and 100) were collected from 1 female donor. Saliva cells (10, 25 and 50) were collected from a single male donor. Vaginal secretion and saliva cells were deposited onto Gel-film® by rolling a swab (see body fluid samples for collection details) over the Gel-film® surface before the swab was allowed to dry. For all samples a “0” cell sample was collected from an apparent blank area on the Gel-film® to test for the presence of “cell-free” DNA. The cells/bio-particles were collected from Gel-film® using a Leica 205C Stereomicroscope. As the sample was viewed under the stereomicroscope, a 3M™ water-soluble wave solder tape (5414 transparent) on the end of a tungsten needle was used to collect the bio-particles. The 3M™ water-soluble adhesive was adhered to a clean glass microscope slide using double sided tape and collected on the end of a tungsten needle while viewing the slide under the stereomicroscope. The collected bio-particle and water-soluble adhesive was then transferred into a sterile 0.2ml PCR flat-cap tube containing either 3.55µl nuclease free water or a 3.55µl aliquot of a lysis buffer solution (for 10 µl: 1x buffer-silver, 5% RNAGEM™ and nuclease free water) (ZyGEM RNAGEM™ tissue kit, VWR). The samples were lysed at 75°C for 5 minutes using a 9700 GeneAmp® PCR system thermal cycler.

**cDNA Synthesis**

Touch bio-particles, vaginal secretion cells, saliva cells and extracted total RNA for each body fluid (positive control) were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems by Life Technologies; 0.5 µl RT Buffer, 0.2 µl dNTPs, 0.5 µl random primers, 0.25 µl Multiscribe reverse transcriptase) and the 3.55µl cell lysate (see
above) or extracted total RNA (1ng input). The samples were incubated on a 9700 GeneAmp® PCR system at 25°C for 10 minutes (primer extension), 37°C for 2 hours (cDNA synthesis) and 85°C (reverse transcriptase inactivation). A reverse transcription negative control containing the reaction mixture and nuclease free water was performed for each sample.

*mRNA Biomarker Pre-Amplification*

Five microliters of the reverse transcribed touch bio-particles, vaginal secretion cells, saliva cells and extracted total RNA (positive control) were pre-amplified with their respective unlabeled body fluid-specific primers: touch bio-particle – LCE1C [22]; saliva – HTN3 [23]; vaginal secretions – MYOZ1 [24], IL19 [24] and NOX01 (unpublished). All primers were obtained from Invitrogen by Life Technologies. Each 25 μl reaction included: 12.5 μl QIAGEN Multiplex Master Mix, 2.5 μl unlabeled primer mix (1μM), the reverse transcribed sample (5 μl) and 5 μl of nuclease free water. Table 3 lists the sequences of the body fluid-specific primers used. All assays were run on a 9700 GeneAmp® PCR System using the following cycling conditions: 95°C 5 min, followed by 14 cycles of 95°C 15 sec, 60°C 2 minutes. A negative control (nuclease free water) was included with each reaction.

*High Resolution Melt (HRM) Analysis*

The pre-amplified samples were amplified and detected using High resolution melt (HRM) analysis. HRM assays were performed using the Type-It® HRM PCR kit (QIAGEN) and run on the Rotor-Gene® Q real time PCR instrument (QIAGEN). Each 25 μl reaction included: 12.5 μl Type-It® HRM reagent, 1.75 μl body fluid-specific unlabeled primer mix, 5 μl of the sample (pre-amplified cDNA, see above) and 5.75 μl nuclease free water. For the singleplex HRM assays a 10μM primer mix was used for HTN3, MYOZ1, IL19 and NOX01 and a 40μM
primer mix was used for LCE1C. For the triplex HRM assay, the primer mix consisted of: for 10µl; 2.38µl 10µM HTN3, 3.81µl 40µM LCE1C and 3.81µl 10µM NOX01. The following cycling conditions were used: 95°C 5 min, followed by 45 cycles of 95°C 10 sec, 57°C 40 sec, 72°C 20 sec. HRM analysis was performed using a 65–98°C temperature range. The positive control consisted of 3µl of 25ng RT (cDNA) from vaginal secretions, saliva and total skin (Stratagene/Agilent Technologies) samples. A HRM negative control containing the reaction mixture and nuclease free water was performed for each sample.

*DNA/RNA Co-Isolation of Cells/Bio-Particles*

Touch bio-particles, vaginal secretion cells and saliva cells were collected from the Gel-film® surface while viewing under the stereomicroscope using the micro-manipulation technique described in the touch DNA section. The bio-particle/cells were transferred into a 5.1 µl of a lysis buffer solution (ZyGEM RNAGEM™ tissue kit or ZyGEM forensicGEM™ saliva kit) in the bottom of a 0.2 ml PCR flat-cap tube. The ZyGEM RNAGEM™ tissue kit lysis buffer consisted of (10µl) 1x buffer-silver, 5% RNAGEM™ and nuclease free water. The ZyGEM forensicGEM™ saliva kit lysis buffer consisted of (10µl) 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water. Samples were lysed at 75°C for either 5 minutes (RNAGEM™ lysis buffer solution) or 15 minutes (forensicGEM™ lysis buffer solution). A 1.5µl aliquot of lysate was amplified using Identifiler® Plus: 95°C 11 min (hot start and lysis enzyme inactivation); 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a 9700 GeneAmp® PCR system thermal cycler. A positive control (9947a) and negative control (sterile Millipore water) were included with each amplification. The remaining cell lysate (3.55µl) was
reverse transcribed as described above. All pre-amplification and product detection (HRM) reactions were performed as described above.

**Household Dust**

*Dust Bunny Collection*

Dust bunny samples were collected from a variety of private dwellings and public places. The samples were stored at room temperature in manila envelopes and labeled with the collection date, city and specific room within the dwelling that the sample was collected from. Five of the dust bunnies analyzed were from anonymous donors from the state of New York (termed “New York dust bunnies”) collected between 2008-2010. Samples were collected from the private dwellings of lab members, locations throughout the National Center for Forensic Science (NCFS) building and from the University of Central Florida (UCF) Engineering building (termed “Florida dust bunnies”). Additionally, an “international dust bunny” was collected from the departure lounge of the Frankfurt Airport, Germany. All “new” samples (not including “New York dust bunnies”) were collected using disposable plastic tweezers (ThermoFisher Scientific) to prevent contamination by the collector. For all samples, the dust specimen was stored in small manila envelopes at room temperature. The envelopes were taped closed and labeled with the collection date, location and a general description of the immediate area of the collection location (e.g. carpeted floor, under fridge, on electrical cords).

*Whole’ Dust Bunny Samples Preparation*

DNA was extracted from a cut portion of 3 dust bunnies in order to compare standard methods of DNA isolation and analysis with the micro-manipulation technique. Dust bunnies collected from underneath a donor’s bed (“Florida bedroom #2,” collected 2014) and from a
donor’s bedroom (“New York bedroom #4,” collected 2008) were cut into 3 approximately equal portions using sterilized scissors in a PCR amplification hood with the blower disabled. The dust bunny collected from the Frankfurt Airport, Germany was cut into 2 approximately equal portions. Portion sizes were visually estimated and stored in separate manila envelopes until needed. The remaining portions were either wholly extracted for DNA or broken up for genetic analysis of individual bio-particle analysis using the micro-manipulation technique (see micro-manipulation sample preparation section).

**Standard Organic DNA Extraction of ‘Whole’ Dust Bunny**

A standard organic DNA extraction was performed on half of the dust bunny collected from the departure lounge of the Frankfurt Airport, Germany. The specimen was incubated overnight at 56°C in 400µl Stain Extraction Buffer (TRIS, NaCl, 20% SDS and 0.5M EDTA) and 13µl Proteinase K. The dust specimen was transferred to a DNA IQ™ spin basket, re-inserted into the extraction tube and spun down at 14,000 rpm for 5 minutes. The organic and aqueous (DNA containing) phases were then separated by adding 400µl phenol/chloroform/isoamyl alcohol (P/C/IAA, 25:24:1, pH 6.6), mixed by inversion and centrifuged at 14,000 rpm for 5 minutes. The top (DNA containing) layer was then transferred to a new 1.5mL tube. The DNA was precipitated at -20°C for 1 hour with 1mL cold absolute (100%) ethanol. The DNA was then pelleted by centrifugation at 14,000 rpm for 15 minutes. The ethanol was removed and the DNA pellet was washed with 1mL 70% ethanol then dried in a vacuum centrifuge for 5 minutes. The DNA was re-solubilized in 25µl TE\(^{-4}\) (10mM Tris, 0.1mM EDTA, pH 7.5) overnight in a water bath at 56°C.
**DNA Isolation of ‘Whole’ Dust Bunnies with QIAamp DNA Investigator Kit**

DNA was extracted from the dust bunny portions using QIAamp® DNA Investigator kit according to the manufacturer’s recommended conditions. The sample was incubated in 400µl Buffer ATL and 20µl proteinase K at 56°C for 1 hour in a 1.5 mL Safe Lock tube extraction tube. The sample was vortexed and spun down every 10 minutes to improve lysis during the 1 hour incubation. 400µl of Buffer AL was then added and the sample was mixed by pulse-vortexing for 15 seconds. The sample was then incubated for 10 minutes at 70°C, vortexing briefly every 3 minutes for complete lysis. Using sterilized tweezers, the dust bunny was then transferred to DNA IQ™ spin basket in the same 1.5 mL Safe Lock tube extraction tube and spun down at 14,000 rpm for 5 minutes to remove all liquid from the sample. The dust bunny and spin basket were then discarded and the entire lysate was transferred to a QIAamp® MinElute® spin column which selectively binds DNA. Contaminates were then removed by washing the sample with 500µl Buffer AW1, 700µl Buffer AW2 and 700µl 100% ethanol. The membrane of the column was dried by centrifuging the sample at 14,000 rpm for 3 minutes then incubation at room temperature for 10 minutes. The sample was eluted into 20µl of buffer EB for a more highly concentrated eluate (recommended by the manufacturer for increased DNA concentration). An extraction blank was included with each extraction as a negative control.

**Quantitation of Human DNA in ‘Whole’ Dust Bunnies**

DNA was quantified with the Quantifiler® Human DNA Quantification kit according to the manufacturer’s conditions. All quantitations were performed on an ABI Prism 7000 real time PCR instrument.
**Micro-manipulation Sample Preparation**

To prepare the dust bunny for micro-manipulation collections, the specimen was broken up in a PCR amplification hood with the blower disabled to simulate a ‘dead air box.’ The specimen was teased apart over wax weighing paper (6” x 6” ThermoFisher Scientific) using sterilized tweezers to disperse biological material that was entrapped in the conglomerate. As material fell out of the dust bunny, exposed Gel-film® adhered to a glass microscope slide support (same specifications as described in touch DNA section) was touched to the wax paper to collect the material. The process of teasing the dust bunny and collecting the material on Gel-film® was repeated until the sample was sufficiently dispersed over multiple Gel-film® slides. All samples were stained with Trypan blue (0.4%) for 1-2 minutes then rinsed briefly with a gentle flooding with sterile Millipore water. The sample slides were air-dried at room temperature and stored at room temperature in microscope slide boxes protected from light.

**Dust Bunny Bio-particle Collection**

Bio-particle collection was conducted using a Leica 205C Stereomicroscope. Images of the collected individual bio-particles were taken before and after bio-particle collection at a 250X magnification with trans-illumination. The bio-particles were measured using Leica Application Suite LAS v4.3 software. For each sample, 20 bio-particles were collected. A classification of ‘nucleated’, ‘clumped’, or ‘fluid-like’ was assigned and recorded for each bio-particle based on the image of the bio-particle. Explanations of the descriptors are given in the touch bio-particle collection section. For all samples a “0” cell sample was collected from an apparent blank area on the Gel-film® to test for the presence of “cell-free” DNA. As the sample was viewed under the stereomicroscope a 3M™ water-soluble wave solder tape (5414
transparent) on the end of a tungsten needle was used to collect the bio-particles. The 3M™ water-soluble adhesive was adhered to a clean glass microscope slide using double sided tape and collected on the end of a tungsten needle while viewing the slide under the stereomicroscope. The collected bio-particle and water-soluble adhesive were then dissolved into Identifiler® Plus Master Mix in the bottom of a sterile 0.2ml PCR flat-cap tube which consisted of 2.2µl PCR mix, 1.1µl Primer mix and 0.2µl (1U) AmpliTaq® Gold polymerase.

One-step Lysis/STR Amplification of Isolated Bio-particles from Dust Bunnies

Following bio-particle collection, a lysis buffer solution was prepared (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) and a 1.5µl aliquot was added to the sample. Samples were amplified using a modified Identifiler® Plus protocol: 75°C 15 min (lysis step) 95°C 11min (hot start and lysis enzyme inactivation); 34 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using a9700 GeneAmp® PCR system thermal cycler. A positive control (9947a) and negative control (sterile Millipore water) was performed with each amplification.

Detection of PCR Products

One microliter of amplified DNA product was added to 9.7µl Hi-Di™ formamide and 0.3µl of GeneScan™ 500 LIZ® size standard. Samples were analyzed using capillary electrophoresis (CE) with the ABI Prism 3130 Genetic Analyzer using Module G5 (16 second injection, 15kV, 60°C) and analyzed with GeneMapper® analysis software v4.0. A peak height detection threshold of 50 RFUs was used for allele detection.
CHAPTER THREE: RESULTS

Method Development of Enhanced STR Amplification Strategies for Touch DNA Sources

Increased PCR Cycle Number Amplification of Low Template Samples

Before testing enhanced STR amplification strategies with isolated bio-particles from touch samples, various amplification conditions were tested with buccal epithelial cells isolated using micro-manipulation and amplified with the Identifiler® Plus amplification kit (15 STR loci and Amelogenin detection). Using a standard (25µl) amplification reaction volume, 2 sets of samples containing 1, 2, 5 and 10 buccal cells from a single donor were collected using the micro-manipulation technique into 10 µl of sterile Millipore water and the amplification mix (15µl) was subsequently added to the tube. A “0” cell sample was also collected for both sets (and all subsequent micro-manipulation collections) to test for the presence of ‘cell-free’ DNA. Amplification cycles of 29 and 34 were tested according to the manufacturer’s conditions. Manufacturer recommendations suggest use of a 29 cycle amplification protocol for increased sensitivity when the total DNA input is <0.5ng. Reference profiles were obtained for all sample donors involved in research conducted for this thesis. Any profiles obtained from isolated cells/bio-particles were therefore verified with the reference profile of that donor. When interpreting STR profiles, STR loci where the donor has a homozygous genotype a single allele peak is counted twice to represent both copies of the detected allele. In all cell samples the increased (34) cycle number resulted in increased allele detection (Figure 1). For the 29 cycle amplification, 2 alleles were observed in the 5 cell sample and no profile was detected in the 1, 2 or 10 cell samples. For the 34 cycle amplification, 2 alleles were detected for the 1 cell sample, 1 allele was detected for the 2 cell sample, 16 alleles were detected in the 5 cell sample and 15
alleles were detected in the 10 cell sample. Figure 2 shows the DNA profile recovered from the 5 cell sample amplified with 34 cycles. In both sets no alleles were detected in the “0” cell sample.

Optimized Cell Lysis

In the Direct PCR amplification of buccal cells described above the cells are lysed during the 95°C hot start. A lysis step using forensicGEM™ saliva kit was added to the beginning of the amplification program to determine if it would improve the efficiency of the cell lysis. Buccal cells (1, 2, 5, 10 and 0) from the same donor used above were collected into 10µl of forensicGEM™ lysis buffer saliva kit (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water). Samples were amplified with a modified Identifiler® plus protocol using 34 PCR cycles with a 15 minute 75°C lysis step added before the 95°C hot start. The added lysis step improved profile recovery in all samples compared to the samples amplified without the added lysis step. Figure 3 compares the number of alleles detected in the cells amplified with and without the added lysis step (both sets amplified with 34 PCR cycles). In the set with the added lysis step 7 alleles were observed in the 1 cell sample, 12 were observed in the 5 cell sample and full (30 alleles) were observed in both the 5 and 10 cell samples. Figure 4 shows the full STR profile detected from the 10 cell sample tested with the added lysis step. In the “0” cell sample, a STR profile with 10 alleles was observed. This could have occurred due to inadvertent collection of biological material while collecting from a blank area on the Gel-Film®. Additional studies testing for ‘cell-free’ DNA on the prepared Gel-Film® slides was conducted.
Micro-Volume Reaction Mixture Validation

A micro-volume reaction mixture was tested and compared to the standard reaction mixture volume using buccal cells amplified with the one-step lysis/STR amplification protocol using 34 PCR cycles. For this experiment a set of buccal cells (1, 2, 5, 10 and 0) from 5 donors (4 female, 1 male) were tested using the standard (25µl) reaction mixture and a 3.5µl micro-volume reaction mixture. Cells amplified with the micro-volume reaction mixture were collected into 2.1µl Identifiler® Plus amplification mix (1.4µl Master Mix and 0.7µl Primer mix) and a 1.5µl aliquot of the lysis buffer solution (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) was added to the tube following sample collection. Part A of Figure 5 shows the average number of alleles detected in each of the cell numbers tested. Average profile recovery was improved in the 3.5µl reaction mixture for the 1, 2 and 10 cell samples compared to the standard reaction volume. The 5 cell samples, however, had improved average profile recovery in the 25µl reaction mixture. In 3 of the 5 donors full (30 alleles) STR profiles were observed for the 5 cell sample amplified in the 25µl reaction mixture. Both sets of reaction mixtures yielded, on average, probative DNA profiles with >20 alleles detected. In addition to a moderate improvement in profile recovery, the 3.5µl reaction volume resulted in increased peak heights (RFUs) of the detected alleles in all samples (Figure 5, part B).

STR Profiling of Isolated Bio-Particles – Micro-Volume Validation

3.5µl Micro-Volume vs. Standard (25µl)

The one-step lysis/STR amplification method was used to analyze bio-particles collected from a touched coffee cup (grip portion held by donor) with the 3.5µl and 25µl reaction volumes. One, 10, 25, 50, 100 and 0 bio-particles were collected using micro-manipulation. Figure 6
compares the number of alleles detected for each bio-particle number tested. The 25µl set had very little profile recovery; 1 and 2 alleles were observed for the samples containing 10 and 100 bio-particles, respectively. The observed profiles for the 10 and 100 bio-particles amplified in the 25µl reaction volume are shown Figures 7. The 1, 25, 50 and 0 bio-particle samples had no profile recovery for the 25µl reaction mixture. The profile recovery for the bio-particles amplified in 3.5µl reaction volume was improved; 8, 12 and 10 alleles were detected for the 10, 25 and 100 bio-particles collected, respectively. The recovered STR profiles of the 10 and 100 bio-particles amplified in the 3.5µl reaction mix are shown in Figure 8.

3.5µl One-Step Lysis /STR Amplification – Single Source Touch DNA

To analyze the utility of the one-step lysis/STR amplification protocol with single or few isolated bio-particles from touch DNA sources, the method was tested on bio-particles recovered from 5 worn clothing items from 5 different donors. Samples included: inside sweater sleeve, inside the back of cotton shirt, inside the collar of a polyester shirt, inside a pant leg (denim) and the inside portion of an elastic waistband on exercise shorts (Table 1). For each sample, (and all subsequent touch DNA bio-particle collections) 20 ‘single’ and 20 ‘clumped’ (an apparent conglomerate of many cells) bio-particles were collected (1 bio-particle/tube) using micro-manipulation. Images were taken of each bio-particle prior to its collection so the morphology of any samples which produced profiles could be analyzed. Nucleated bio-particles were the primary target of the collections followed by large clumped bio-particles and any bio-particles that appeared ‘fresh’ or non-degraded.

DNA profiles (mainly partial profiles) were recovered from only ~2-3 of the single and clumped bio-particles for all items except the shirt collar, which had on average, the highest
profile recovery. For the shirt collar sample, DNA profiles (~15-70% profile recovery) were obtained for 9/20 single bio-particles and 4/20 clumped bio-particles. In general, the shirt collar sample appeared to have more nucleated and large clumped bio-particles, indicating that more biological material was present on the sample. The allele recovery results for the single and clumped bio-particles collected from the shirt collar sample are shown in Figure 9, ordered highest allele recovery to lowest allele recovery for the single bio-particles (left) and the clumped bio-particles (right). Figure 10 shows the DNA profile (and corresponding bio-particle image, left) of a bio-particle recovered from the shirt collar sample.

Micro-Volume Reaction Mixture Modifications

Increased split peaks and out of bin alleles were frequently observed in the obtained DNA profiles obtained from isolated bio-particles using the 3.5µl one-step lysis/STR amplification protocol. These stochastic effects, typically caused by inefficient non-template addition, were thought to have occurred due insufficient amounts of AmpliTaq® Gold polymerase in the reaction mixture and the increased (34) PCR cycles [25]. An additional unit (U) of AmpliTaq® Gold was added to the mixture to improve the efficiency of non-template addition. Two additional micro-volumes were tested using bio-particles collected from 4 of the 5 clothing items described above. The first micro-volume (`3.5µl`) consisted of a total reaction volume of 3.5µl with an additional 0.2µl (1U) AmpliTaq® Gold and a reduced lysis buffer solution aliquot of 1.2µl. The 2nd micro-volume reaction mix (`5µl`) consisted of slightly more Identifiler® Plus amplification mix (3.5µl; 2.2µl Master mix, 1.1µl primer mix and 0.2µl AmpliTaq® Gold) and a 1.5µl aliquot of the same lysis buffer solution. Twenty single and 20 clumped bio-particles (1 bio-particle/tube) were collected from the inside of a shirt collar, the inside of the back of a shirt,
the inside of a sweater sleeve and the inside portion of the elastic waistband of exercise shorts. The quality of the STR profiles recovered (# of alleles detected) for the bio-particles amplified with each of the 3 micro-volumes is compared in Figure 11. The ‘5µl’ reaction volume resulted in the highest number of highly probative or full profiles (>20 alleles) for both the single and clumped bio-particles. The original 3.5µl micro-volume (no added AmpliTaq® Gold) had ~1% of singles (1) and ~3% of clumped bio-particles (2) with highly probative profiles. The ‘3.5µl’ reaction mixture showed improvement over the 3.5µl reaction mixture with ~6% of singles (5) and ~7% of clumped bio-particles (6) with highly probative profiles. The ‘5µl’ reaction mixture had 10% of singles (8) and 15% of clumps (12) with highly probative STR profiles.

**STR Profiling of Isolated Bio-Particles - Application to a Variety of Touch DNA Sources**

*Touched Objects – Single Source Touch DNA*

Touch DNA was collected on Gel-Film® adhered to glass microscope slide supports from 4 touched items. The items included the armrest of an office chair (single user of chair), the grip of a coffee cup, a cell phone (face of phone) and a car steering wheel (Table 1). All touched surfaces were selected in part based on the unlikelihood that it would be touched by a donor other than the owner of the item. For each item, 20 single and 20 clumped bio-particles (1/tube) were collected into 3.5µl Identifiler® Plus amplification mix (5µl+ described above) using micro-manipulation. Figure 12 shows the allele recovery for all single and clumped bio-particles for all 4 objects tested. All observed profiles were verified with the known reference profile of the donor of the touched item. The steering wheel had the highest allele recovery for both the single and clumped bio-particles. Twenty five percent of the single (5) and 75% of the clumped bio-particles collected from the steering wheel sample had highly probative STR profiles (>20 alleles
detected). Of those highly probative profiles, 2 of the single and 10 of the clumped bio-particles were full (30 alleles) DNA profiles. An STR profile recovered from a single bio-particle isolated from the steering wheel sample in shown in Figure 13. Several highly probative profiles were also recovered from the coffee cup sample. Ten percent of the single bio-particles (2) and 45% of the clumped bio-particles (9) collected from the coffee cup sample had highly probative STR profiles. Fewer probative profiles were observed in the chair armrest and cell phone samples but for each sample a DNA profile was recovered. One clumped bio-particle recovered from the chair armrest produced a full (30 alleles) STR profile. An STR profile with 25/30 alleles was observed in one single bio-particle collected from the cell phone sample.

Saliva Cells vs. Touch Bio-Particles – Comparison of Profile Recovery

In order to evaluate if cells originating from saliva (buccal epithelial cells) would result in higher profile recovery than skin epithelial cells, two samples were tested that contained areas where both saliva contact and touch contact occurred (geographically separated on the same object); a cigarette and a pen that had evidence of “chewing” on the cap. The cigarette sample was prepared by rolling the filter portion (saliva contact) onto a Gel-Film® prepared slide. A 2nd Gel-Film® slide was prepared rolling the portion held by the donor onto the Gel-film® surface. The pen sample was prepared by rolling the cap (saliva contact) and the grip of the pen (touch contact) onto separate Gel-Film® slides. Twenty single and 20 clumped bio-particles were collected from both regions of the cigarette and the pen. The STR profiling results for all samples collected from the cigarette are shown in Figure 14. All observed profiles were verified with the known reference profile of the donor of the item. The number of samples with profile recovery was significantly higher for the section of the cigarette that had contact with the donor’s
saliva. From the filter portion of the cigarette full profiles were observed in 5 clumped bio-particles. Fifty five percent of the clumped bio-particles (11) and 5% of the single bio-particles (1) from the cigarette filter had highly probative profiles, while only 2 single bio-particles recovered from the touched portion had probative profiles (>20 alleles). The distribution of the profile quality (number of alleles detected) for the samples collected from the pen are shown in Figure 15. The number of samples with profile recovery was also significantly higher for the “chewed” pen cap compared to the grip of the pen where touch contact occurred. From the pen cap 85% (17) of the clumped bio-particles resulted in full STR profiles and 10% (2) single bio-particles resulted in full profiles. In fact, for every clumped bio-particle tested from the pen cap some profile recovery was observed; 95% (19) clumped bio-particles had allele detection above 17 alleles and 2 alleles were observed in 1 clumped bio-particle. Allele detection from the grip of the pen where touch contact was made was much lower; the highest number of alleles detected was 7.

**Touch DNA Recovery from Male and Female Donor Sources**

To evaluate if males or females have higher levels of DNA profile recovery from isolated bio-particles, samples were collected from direct skin samples taken from each sex. Gel-film® was touched directly to the surface of the skin of an index finger, thumb and forearm of a male and female donor (different donors tested for each direct skin location; total 3 males and 3 females tested). Twenty single and 20 clumped bio-particles were collected from each sample (1/tube) using micro-manipulation for a total of 120 bio-particles (60 single and 60 clumped bio-particles) collected from the male and female donors. As shown in Figure 16, the bio-particles collected from the male donors produced more highly probative (21-29 alleles) and full (30
alleles) STR profiles than the bio-particles collected from the female donors. From the 3 male donors, full STR profiles were observed in 8 bio-particles (3 clumped and 5 single) while only 1 full profile was observed in a single clumped bio-particle from the 3 female donors tested. Additionally, 22% (13) of the clumped and 3% (2) of the single bio-particles recovered from a male donor were highly probative. Figure 17 shows a clumped bio-particle collected from a male donor’s thumb and the full DNA profile detected.

Trypan Blue Staining for Viable Bio-Particle Identification

Examination (under a stereomicroscope) of touch bio-particle samples collected onto Gel-film® and stained with Trypan blue (0.4%) revealed that not all bio-particles take up the stain and therefore remain unstained (Figure 18). Trypan blue stains ‘dead’ cells by entering through a perforated membrane and binding to intracellular proteins in the cytoplasm. Touch DNA samples contain dead and dying keratinocytes (i.e. corneocytes) originating from the outer epidermal layer of skin so it is not unexpected for dead cells to be observed in touch DNA samples [26]. To evaluate if the unstained bio-particles would result in higher DNA profile recovery than bio-particles that took up the stain, 20 stained and unstained clumped bio-particles (1/tube) were collected by micro-manipulation and analyzed with the one-step lysis/STR amplification method. A male thumb print sample was used since it is representative of the type of touch DNA that might be found at a crime scene (fingerprint). The number of STR alleles recovered from each of the stained and unstained clumped bio-particles is shown in Figure 19. Highly probative (35%, 7 clumps) or full (15%, 3 clumps) were observed in 50% of the stained bio-particles tested. Figure 20 shows an example of a full STR profile recovered from a stained bio-particle. As can be seem from the bio-particle image in Figure 20, a “fluid-like” material is
visible outside of the defined shape clumped bio-particle. Although it is unclear what this material actually is, if the bio-particle is in the process of cell breakdown it could be cellular material that has not yet fully dispersed. Throughout the course of the research for this thesis this “fluid-like” material was observed in additional touch samples. Initial testing resulted in typically high profile recovery so further investigation into the nature of this material was conducted and is described in “fluid-like” material section. No probative STR profiles were recovered from the unstained bio-particles. The maximum number of alleles recovered from an unstained clumped bio-particle was 12 alleles, a 40% profile recovery (Figure 21).

“Cell-Free” DNA

To further evaluate the source of the DNA that has been recovered from touch DNA samples on Gel-film® slides, “cell-free” samples were tested. “0” cell samples were collected to determine if non-cellular DNA, which could have originated from perforated or burst cells, was present on the prepared Gel-film® slides. For all micro-manipulation collections preformed (33 collections), a “0” cell sample was collected by touching the water soluble adhesive to an apparently empty or “cell-free” area of the Gel-film®. Of the 33 “0” cell samples collected, 27 (~82%) showed no allele detection and 5 (~15%) samples had one peak, which could have been due to allelic drop-in. The remaining sample had 3 alleles detected. This initial study shows that “cell-free” DNA is not abundant on the Gel-film surface®.

To ensure that non-cellular DNA was not missed in the limited study described above, multiple “0” cell samples were collected from the same Gel-film® slide. Twenty “0” cell samples were collected from 4 worn clothing samples: shirt back, sweater sleeve, shirt collar and shorts waistband (total N=80). The amount of drop-in that was detected is summarized in Figure
22. For all 4 clothing items tested, the majority of the “0” cell samples produced no drop-in. Of the 80 samples tested, 14 had 1 drop-in peak, 3 samples had 2 drop-in peaks, and 1 sample had 7 alleles. Due to the small size of the bio-particles collected and the crowding of bio-particles on some Gel-film® samples, inadvertent collection of material is possible and could explain allele detection from apparently “cell-free” areas.

“Fluid-Like” Material

To further evaluate the “fluid-like” material that was observed on the Gel-film® samples, additional collections were conducted targeting this material. Figure 23 shows images taken with a stereomicroscope of this “fluid-like” material found on a worn shirt collar and a direct skin sample from a male donor’s neck. Ten samples of “fluid-like” material were collected via micro-manipulation from 4 touch samples; a shirt collar from a male donor, a female donor’s fingertips, a female donor’s forearm and a male donor’s neck. A different donor was used for each sample collected. Figure 24 shows the number of alleles observed for each of the samples collected. The male shirt collar sample had the highest amount of probative and full STR profile recovery. Full profiles were observed in 7 of the 10 “fluid-like” materials tested. Figure 25 shows an example of a full profile recovered and the associated “fluid-like” material collected from the male shirt collar sample. As seen in the image in Figure 25, what appear to be several nuclei are visible within the fluid. The presence of several nuclei could help explain the high rate of profile recovery from this type of sample.

STR Profiling of Isolated Bio-Particles – Simulated Physical Assault Mixtures

In order to evaluate if the developed one-step lysis/STR amplification method could be used to detect a single source DNA profile of a male in mixed touch DNA sources, various
simulated physical assault scenarios were tested. Three physical assault scenarios were
simulated with 3 male (termed the “assailants”) and 3 female (termed the “victims”) donor sets;
some donors were repeated between scenarios. The physical assault scenarios are described in
detail in the methods section. For every scenario tested, 20 single and 20 clumped bio-particles
were collected from each donor set. The goal of this study was to detect the male “assailant’s”
DNA. Table 2 summarizes the donor source of the single and clumped bio-particles recovered
from the 4 mixture scenarios. The number of bio-particles with profiles (the majority of which
were partial profiles) matching the known reference profile of the male donor are highlighted in
grey. For all donor sets of all mixture scenarios, at least 1 bio-particle (single or clumped)
produced a DNA profile identifying the male “assailant.” The quality of the observed profiles is
shown in Figure 26, a box plot distribution of the number of alleles detected that matched the
male donor. In every scenario tested (but not necessarily every donor set) a highly probative
profile (>21 alleles) matching the male donor was detected.

Comparison of Standard Analysis of Touch DNA from Mixed Sources

For three of the physical assault scenarios (wrist grab, shirt sleeve grab and “scratching”)
standard touch DNA samples were collected to compare the results directly to the individual bio-
particle analysis technique. Three donor sets were tested for the wrist grab and shirt sleeve grab
scenarios by swabbing the female donor with a wet (sterile Millipore water) swab following the
simulated assault. The scrape stick used to collect material from the underside of the female
donor’s nails following the “scratching” scenario was cut near the end containing the collected
material into an extraction tube. DNA was extracted using the QIAamp® DNA Investigator kit.
The quantity of DNA present in the samples was assessed using Quantifiler® Human DNA
quantitation kit and amplified (1 ng target, 28 PCR cycles) using Identifiler® Plus. The results were then compared to the known reference profiles for the donors. In the first donor set of the wrist grab scenario, a partial profile matching the female “victim” was detected, therefore providing no probative information about the male “assailant” (Figure 27). In the 2nd donor set of the wrist grab scenario, a very low partial profile (6/30 alleles) was detected matching the female “victim” therefore providing no probative information about the male “assailant.” In the 3rd donor set of the wrist grab scenario, a partial profile (16/30 alleles) was detected matching the male donor show in Figure 28. However, the peak heights of all except 2 of the alleles detected fell below 100 RFUs, which is below threshold values set by most crime labs.

For the shirt sleeve grab mixture scenario, in the first donor set an admixed DNA profile with full profile matching both donors (Figure 29). In the 2nd donor set of the shirt sleeve grab scenario an inconclusive partial profile with 4 alleles detected. Of the 4 alleles detected, 2 could have originated from the male or female donor, 1 matched the female donor and another matched the male donor. The 3rd donor set for the shirt sleeve grab mixture sample resulted in no profile detection.

For the scrape stick sample a partial profile matching the female donor was detected (Figure 30), therefore, no probative information about the male “assailant” was obtained. Therefore, in the mixture scenarios tested above, no touch DNA mixture samples tested resulted in a single source, probative DNA profile identifying the male “assailant” in the sample.

Wrist Grab Scenario

The results of the 3 micro-manipulation collections performed for the wrist grab mixture scenario are shown in Figure 31. The colors of the individual bars indicate the donor source of
the bio-particle; blue corresponds to a bio-particle belonging to the male donor, green corresponds to the female donor and yellow indicates that the source could not be determined. For donor set 1, 2 full and 2 highly probative profiles (29/30 and 21/30 alleles) matching the male donor was detected. For donor set 2, a highly probative (23/30 alleles) profile matching the male donor was observed. For donor set 3, 1 full and 1 highly probative (29/30 alleles) profile matching the male donor was obtained. The male profile detected from a clumped bio-particle from donor set 1 is shown in Figure 32. For all 3 donor sets, no bio-particles tested produced mixed DNA profiles.

**Shirt Sleeve Grab Scenario**

The results of the 3 micro-manipulation collections preformed for the shirt sleeve grab mixture scenario are summarized in Figure 33. For donor set 1, 1 highly probative profile (23/30 alleles) matching the male donor was detected. For donor set 2, a semi-probative (18/30 alleles) matching the male donor was recovered from 1 clumped bio-particle. For donor set 3, 2 full and 1 highly probative profile (25/30 alleles) matching the male donor was obtained. The male profile detected from a clumped bio-particle from donor set 3 is shown in Figure 34. Again, for all 3 donor sets, no bio-particles tested resulted in mixed DNA profiles.

**“Choking” Scenario**

The results of the micro-manipulation collections from the 3 donor sets of the “choking” mixture scenario are summarized in Figure 35. For donor set 1, a semi-probative profile (16/30 alleles) matching the male donor was observed in 1 single bio-particle. In donor set 2, 13 highly probative profiles (>20 alleles) matching the male donor were observed (3 single and 10 clumped). Of all bio-particles collected from donor set 2, 28 had profiles matching the male
Donor set 3 from the choking scenario had the lowest profile recovery of all donor sets from all scenarios tested. A partial profile (6 alleles) matching the male donor was observed in 1 single bio-particle. A highly probative male profile detected from a clumped bio-particle from donor set 2 is shown in Figure 36. Again, for all 3 donor sets, no bio-particles tested resulted in mixed DNA profiles.

*Sexual Assault Scenario*

The results of the micro-manipulation collections from the 3 donor sets of the sexual assault mixture scenario are summarized in Figure 37. In this scenario 6 bio-particles (3 from donor set 1 and 3 from donor set 3) resulted in “mixed” profiles. In 5 of the 6 “mixed” profiles <30 alleles total were detected because of allelic and locus drop-out. In this experiment, samples were considered “mixed” not just if >30 alleles were observed, but also if the profile contained some alleles that matched only the female donor and some alleles that matched only the male donor. For donor set 1, a highly-probative profile (26/30 alleles) and a full profile matching the male donor were observed in a single and clumped bio-particle, respectively. In donor set 2, 1 highly probative profile (27/30 alleles) and 2 semi-probative profiles (18/30 and 12/30 alleles) matching the male donor were observed. Of all bio-particles collected from donor set 2, 28 had profiles matching the male donor. In donor set 3, a semi-probative profile (16/30 alleles) matching the male donor was recovered from 1 clumped bio-particle. However, a mixed profile containing a full profile from the male donor was observed. The mixed profile and associated clumped bio-particle recovered from donor set 3 are shown in Figure 38. This mixture contains all 30 alleles for both the male and female donor and is therefore interpretable.
“Scratching” Scenario

For this experiment 40 clumped bio-particles were collected instead of the typical 20 single and 20 clumped bio-particles. After viewing the stained (Trypan blue, 0.4%) sample under a stereomicroscope it was observed that the morphology of the bio-particles differed from that of the bio-particles recovered from the other touch DNA sources. The bio-particles appeared to be less transparent (a dark blue or black color as opposed to the light blue color typically observed) and more clumped together (Figure 39). This is likely due to the force put on the bio-particles during the simulation as well as when the bio-particles were collected with the scrape stick. Therefore, clumped bio-particles were exclusively targeted. The results of this experiment are summarized in Figure 40. In this sample type more mixtures (14 clumped bio-particles) were observed than in the other previously tested mixtures. This is not completely unexpected due to the nature of the mixture. As the female donor scratches the male donor, forced is applied to the collected bio-particles and biological material that was originally spatially separated could have become compacted. However, 1 highly probative single source profile (24 alleles) matching the male donor was observed (Figure 41).

RNA Profiling of Isolated Bio-Particles for Identification of Tissue Source of Origin

Identification of gene specific RNA biomarkers

RNA biomarkers specific for saliva and skin and a candidate set for vaginal secretions were identified using literature searches. Histatin 3 (HTN3) was selected for saliva [21]. The gene encoding group 1 of late cornified envelope (LCE1C) was chosen as the skin biomarker [22]. Table 3 lists the three vaginal secretion specific genes that were evaluated; interleukin 19 (IL19), myozenin 1 (MYOZ1) [24] and NOX01 (unpublished).
**Optimized RNA Profiling Strategies**

An RNA profiling assay using high resolution melt (HRM) temperature analysis of mRNA biomarkers was evaluated for its ability to sensitively and specifically detect single or few isolated cells/bio-particles. Based on the success of the micro-volume reaction used for DNA analysis of individual bio-particles, micro-volume RNA reaction mixtures were tested for use in body fluid analysis of isolated cells/bio-particles. An optimized lysis step using ZyGEM’s RNAGEM™ tissue lysis kit was also evaluated. The HRM assay was modified from a protocol for tissue source identification using total RNA extracted from various body fluid sources [27].

A micro-volume direct-RT reaction (Applied Biosystems High-Capacity kit; see methods section for reagent volumes) was used to synthesize cDNA from isolated cells/bio-particles collected via micro-manipulation. The samples were incubated on a 9700 GeneAmp® PCR system at 25°C for 10 minutes (primer extension), 37°C for 2 hours (cDNA synthesis) and 85°C (reverse transcriptase inactivation). The amplification step was conducted on a Real-Time PCR instrument, the Rotor-Gene® Q. The samples were amplified using QIAGEN’s Type-It® HRM kit (see methods section for reagent volumes) with a PCR cycle number of 45 instead of the manufacturer recommended 40 cycles to increase sensitivity. The amplicons were then melted over a range of 73°C-98°C to determine their melt temperature to identify the body fluid source of the bio-particles [27].

Preliminary sensitivity tests were conducted using isolated vaginal secretion cells due to the anecdotal observance that vaginal cells are typically nucleated and therefore likely to contain more genetic material than touch bio-particles. Figure 42 shows images of vaginal cells from a swab that was rolled over gel-film to deposit the material for micro-manipulation collections. A
pre-amplification protocol using the QIAGEN Multiplex PCR kit was suggested by the manufacturer to improve sensitivity of RNA profiling [28]. To evaluate if the added pre-amplification step increased the detection of tissue specific RNA in isolated cells, 2 sets of 10, 25, 50 and 100 vaginal cells were tested. Vaginal cells were used because they typically contained more “nucleated” cells and were assumed to contain more genetic material than touch DNA samples. Cells were collected using micro-manipulation into 3.55 µl RNAGEM™ lysis buffer solution (for 10µl: 1x buffer-silver, 5% RNAGEM™ and nuclease free water) and lysed at 75°C for 5 minutes to release RNA from the cells. Following cDNA synthesis, samples were then either amplified using a singleplex NOX01 HRM assay (set 1) or first amplified with the pre-amplification protocol (singleplex NOX01, protocol provided by manufacturer) followed by the singleplex NOX01 HRM assay (set 2). Figure 43 shows the melt plots of the samples along with the melt temperatures of the detected products. In set 1, NOX01 was only detected in the 100 cell count sample. In set 2, however, NOX01 was detected in all cell count samples tested. Neither sample set had NOX01 detected in any of the negative control samples (collection blank, RT blank and amp blanks).

To evaluate if the lysis step improved RNA detection, cells lysed in nuclease free water were compared to cells with the added lysis step for the 3 vaginal secretion biomarkers (NOX01, MYOZ1, IL19). For each vs biomarker, 2 sets of 10, 25, 50 and 100 vaginal cells were collected into either 3.55µl of nuclease free water or 3.55µl ZyGEM RNAGEM Tissue™ lysis solution. The cells were then lysed at 75°C for 5 minutes on a 9700 GeneAmp® PCR system. CDNA synthesis, singleplex pre-amplification, singleplex amplification and HRM analysis (described above) was then preformed. The added lysis step resulted in improve amplicon detection for the
MYOZ1, but the NOX01 and IL19 sets showed similar results for both the water lysis and RNAGEM™ lysis (Figures 44-46). Evaluation of the melt plots showed that biomarkers NOX01 and IL19 had the highest RNA detection in the lower cell counts (10 and 25 cells) tested. In order to test the sensitivity of the protocol, reduced cell counts (1 and 2 cells) were tested with the biomarkers NOX01 and IL19 due to the improved peak height and appearance observed in Figure 47. All cell counts tested had detection of NOX01 and IL19.

Touch bio-particles were then tested for the presence of mRNA skin bio-marker LCE1C (late cornified envelope gene) using the same RNAGEM lysis, cDNA synthesis, singleplex pre-amplification, singleplex amplification and HRM analysis used to evaluate the vaginal cell candidates described above. Touch bio-particles were isolated using micro-manipulation from a shirt collar sample of a male donor. Ten, 25 and 50 single and 1, 5, 10 and 20 clumped bio-particles were collected. A “0” cell sample from an apparent blank area on the Gel-film® slide was also collected to test for the presence of cell-free RNA. Commercially obtained (Stratagene/Agilent Technologies) total skin RNA extracts were used as positive controls. cDNA synthesis of total skin RNA extracts is described in methods section. Figure 48 shows the melt plots of the amplicons detected using the HRM analysis method. All touch bio-particle numbers tested except the 10 single bio-particle sample, which had LCE1C detection.

To test for the presence of three types of epithelial cells that could be encountered in forensic casework (skin, saliva and vaginal secretions), an HRM epithelial cell triplex assay was evaluated. HTN3 was selected as the saliva epithelial cell biomarker. Previous analysis of the biomarker using whole sample extracts had sample detection with an expected Tm of ~78°C. NOX01 was chosen as the vaginal cell biomarker due to the Tm of IL19 (~81.5°C) being close to
that of LCE1C (Tm~82°C) and therefore would not be suitable for use in a multiplex reaction containing LCE1C [27]. The triplex HRM assay was evaluated using QIAGEN’s Type-It® HRM kit containing a primer mixture of the forward and reverse primers for HTN3, LCE1C and NOX01 (12.5 µl Type-It® HRM reagent, 1.75 µl body fluid specific primer and 5.75 µl nuclease free water). Following the singleplex amplification of skin bio-particles (10, 25 and 50 singles and 5, 10 and 20 clumps) and saliva and vaginal cells (10, 25, 50 and 100 cells) with their respective RNA biomarker (after collection via micro-manipulation and cDNA synthesis) the HRM triplex amplification was performed on the Rotor-Gene® Q and the melt temperatures of the amplicons was obtained. Figure 49 shows the melt plots for all samples tested. LCE1C was detected in all skin bio-particle counts except the 10 and 25 single bio-particles, HTN3 was detected in the 10 saliva cell sample and all vaginal cell counts had NOX01 detection. HTN3 detection in the “0” cell saliva sample could have occurred due to inadvertent collection of cellular material during the micro-manipulation step. However, HTN3 detection in the reverse transcriptions blank (RTB+) for the saliva cell set indicates that further work validating the use of HTN3 as a biomarker for saliva cell identification is needed. The lack of cross reactivity in the skin and vaginal cell markers demonstrate the potential for an assay to differentiate between the two cell types in low template samples.

A co-isolation method was evaluated to obtain both an STR profile of the sample donor as well as the tissue source of origin. The singleplex HRM assays were used initially for the tissue source identification pipeline. Based on the success of the individual DNA and RNA micro-volume reactions, the DNA (ForensicGEM™) and RNA (RNAGEM™) micro-volume
lysates were evaluated to determine which would be more compatible for DNA and RNA recovery.

A co-isolation method was evaluated on isolated skin bio-particles and vaginal cells. Skin bio-particles (10, 25 and 50 single and 1, 5, 10 and 20 clumped) and vaginal cells (1, 2, 5, 10, 25, 50) were collected using the micro-manipulation technique into 5µl of a DNA buffer solution (for 10µl: 2.1x buffer-blue, 10% forensicGEM™ reagent, sterile water) and 5µl of an RNA buffer solution (for 10µl: 1x buffer-silver, 5% RNAGEM™ reagent, sterile water.) A “0” cell sample was also collected from both sources to test for “cell-free” DNA and RNA. The entire lysate was lysed at 75°C for 15 minutes for the DNA lysis set or 75°C for 5 minutes for the RNA lysis set. To keep the micro-volume reactions consistent with what was used in the separate DNA and RNA profiling assays, 1.5µl of the lysate was used for DNA analysis and 3.55µl was used for RNA analysis. No further modifications were made to the existing DNA and RNA profiling methods. The recovery results are summarized for the vaginal secretion cells (Figure 50) and the touch bio-particles (Figure 51). In both sample types, the RNA and DNA lysis mixture appeared to be compatible with the DNA analysis pipeline. The STR profile recovery was similar for the DNA and RNA lysis set for the vaginal cells; in both cases full (30 alleles) were observed in the 25 and 50 cell counts. For the touch bio-particles, STR profiles were observed in all samples tested (with full profiles for the 10 and 50 single bio-particles and 1 clumped bio-particle) with the RNA lysis mixture. Figure 52 shows the melt plot of the detected NOX01 amplicon and the partial STR profile (16/30 alleles) recovered from 2 vs cells analyzed with the co-isolation method. Figure 53 shows the melt plot of the detected LCE1C amplicons and the full STR profile recovered from 50 single bio-particles from a male shirt collar analyzed
with the co-isolation method. Further evaluation of the co-extraction method would be necessary; possibly through the evaluation of various PCR cycling conditions and the development of a triplex pre-amplification protocol.

Enhanced Genetic Analysis of Biological Material in Household Dust

Optimized Recovery Strategies of Human Bio-particles from Dust Bunnies

Due to the success of the micro-manipulation collection technique and one-step lysis/STR amplification protocol (34 PCR cycles) developed for the analysis of isolated bio-particles from sources of touch DNA evidence, the method was evaluated for its use with biological material isolated from dust bunnies. See the methods section for a detailed description of the preparation of dust bunny Gel-film® samples. Genetic analysis was performed on 20 dust bunnies. Fourteen of the dust bunnies were collected “locally” from private dwellings (e.g. areas within donor’s homes) and public places (e.g. Engineering Building on the University of Central Florida main campus and areas within the National Center for Forensic Science (NCFS)). An “international dust bunny” was obtained from the departure lounge of the Frankfurt Airport, Germany; and the remaining 5 dust bunnies (termed the “New York dust bunnies”) tested were collected from donors in the state of New York between 2008 and 2010. Images of the dust bunnies analyzed are shown in Figure 54.

One-Step Lysis/STR Amplification of Isolated Bio-Particles from Dust Bunnies

For each dust bunny tested, multiple collections were performed until a minimum of 2 distinct, probative (>20 alleles) profiles was observed or until the biological material appeared to be reasonably exhausted. With the exception of the first 4 dust bunnies tested in the initial stages of evaluating this method, collections of 20 single or clumped bio-particles were collected at a
A “0” cell sample was collected with every collection from a blank area on the Gel-film slide as a negative control. Figure 55 shows the distribution of the quality of all DNA profiles observed from the bio-particles collected from the 20 dust bunnies shown in Figure A. DNA profiles with 21-30 alleles were detected in 22 samples (2%), 37 (4%) had 11-20 alleles, 212 (22%) had 1-10 alleles and 42 (4%) only had detection at Amelogenin (gender). Ten of the samples produced DNA profiles with >30 alleles, indicating the presence of a mixture. Figure 56 shows a box plot distribution of the number of alleles detected for all ‘successful’ bio-particles (excluded bio-particles with no profile detection) from each of the 20 dust bunnies tested. In this figure, the maximum number of alleles detected in a bio-particle for each dust bunny is indicated by either the top whisker/top edge of the bar, an open circle (o) or an asterisk (*). An open circle (o; outlier) indicates any data point between 1.5 and 3 box lengths from the upper or lower edge of the box and an asterisk (*; extreme outlier) indicates any data point more than 3 box lengths from the upper or lower edge of the box. Of the 20 dust bunnies tested, a probative (>20 alleles) profile was observed in at least 1 bio-particle from 10 of the dust bunnies. An image of a bio-particle collected from a “New York dust bunny” (Bedroom, collected in 2008) and the probative profile that was recovered is shown in Figure 57. A nucleated single bio-particle collected from a dust bunny found in a donor’s living room (collected January 2014 is shown in Figure 58 along with the probative profile (27/30 alleles) that was recovered. In this profile an extraneous peak (i.e. 3 alleles) was observed at the vWA locus; however, had a known reference profile been available, the resulting profile still could have been used to identify the donor source of the bio-particle. In some cases known reference profiles were available for the known residents or habitual occupants of the dwelling. Profiles matching known reference profiles were identified in 5 of the dust bunnies tested. An example of a bio-particle and the
observed probative profile (20/30 alleles) matching the known reference profile of the habitual occupant of the dust bunny collection location is shown in Figure 59.

Additionally, in 5 of the dust bunnies tested, probative profiles from 2 distinct donor sources were obtained. Figure 60 and Figure 61 show an example where both the known habitual occupants were observed in bio-particles isolated from a dust bunny collected from the donor’s bedroom (“Florida bedroom #2”).

Standard DNA Isolation vs. Enhanced Method for Genetic Analysis of Dust Bunnies

In order to compare the developed enhanced genetic analysis technique to standard methods of DNA analysis used in operational crime labs, 3 of the 20 dust bunnies tested were sectioned into pieces for comparative genetic (standard method vs enhanced) analysis of the same sample. The Frankfurt Airport, Germany (the ‘Frankfurter’) dust bunny was cut into 2 sections and a dust bunny from a donor’s bedroom collected 2014 (“Florida bedroom #2”) along with a “New York dust bunny” from a donor’s bedroom collected 2008 (“New York Bedroom #4”) were cut into 3 sections (see methods for details of sample prep). A manual organic extraction was performed on half of the ‘Frankfurter’ dust bunny and enhanced DNA analysis was performed on the other half. The human DNA concentration was quantified with the Quantifiler® Human DNA quantitation kit and a total of 0.269ng/µl was obtained. The extract was amplified (1ng input, 29 PCR cycles) using AmpFISTR® Identifiler® Plus amplification kit. The complex mixed DNA profile that was obtained from the whole extraction portion of the ‘Frankfurter’ along with an image of the portioned dust bunny is shown in Figure 62. Four micro-manipulation collections were conducted on the ‘Frankfurter’ dust bunny; 20 bio-particles were collected in the first 3 collections and 10 were collected in the last collection. While mixed
DNA profiles were detected more often than was observed in any previous bio-particle collection from dust bunnies, 2 distinct, full profiles (30 alleles) were detected. The observed profiles and their associated images are shown in Figure 63 and Figure 64. Comparison of the 2 single source profiles to the admixed profile obtained from the whole extraction section shows that neither profile is fully observed in the mixture, and therefore would have gone undetected had standard methods of DNA analysis been used. For the dust bunny from a donor’s bedroom (collected 2014) and the “New York dust bunny” (collected 2008) 1/3 of the specimens were extracted using QIAGEN’S DNA Investigator kit (see methods section), 1/3 was analyzed with enhanced genetic analysis and the remaining 1/3 reserved for micro-chemical analysis of the non-biological material such as dirt, or fibers in the samples (data not shown). The human DNA concentration was quantified with the Quantifiler® Human DNA quantitation kit and a total of 0.579ng/µl and 0.43ng/µl was obtained for the “Florida bedroom #2” and the “New York bedroom #4” dust bunny, respectively. The extracts were amplified (1ng input, 29 PCR cycles) using AmpFISTR® Identifiler® Plus amplification kit. In both samples, mixed DNA profiles were obtained (Figure 65 and Figure 66). The 2 single-source, probative profiles matching habitual occupants in bio-particles collected from the “Florida bedroom #2” dust bunny are described in the previous section. Alleles matching both habitual occupants were observed in the mixed DNA profile obtained from the extracted portion of the specimen. The enhanced genetic analysis of the “New York bedroom #4” dust bunny resulted in detection of a semi-probative profile (19/30 alleles) (Figure 67). Alleles in the profile obtained from the bio-particle were present in the mixed DNA profile obtained from the extracted portion of the specimen.
As can be seen in Figures 55 and 56, DNA profiles containing >30 alleles were detected in some samples. It should be noted that in some cases where partial profiles were obtained, >2 alleles were detected at some loci and could also be considered mixtures. Of the 323 bio-particles that produced any profile recovery, 22 (7%) had detection of >2 alleles at some loci, indicating potential DNA mixtures (Figure 68). However, of those potential DNA mixtures, 12 samples had extraneous peaks at only 1 or 2 loci and could have been caused due to random drop-in caused by the increased amplification cycles used. Only 10 (3%) samples had extraneous peaks at more than 2 loci and were therefore designated as “true mixtures.”
CHAPTER FOUR: DISCUSSION

Enhanced Analysis of Touch DNA Evidence

In forensic casework it is often necessary to analyze evidence containing increasingly smaller amounts of biological material left behind by those involved in criminal cases. Touch DNA evidence, considered to be the result of DNA transferred from a donor’s shed skin cells through casual contact, is often present at crime scenes and could be used to detect a person’s presence after they have left the crime scene [13,26,29]. Locard’s exchange principle states that ‘every contact leaves a trace’, and therefore collection and analysis of this ‘trace’ could be a valuable tool for forensic investigators [1]. Current touch DNA analysis methods utilize a ‘blind-swabbing’ technique of applying a cotton swab or adhesive tape to an area of interest followed by a DNA extraction of the biological material [10,30,31]. While this method has been successful, the technique co-samples cellular material that was originally geographically separated on an item or person. The result is often admixed DNA profiles that can be difficult to interpret. Analysis of individual bio-particles isolated from a touch DNA sample using the developed one-step lysis/STR amplification protocol provides a ‘smart’ (specific, measurable, attainable, realistic and timely) method for obtaining single-source, probative DNA profiles.

In order to circumvent challenges encountered with the ‘blind-swabbing’ technique, a strategy for the isolation of bio-particles from ‘touch DNA’ was developed. The technique was the results of an adaptation of a method for the localization and collection of sperm cells [32]. Individual bio-particles are easily collected from the Gel-film® surface using a water-soluble adhesive on a tungsten needle. The adhesive, along with the collected bio-particle, then readily dissolve in the PCR amplification mix. The collection process can be monitored under the
stereomicroscope and images of both the target bio-particle prior to collection as well as immediately following can be taken to document to successful isolation of the intended material. This transfer technique requires minimal training and practice and can be incorporated into any operational crime lab without the need to purchase any expensive equipment or reagents that are not typically already utilized by the lab. In fact, McCrone’s Group of College Microscopy offers a 2 hour course to learn micro-manipulation and isolation technique for use with sperm cells [32]. Modifications to the standard protocol for STR analysis including an increased (34) amplification cycle number, micro-volume reaction mixture and added cell lysis step improved the rate of profile recovery from low template samples (Figures 1, 3 and 6). The results of this thesis demonstrate that full or probative single source profiles can be recovered from isolated single or clumped bio-particles found in touch DNA evidence. Importantly, it has been demonstrated that even clumped bio-particles rarely result in admixed DNA profiles and are a good target when utilizing this analysis method.

Analysis of profile recovery from objects where both “saliva contact” and “touch contact” occurred on the same object showed that the developed technique can be used for analysis of non-skin fluids and tissues that could be encountered in forensic casework. Samples where casual transfer of saliva (e.g. cigarette filter or “chewed” pen cap) occurred were tested to determine if the method would be successful. The results demonstrated that samples where saliva contact occurred resulted in higher levels of profile recovery than on portions of the same object that were only touched. Therefore, during the collection of forensic evidence it could be beneficial to sample from an object where it could be reasonably assumed that saliva contact could occur (e.g. pen cap with evidence of “chewing” or the rim of a drinking glass).
A comparison of the rate of profile recovery of bio-particles collected from direct skin of male and female donors showed that bio-particles from males typically produced more probative DNA profiles. The fact that males may leave behind more of their DNA than females could be beneficial for investigators. Males are more often the perpetrators of violent crimes than women. In 2013, males were responsible for 79.9% of all violent crime according to the FBI [33]. This could provide investigators with more “biological breadcrumbs” than would be typically left behind by a female.

Despite the fact Trypan blue (0.4%) stains dead and dying cells, it has been demonstrated that bio-particles that took up the stain had increased profile recovery over bio-particles that remained unstained in its presence. While it may seem counter-intuitive to target cells in the process of cell death, cells found in shed skin are typically dead and dying keratinocytes (i.e., corneocytes) and therefore nuclear (i.e., DNA) material may still be present in cells/bio-particles that contain damaged membranes or appear nonnucleated [26,34]. It is likely that unstained bio-particles in touch DNA samples are void of all cellular material and not living cells with intact membranes preventing the uptake of Trypan blue.

Another factor contributing to the ‘mystery’ of the nature of touch DNA is the possible presence of “cell-free” DNA that could have originated from necrosis, apoptosis and secretion from biological fluids [35,34]. Research suggests that “cell-free” DNA is present in touch DNA and originates from serum and sweat and is through “sweaty hands” [36]. However, sampling of 80 “cell-free” areas on Gel-film® surfaces (20 areas; 4 clothing items) resulted in very little DNA detection. Without knowledge of cellular origin, the significance of touch DNA evidence could be called into question in court, resulting in it an admissibility decision due to the ambiguity of
the source and therefore the significance of the sample. Further analysis of the presence of DNA in secreted fluids, particularly sweat, should be conducted to further evaluate if and where “cell-free” DNA is originating from in touch DNA evidence.

The occasionally observed “fluid-like” material has been shown to provide robust STR profiles. It is therefore recommended that the analyst target and collect this material type whenever possible. The true nature of this “fluid-like” material is still unknown, however. It is possible that the material originates from the process of cell-death itself; cellular material may leak out of perforated cell membranes from many cells within an immediate area and conglomerate into one unit. Further examination of “fluid-like” material should be conducted in order to identify its tissue of origin.

Individual analysis of bio-particles isolated from mixed sources where assaults were simulated resulted in the identification of a male “assailant” in all scenarios tested. Probative or full STR profiles matching the male “assailant’s” known reference profile were obtained in all 5 scenarios tested (but not necessarily all donor sets; see results section). This demonstrates the improvement of the enhanced analysis method over standard ‘blind-swabbing’ techniques, which produced no probative single source profiles originating from the male “assailant.” The ability to obtain a probative, single-source DNA profile matching a male “assailant” even in the presence of the female “victim’s” DNA was a critical goal of this project. Mixed DNA profiles, while potentially interpretable depending on the profile, are often complex and vulnerable to questioning in court. Even if deemed admissible, the mere implication of a DNA profile as non-reliable and could result in its consideration as weak evidence by a jury. While mixed DNA profiles were occasionally observed using the enhanced method; the strategy permits repetitive
sampling that is both simple and cost effective. A touch DNA sample collected with Gel-film® could theoretically be sampled until the genetic material (i.e., bio-particles) have been exhausted, providing a large sample set available for testing. The ability to sample, test and repeat is not generally possible using standard ‘blind-swabbing’ techniques. The micro-volume amount of required amplification mix (3.5µl) and lysis buffer solution (1.5µl) make the method extremely inexpensive compared to standard DNA analysis methods. Reagents and equipment for DNA extraction and quantitation are not required; additionally valuable time is saved bypassing these analysis steps.

In addition to the issues surrounding the genetic analysis of touch DNA samples, the inability to definitively determine the tissue source of touch DNA as shed skin cells and not, for example, saliva permits the significance of the detected DNA profile to be challenged in court. In the case of saliva, a suspect could potentially claim in court that his detected DNA was the result of saliva deposited through speaking and not physical contact [19]. Additionally, the identification of vaginal secretions as the tissue source of an obtained DNA profile could be used to prove the occurrence of a sexual assault crime, one which would have different criminal charges than a physical assault [37]. Preliminary evaluation of a technique for sensitive detection of mRNA biomarkers specific for forensically relevant tissue sources has been evaluated here. Synthesis of cDNA from mRNAs present in specific body fluids has been demonstrated using a multiplex reverse transcription-polymerase chain reaction (RT-PCR) [21,23,38,39]. The developed technique used selective amplification of body fluid specific biomarkers to detect forensically relevant body fluids on evidentiary items that contain limited quantities of biological material. mRNA profiling provides increased sensitivity compared to
conventional methods of body fluid identification, for which there are only tests for saliva, semen and blood [40]. Biomarkers specific for vaginal secretions, saliva and skin have been identified for use in mRNA profiling assays [23,24,41]. Here the sensitivity of the mRNA profiling technique has been increased for its application to isolated epithelial cells from vaginal secretions, saliva and skin using a micro-volume reaction mixture, an added lysis step (similar to the one used in the one-step lysis/STR amplification) and a pre-amplification step. The adaptation of the High Resolution Melt (HRM) analysis method for identification of mRNA amplicons based on specific melt temperatures provided a fast and inexpensive technique sensitive enough for use with single or few isolated cells/bio-particles [27]. The evaluated triplex assay testing for the 3 epithelial cell types could be used to determine the tissue source of a touch DNA sample. Confirmation of vaginal secretion or skin cell transfer instead of saliva transfer could provide supporting evidence of physical contact in cases involving assault. Additionally, it would be beneficial to determine donor source and tissue source from the same forensic sample. A co-isolation method of DNA and RNA from biological stains has been demonstrated [42]. Here the enhanced methods for separate DNA and RNA analysis were easily integrated for a comprehensive evaluation of single or few isolated cells/bio-particles. The use of the RNAGEM™ lysis buffer solution was able to sufficiently lyse the samples without hindering the enhanced DNA analysis method. Using the co-isolation technique, a probative STR profile (24/30 alleles) and the vaginal secretion cell specific biomarker NOX01 were detected in a sample containing 2 vaginal cells. While further method validation is needed, the techniques could potentially determine the tissue and donor source simultaneously in evidentiary samples containing extremely small amounts of biological material.
Genetic Analysis of Household Dust

Household dust, and in particular dust bunnies, is a trace material that is widely overlooked by forensic investigators despite its pervasive presence at crime scenes. A dust sample is a collection of materials from an immediate environment; fibers from textiles, dirt carried in from the outdoors and shed skin cells unknowingly left by visitors [43]. Therefore, a dust specimen is a unique snapshot of the area it was collected from, and could provide investigators with valuable information about the scene of a crime. Dust is easily transported from its original location; a suspect could unknowingly leave a crime scene with it attached to his or her clothing or shoes, or a heavy body taken from a homicide scene could pick it up as it is dragged across a room. Genetic analysis of biological material isolated from a dust bunny could identify persons of interest in a case and lead investigators to a crime scene where more valuable evidence could be recovered.

In order to recover biological material from dust bunnies the conglomerate structure was deconstructed to isolated individual bio-particles. Dust bunnies were easily teased apart over wax paper and particulate material was collected onto adhesive Gel-film® as it fell out. Once on Gel-film®, the samples were stained for visualization with Trypan blue (0.4%) in the same manner as touch DNA samples. The micro-manipulation technique was then utilized to analyze individual bio-particles. The one-step lysis/STR amplification proved to be a successful technique for recovery of DNA profiles from bio-particles isolated from a dust bunny. As a comparison, genetic analysis was performed on dust bunnies using standard methods. While extraction of ‘whole’ (dust bunnies were previously portioned for comparative analysis) samples did result in DNA recovery, complex admixed profiles were detected, as was expected. ‘Whole’
dust bunny extraction dually served as a proof of concept (DNA was in fact present) and proof that enhanced analysis methods are necessary for probative DNA profiles to be recovered. A total of 20 dust bunnies collected from various private dwellings and public locations and over 2 time periods from 2008-2010 and 2014-February 2015. Of the 20 dust bunnies tested, 14 (70%) produced at least 1 probative (>20 alleles) single-source DNA profile. The ability to detect probative, single-source DNA profiles from multiple (at least 2) donors within a dust bunny was the ultimate goal of these experiments. While this goal was not achieved in all dust bunnies, the ability to identify multiple donors from 1 sample was demonstrated 5 times, representing a 25% success rate.

Using ‘smart’ analysis, human DNA can be identified to potentially enable investigators to identify a location of a crime scene from a dust bunny associated with a case. The results of this enhanced method for genetic analysis of dust bunnies demonstrate that probative DNA profiles can be obtained from this under-utilized trace material.
CHAPTER FIVE: CONCLUSION

The goals of this research were to develop an enhanced analysis method for LTDNA samples found in touch DNA and household dust. Currently, standard methods used for touch DNA analysis regularly fall short of providing probative data and there is essentially no standardized DNA analysis method for household dust. Biological samples containing genetic material from multiple donors are regularly encountered in forensic casework, so there is a need to a technique that can separately analyze the individual sources of the DNA. By isolating individual bio-particles from touch DNA and household dust bunnies, probative, single-source DNA profiles have been obtained. The micro-manipulation technique described here requires little training and no additional equipment not already found in operational crime laboratories. Through the use of an optimized cell lysis step, a concentrated micro-volume reaction mixture and an increased amplification cycle number, the protocol is sensitive enough to obtain full or probative STR profiles from as little as 1 cell. Also, it has been demonstrated that clumped bio-particles and the observed “fluid-like” material are a good target for DNA analysis and very rarely produced admixed DNA profiles. It is often desirable to detect a profile of a minor contributor in a DNA mixture. The standard ‘blind-swabbing’ technique typically results in the major contributor’s DNA masking any alleles belonging to the minor donor and no probative data is obtained. The one-step lysis/STR amplification protocol allows for isolation of many bio-particles from 1 touch DNA, providing many more opportunities to detect the DNA of the minor contributor. Further research analysis of bio-particles from a variety of touch DNA sources will provide insight into the nature of this biological material and will provide forensic scientists with a means of better understanding touch DNA.
The ability to obtain probative DNA profiles from accumulated dust has also been demonstrated here. Dust, which can be found at any crime scene, could provide a wealth of information that could be useful to investigators. The ability to identify a donor in dust found to be associated with a crime could point investigators to a person of interest. Additionally, if the inorganic material found in a dust bunny was simultaneously analyzed to determine characteristics of where the specimen originated from, it could even identify a specific room.

The results of this research demonstrate how valuable DNA evidence is present and available for analysis in even the most compromised samples; one only needs to know where to look.
Figure 1: Comparison of Profile Recovery from Buccal Cells Using 29 and 34 PCR Cycles. Standard volume (25µl), Identifiler Plus
Figure 2: Profile Recovered from 5 Buccal Cells
Standard volume (25µl), Identifiler Plus 34 cycles. LDO indicates locus drop-out. ADO indicates allelic drop-out. Allele numbers and relative fluorescence units (RFUs) are listed below each allele. X-axis represents size in base pair and y-axis represents peak height or RFU values.
Figure 3: Effect of Optimized Lysis Step on Profile Recovery
Isolated buccal cells. Standard volume (25µl), Identifiler Plus, 34 cycles
Figure 4: Profile Recovered from 10 Buccal Cells with Optimized Lysis Step
Standard volume (25µl), one-step lysis/amplification (Identifiler Plus, 34 cycles)
Figure 5: Profile Recovery and Peak Height Intensity from Buccal Cells
Five donors were tested (4 female, 1 male). One-step lysis/amplification (Identifiler Plus, 34 cycles). Comparison of standard and micro-volume (3.5µl) reaction volume. Part A) average number of alleles detected. Part B) average peak height (RFUs) for all genuine alleles in STR profile. Bars indicate standard error.
Figure 6: Profile Recovery from Bio-Particles Using the 25µl and 3.5µl Reactions

Bio-particles were collected onto Gel-film® from the grip of a donor’s personal coffee cup. Bio-particles were counted while viewed under the stereomicroscope as they were picked up with the water-soluble adhesive. One-step lysis/amplification (Identifiler Plus, 34 cycles).
Figure 7: Profiles Recovered from 10 and 100 Bio-Particles Using 25µl Reaction
Figure 8: Profiles Recovered from 10 and 100 Bio-Particles Using 3.5µl Reaction
Figure 9: Profile Recovery from Bio-Particles from Inside of a Shirt Collar
One-step lysis/amplification reaction (‘3.5μl’), Identifiler Plus, 34 cycles. Listed from highest to lowest allele recovery. Single bio-particles indicated in light blue (left) and clumped bio-particles indicated in dark blue (right).
Figure 10: Profile Recovered from an Individual Bio-Particle from a Shirt Collar
One-step lysis/amplification reaction (’3.5µl’), Identifiler Plus, 34 cycles. Image shows collected bio-particle

20/30 alleles detected
Figure 11: Comparison of 3 Micro-Volume One-Step Lysis/Amplification Reactions (Identifiler Plus, 34 cycles) N=80 for all graphs
Figure 12: Comparison of Profile Recovery from Bio-Particles from Touched Objects

One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Listed from highest to lowest allele recovery. Single bio-particles indicated in light blue (left) and clumped bio-particles indicated in dark blue (right). Part A) chair arm rest, part B) coffee cup, part C) cell phone and part D) steering wheel.
Figure 12: Comparison of Profile Recovery from Bio-Particles from Touched Objects
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Listed from highest to lowest allele recovery. Single bio-particles indicated in light blue (left) and clumped bio-particles indicated in dark blue (right). Part A) chair arm rest, part B) coffee cup, part C) cell phone and part D) steering wheel.
Figure 13: Profile Recovered from an Individual Bio-Particle from a Steering Wheel

One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.
Figure 14: Profile Recovery from Bio-Particles Isolated from Cigarette
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Listed from highest to lowest allele recovery. Single bio-particles indicated in light blue (left) and clumped bio-particles indicated in dark blue (right). Part A) saliva contact, part B) touch contact.
Figure 15: Profile Quality Assessment from Bio-particles Isolated from Pen
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Part A) saliva contact, Part B) touch contact. N=20 for each graph
Figure 16: Profile Recovery Compared between Bio-Particles from Male and Female Donors
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Direct skin samples (Gel-film® touched directly to skin surface) from index finger, thumb and forearm collected from 1 male and 1 female donor (different donors used for each area tested, N=60). Twenty single and 20 clumped bio-particles were tested from each area.
Figure 17: Profile Recovered from Bio-Particle from a Male Donor’s Thumb

One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.

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30/30 alleles detected
Figure 18: Trypan Blue Stain Negative Bio-Particles
Examples of bio-particles that resisted Trypan blue staining.
Figure 19: Profile Recovery from Trypan Blue Stained and Unstained Bio-Particles
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Bio-particles collected from a direct skin sample from a male donor’s thumb. Listed from highest to lowest allele recovery. Stained bio-particles indicated in dark blue (left) and unstained bio-particles indicated in grey (left).
Figure 20: Profile Recovered from a Trypan Blue Stained Bio-Particle

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30/30 alleles detected
**Figure 21: Profile Recovered from a Trypan Blue Stain Negative Bio-Particle**


12/30 alleles detected
Figure 22: Prevalence of “Cell-Free” DNA on Gel-Film® Surface

Collected from 20 “cell-free” areas (no apparent cellular material present) from 4 clothing item samples. One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. Twenty “0” cell samples collected from cell-free areas on Gel-film® surface, so a total of 80 areas tested. Possible profile indicates any sample where >2 peaks were detected.
Figure 23: “Fluid-like” Material
“Fluid-like” material observed on Gel-film® surfaces, which typically produced high levels of profile recovery.
Figure 24: Profile Recovery from “Fluid-Like” Material
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. “Fluid-like” material was collected from a male shirt collar (blue), female fingertip (green), female forearm (maroon) and male neck (light blue). N=10 for each sample.
Figure 25: Profile Recovered from “Fluid-Like” Material
Bio-particle collected from a male shirt collar sample. One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.
Figure 26: Profile Quality Assessment of Male DNA in Simulated Mixture Samples

One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles. N values can be found in Table 2; total number of singles (light blue) and clumps (dark blue) for each mixture scenario sourced from the male donor. Bars indicate quartile ranges of allele detection for each mixture scenario. Asterisk (*) indicates an outlier; any sample that is more than 1.5x box lengths from the top line of the box. Top bar/whisker or asterisk indicate the maximum number of alleles detected.
Figure 27: Profile Recovered from ‘Blind-Swab’ of Wrist Grab Scenario
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.
Figure 28: Profile Recovered from ‘Blind-Swab’ of Wrist Grab Scenario
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.
Figure 29: Profile Recovered from ‘Blind-Swab’ of Shirt Sleeve Grab Scenario
One-step lysis/amplification reaction (‘5µL ’), Identifier Plus, 34 cycles.
Figure 30: Profile Recovered from Extracted Scrape Stick - “Scratching” Scenario
One-step lysis/amplification reaction ("5µl+"), Identifiler Plus, 34 cycles.
Figure 31: Donor Source of DNA in Simulated Mixture Samples – Wrist Grab
**Figure 32: Profile Recovered from the Male “Assailant” in Wrist Grab Scenario**

A single bio-particle (left panel) was collected from a simulated physical contact mixture (male donor forcefully grabbing a female’s wrist). The obtained STR profile is shown (5µl+ one-step lysis/amplification, Identifiler Plus, 34 cycles).

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30/30 alleles detected
Figure 33: Donor Source of DNA in Simulated Mixture Samples – Shirt Sleeve Grab
Figure 34: Profile Recovered from the Male “Assailant” in Shirt Sleeve Grab Scenario.
A single bio-particle (left panel) was collected from a simulated physical contact mixture (male donor forcefully grabbing a female’s shirt sleeve). The obtained STR profile is shown (5µl+ one-step lysis/amplification, Identifiler Plus, 34 cycles).
Figure 35: Donor Source of DNA in Simulated Mixture Samples – “Choking”
Figure 36: Profile Recovered from the Male “Assailant” in “Choking” Scenario.
A single bio-particle (left panel) was collected from a simulated physical contact mixture (male donor grabbing a female’s neck). The obtained STR profile is shown (5µl+ one-step lysis/amplification, Identifiler Plus, 34 cycles).

27/30 alleles detected
Figure 37: Donor Source of DNA in Simulated Mixture Samples – Bed Sheets
**Figure 38: Profile Recovered from the Male “Assailant” in Bed Sheets Mixture.**

A clumped bio-particle (left panel) was collected from a simulated sexual assault mixture (bedding where mutual contact was made by male and female donor). The obtained STR profile is shown (5µl+ one-step lysis/amplification, Identifiler Plus, 34 cycles).
Figure 39: Clumped Bio-Particles from “Scratching” Scenario
Female donor scratched male donor’s arm with enough force to temporarily leave a mark. The underside of the female donor’s nails were scraped with a scrape stick. Collected material on the Scrape Stick was deposited onto Gel-film and stained with Trypan blue for visualization.
Figure 40: Donor Source of DNA in Mixture Samples – “Scratching” Scenario
One-step lysis/amplification reaction (‘5µl+’), Identifiler Plus, 34 cycles.
Figure 41: Profile Matching Male “Assailant” in “Scratching” Scenario
A clumped bio-particle (left panel) was collected from a simulated assault mixture (female donor scratched inside of male donor’s arms). The obtained STR profile is shown (5µl+ one-step lysis/amplification, Identifiler Plus, 34 cycles).
Figure 42: Vaginal Secretion Cells on Gel-Film®.
Stained with Trypan blue (0.4%). Viewed under stereomicroscope (250X) with transillumination.
Figure 43: Increased Sensitivity of HRM Analysis with Pre-Amplification.
Vaginal secretion cells were collected using micro-manipulation. RNA was isolated from the recovered bio-cells using a reduced volume direct RT reaction. Part A) vs cells amplified without pre-amplification step. Part B) vs cells amplified with pre-amplification step. Products were then used in a subsequent singleplex NOX01 HRM assay. The melt plots for all samples are shown. Cell counts indicated in darker green and positive controls (total RNA) indicated in light green. X-axis – Tm (°C); Y-axis – (dF/dT). Horizontal line on graph is threshold. The table indicates the Tm value obtained for all samples. Grey cells represent no detection.
Figure 44: Evaluation of Lysis Step with Singleplex NOX01 HRM Analysis.
Vaginal secretion cells were collected using micro-manipulation. RNA was isolated from the recovered bio-cells using a reduced volume direct RT reaction.
**Figure 45: Evaluation of Lysis Step with Singleplex MYOZ1 HRM Analysis.**
Vaginal secretion cells were collected using micro-manipulation. RNA was isolated from the recovered bio-cells using a reduced volume direct RT reaction.
**Figure 46: Evaluation of Lysis Step with Singleplex IL19 HRM Analysis.**

Vaginal secretion cells were collected using micro-manipulation. RNA was isolated from the recovered bio-cells using a reduced volume direct RT reaction.
**Figure 47: HRM Analysis of Reduced Vaginal Secretion Cell Numbers.**

Vaginal secretion cells were collected using micro-manipulation. RNA was isolated from the recovered bio-cells using a reduced volume direct RT reaction, part A) NOX01, part B) IL19. Products were then used in a subsequent singleplex HRM assay.
Figure 48: HRM Analysis of Touch Bio-Particles from a Shirt Collar.
Bio-particles (single (10, 25 and 50) and ‘clumped’ (1, 5, 10, 20)) from a shirt collar were collected using micro-manipulation. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent singleplex LCE1C HRM assay. Purple indicates positive controls (total RNA extracts).
Figure 49: Triplex Epithelial Cell HRM Analysis.
A triplex pre-amplification and HRM detection assay were developed using a biomarker for saliva (HTN3), skin (LCE1C) and vaginal secretions (NOXO1). A representative sample (saliva - blue; skin - peach; vaginal – green) are shown to demonstrate the successful use of the triplex assay for tissue source of identification for isolated cells/bio-particles. Grey indicates no product detection. White indicates no detection only at melt temperature for that amplicon.
Figure 50: DNA/RNA Co-Isolation from Vaginal Cells.
Vaginal cells (1, 2, 5, 10, 25 and 50 cells) were collected using micro-manipulation and analyzed using DNA/RNA co-isolation micro-volume assays. Part A) shows cells lysed with ForensicGEM™ DNA lysis buffer solution and part B) shows cells lysed with RNAGEM™ RNA lysis buffer solution. STR profiles were obtained from DNA lysates (Identifiler Plus, 34 cycles) and the number of alleles observed are shown on the left. RNA was isolated from the recovered cells using a reduced volume direct RT reaction, followed by singleplex NOX01 pre-amplification. Pre-amplification products were then used in a subsequent singleplex HRM assays. The on the right table indicates the Tm value obtained for all samples.
Figure 51: DNA/RNA Co-Isolation from Touch Bio-Particles.

Bio-particles (single (10, 25 and 50) and clumped (1, 5, 10 and 20)) were collected from a male shirt collar sample using micro-manipulation and analyzed using DNA/RNA co-isolation micro-volume assays. Part A) shows bio-particles lysed with ForensicGEM™ DNA lysis buffer solution and part B) shows bio-particles lysed with RNAGEM™ RNA lysis buffer solution. STR profiles were obtained from DNA lysates (Identifiler Plus, 34 cycles) and the number of alleles observed are shown on the left. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplification products were then used in a subsequent singleplex HRM assays. The on the right table indicates the Tm value obtained for all samples.
Figure 52: HRM Analysis and STR Profile from Vaginal Secretion Cells.

Two vaginal secretion cell sample was collected using micro-manipulation. A) RNA was isolated from the recovered cells using a reduced volume direct RT reaction, followed by singleplex NOX01 pre-amplification. Pre-amplifications products were then used in a subsequent singleplex NOX01 HRM assay. The melt plot is shown. X-axis – Tm (°C); Y-axis – dF/dT). Partial STR profile recovered (Identifiler Plus, 34 cycles) is shown.
Figure 53: HRM Analysis and STR Profile from Touch Bio-Particles.
Fifty single bio-particles were collected (into 1 tube) from a male shirt collar sample using micro-manipulation. A) RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent singleplex LCE1C HRM assay. The melt plot is shown. X-axis – Tm (°C); Y-axis – dF/dT). Full STR profile recovered (Identifiler Plus, 34 cycles) is shown.

*pull-up

30/30 alleles
**Figure 54: Whole Dust bunny Images.**

Images taken of whole dust bunny or dust bunny material from various dust bunnies used in one-step lysis/STR amplification analysis method. The location and date of collection is listed below each dust bunny. No available image for “refrigerator dust bunny.” Part A) “New York dust bunnies” collected from 2008-2010. Part B) “Florida dust bunnies” collected from 2014-2015. Collection location and date list below each image. Rulers and paperclip included for size reference.
Figure 55: DNA Profile Quality (Number of Alleles) of Bio-Particles Collected From 20 Dust Bunnies.

976 total bio-particles were analyzed amongst 20 dust bunny samples. The number of alleles for each sample was determined. The percent of samples within each allele number range are provided (number of samples shown below each percentage).
Figure 56: Allele Detection Distributions for Bio-Particles Collected from 20 Dust Bunnies.
The allele recovery distribution for bio-particle samples collected from amongst 20 dust bunnies is shown. Asterisks and open circles represent “outliers” and “extreme outliers”, respectively. The dust bunny collection location and year are listed on the x axis (BR = bedroom, LR = living room). ‘Frankfurter’ indicates dust bunny collected from departure lounge of Frankfurt Airport, Germany. Number of bio-particles with any profile recovery (bold,underlined) and total N for each sample is shown above or on each bar.
**Figure 57: Single Source Profile Detected in “New York Dust Bunny”**

Individual bio-particle collected from a dust bunny (DB) sample from a donor’s bedroom in the state of New York in 2008. Dust bunny sample was stored in manila envelope at room temperature until delivered to the National Center for Forensic Science (NCFS) in Central Florida for analysis in January 2014. Sample was analyzed using a micro-volume one-step lysis/direct PCR reaction. Since no reference profile was available, loci with only 1 observed allele was considered homozygous at that locus if the RFU value was above 500.

22/30 alleles
**Figure 58: Single Source Profile Detected in Individual Bio-Particle from Living Room Dust Bunny.**

Individual bio-particle collected from a dust bunny sample from a donor’s living room in January 2014. Sample was analyzed using a micro-volume one-step lysis/direct PCR reaction. Since no reference profile was available, loci with only 1 observed allele was considered homozygous at that locus if the RFU value was above 500.

*drop-in

27/30 alleles
Figure 59: Single Source Profile Matching Habitual Occupant of Dwelling.
Individual bio-particle (top left) collected from a dust bunny sample from a shelf located in the living room of donor’s home and analyzed using a micro-volume one-step lysis/direct PCR reaction. A post-collection image is provided (top right) to demonstrate successful removal of the target bio-particle. Profile was verified with known reference profile for that donor.
Figure 60: Detection of Habitual Occupant of a Room.
Individual bio-particles (top left) collected from a dust bunny sample from a donor’s bedroom and analyzed using a micro-volume one-step lysis/direct PCR reaction. A post-collection image is provided (top right) to demonstrate successful removal of the target bio-particle. Profile was verified with known reference profile for that donor.
Figure 61: Detection of Habitual Occupant of a Room.
Individual bio-particles (top left) collected from a dust bunny sample from a donor’s bedroom and analyzed using a micro-volume one-step lysis/direct PCR reaction. A post-collection image is provided (top right) to demonstrate successful removal of the target bio-particle. Profile was verified with known reference profile for a known habitual occupant of that room.

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Figure 62: Admixed DNA Profile Observed in Extraction of ‘Frankfurter’ Dust Bunny
Portion of dust bunny extracted using manual organic method shown in boxed outline. Human DNA concentration obtained using Quantifiler™ Human DNA quantitation kit and extract (1ng input, 29 PCR cycles) was amplified using AmpFISTR® Identifiler® Plus kit.

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Figure 63: Single Source Profile Observed in Bio-Particle from ‘Frankfurter’ Dust Bunny.
Bio-particle (top left) collected from a dust bunny from the departure lounge of the Frankfurt Airport in Germany and analyzed using a micro-volume one-step lysis/direct PCR reaction. A post-collection image is provided (top right) to demonstrate successful removal of the target bio-particle. Since no reference profile was available, loci with only 1 observed allele was considered homozygous at that locus if the RFU value was above 500.

30/30 alleles detected

*pull-up
Figure 64: Single Source Profile Observed in Bio-Particle from ‘Frankfurter’ Dust Bunny.
Bio-particle (top left) collected from a dust bunny sample from the departure lounge of the Frankfurt Airport in Germany and analyzed using a micro-volume one-step lysis/direct PCR reaction. Since no reference profile was available, loci with only 1 observed allele was considered homozygous at that locus if the RFU value was above 500.
Figure 65: Admixed DNA Profile Observed in Extraction of Dust Bunny from Donor’s Bedroom.

Portion of dust bunny extracted using manual organic method shown in boxed outline. Human DNA concentration obtained using Quantifiler® Human DNA quantitation kit and extract (1ng input, 29 PCR cycles) was amplified using AmpFISTR® Identifiler Plus kit.
Figure 66: Admixed DNA Profile Observed in Extraction of “New York dust bunny” from Donor’s Bedroom.
Portion of dust bunny extracted using manual organic method shown in boxed outline. Human DNA concentration obtained using Quantifiler® Human DNA quantitation kit and extract (1ng input, 29 PCR cycles) was amplified using AMPFISTR® Identifiler® Plus kit.
Figure 67: Single Source Profile Observed in Bio-Particle from “New York Bedroom #4” Dust Bunny.
Individual bio-particle (top left) collected from a dust bunny sample from the bedroom of a donor in the state of New York in 2008. The specimen was analyzed using a micro-volume one-step lysis/direct PCR reaction. Since no reference profile was available, loci with only 1 observed allele was considered homozygous at that locus if the RFU value was above 500.

18/30 alleles detected
Figure 68: DNA Mixture Prevalence in Bio-Particles Collected from 20 Dust Bunnies.
976 total bio-particles were analyzed amongst 20 dust bunny samples. Samples with more than 2 alleles at 1 or 2 loci were considered potential mixtures (orange on graph). Samples with more than 2 alleles at more than 2 loci were considered true mixtures (red on graph). The percent of samples within each category are provided (number of samples shown below each percentage).
APPENDIX B: TABLES
Table 1: “Single Source” Touch DNA Evidence Samples.
Areas tested using the one-step lysis/STR amplification protocol. Samples were assumed to be “single source” because it was unlikely for DNA originating from a person other than the donor of the item to be present. For each sample, 20 single and clumped bio-particles were collected.

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<tr>
<th>Sample Category</th>
<th>Location</th>
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<tr>
<td>Worn clothing</td>
<td>Shirt collar, sweater sleeve, elastic waistband of shorts, shirt back, denim pant leg</td>
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<tr>
<td>Touched object</td>
<td>Steering wheel, chair arm rest, coffee cup, cell phone</td>
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<tr>
<td>Direct skin</td>
<td>Forearm, thumb, index finger</td>
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<tr>
<td>Saliva/touch contact comparison</td>
<td>Cigarette, pen</td>
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Table 2: Donor Source of Bio-particles from various mixture scenarios
For each mixture scenario, 3 donor sets were tested (3 females, 3 males). 20 single and 20 clumped bio-particles were collected for each donor set. For description of mixture scenario see methods section. Samples were then collected by applying Gel-Pak (x8 retention) adhesive adhered to a microscope slide to the area of mutual contact. N=20. Asterisk (*) indicates clumped bio-particle was contaminated by analyst.

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<th>Mixture Scenario</th>
<th>Donor Set</th>
<th>Donor Source</th>
<th>Male single</th>
<th>Male clump</th>
<th>Female single</th>
<th>Female clump</th>
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<th>No Profile clump</th>
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### Table 3: Tissue Specific mRNA Biomarker Primer Sequences

LCE1C- Late Cornified Envelope Protein 1, NOX01- , IL19- Interleukin 19, MYOZ1- Myozenin 1, HTN3- Histatin 3.

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<th>Body Fluid</th>
<th>Gene</th>
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<th>Size (bp)</th>
<th>References</th>
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APPENDIX C: PRESENTATIONS AND PUBLICATIONS
Presentations


Publication

APPENDIX D: FUNDING
This work was supported by Award Numbers 2010-DN-BX-K139 and 2013-DN-BX-K025 awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. The opinions, findings and conclusions of recommendations expressed in this paper are those of the author and do not necessarily reflect those of the Department of Justice.
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