**National Center for Forensic Science**
University of Central Florida
P.O. Box 162367 · Orlando, FL 32826
Phone: 407.823.4041
Fax: 407.823.4042
Web site: http://www.ncfs.org/

**Biological Evidence**

# Molecular Characterization of Trace Biological Evidence for the Optimized Recovery and Analysis of 'Touch DNA'

FINAL REPORT

September 29, 2014

Department of Justice, National Institute of justice
Award Number: 2010-DN-BX-K139
(1 November 2010 – 30 September 2014)

Principal Investigator:
Jack Ballantyne, PhD
Professor
Department of Chemistry
Associate Director for Research
National Center for Forensic Science
P.O. Box 162367
Orlando, FL 32816-2366
Phone: (407) 823 4440
Fax: (407) 823 4042
e-mail: Jack.Ballantyne@ucf.edu

## ABSTRACT

The ability to obtain genetic profiles of the donor of trace biological or touch DNA evidence has been well established, albeit that many such samples comprise mixtures of two or more donors. However, the true nature of touch DNA evidence has remained elusive, generally perceived to be the result of DNA obtained from shed skin cells yet never confirmed with scientific certitude. This is largely due to the perception that it is not possible to ascertain the tissue source of origin of the biological material in touch DNA evidence. The uncertainty with regard to the source of trace biological material is now being exploited in some criminal proceedings in an attempt to diminish the significance of trace biological evidence. Thus far, research has failed to provide operational crime laboratories with feasible methods to identify the tissue source of origin of touch DNA samples. The current work sought to: 1) provide a comprehensive characterization of the biological material recovered from touch DNA evidence and, uniquely; 2) develop standard and enhanced amplification strategies to recover single source STR profiles of the donor(s) of the recovered micro-particles that are the constituents of touch DNA; and 3) provide molecular-based approaches for the positive identification of a skin tissue source of origin.

In order to better characterize the biological material present in touch DNA evidence, we have developed a "micro-particle atlas" that contains thousands of images of bio-particles recovered from various clothing items, household items and touched objects. These images permitted an evaluation of the morphological features of bio-particles in touch DNA evidence and allowed us to assess the type (e.g. single vs "clumps") and quantity of cellular material present. We are currently attempting to make this atlas available online as this could be a useful reference material for operational crime laboratories in support of their analysis of touch DNA evidence.

We have developed enhanced collection and profiling strategies for the recovery and analysis of bio-particles in touch DNA evidence. Collection strategies include transfer of the biological material from touched objects and surfaces to a low-retention adhesive material and isolation of single or few bio-particles or cells using a water soluble adhesive. The use of the water soluble adhesive permits the transfer of the recovered particles to a lysis buffer or directly into a PCR amplification reaction since the adhesive will dissolve thus releasing the particles into solution. An enhanced one-step micro-volume (5µl) lysis/STR amplification reaction permits the recovery of full or probative STR profiles of the donor of single or few bio-particles. We demonstrate the ability to obtain single source STR profiles from single- and multi-source touch DNA evidence (e.g. worn clothing items and other household items, touched/handled objects and surfaces, skin/skin mixtures). We also demonstrate the ability to apply the developed collection and micro-volume profiling strategies to detect the male donor in simulated physical assault mixture samples.

Numerous reports state that the tissue source of origin of touch DNA evidence cannot be determined due to the small amount of biological material present, while many others conclude that the DNA profiles are obtained from shed skin cells as opposed to, say, saliva traces without any scientific basis for this assertion. Proper identification of the biological material present might be crucial to the investigation and prosecution of a criminal offense and a misrepresentation of the nature of the evidence can have undue influence on the perception of the circumstance of the crime. We have identified numerous highly specific mRNA biomarkers for

the identification of skin, including LCE1C (late cornified envelope 1c) which is well suited for the identification of skin as the tissue source of origin of touch DNA samples. We have developed micro-volume cell lysis/reverse transcription reactions, combined with subsequent RNA analysis using capillary electrophoresis (CE) or high resolution melt analysis (HRM) body fluid identification assays, to provide the ability to identify the body fluid origin of individual or few isolated bio-particles. We have also developed an initial DNA/RNA co-isolation strategy to permit tissue source identification and STR profiling from the same sample including bio-particles from touch DNA evidence.

The developed DNA/RNA profiling methods, therefore, provide a comprehensive 'smart' molecular based approach to the characterization, analysis and interpretation of trace biological material recovered from touch and contact samples.

**TABLE OF CONTENTS**

## EXECUTIVE SUMMARY

1. In forensic casework analysis it is sometimes necessary to obtain genetic profiles from increasingly smaller amounts of biological material left behind by persons involved in criminal offenses. The ability to obtain profiles from trace biological evidence is routinely demonstrated with so-called 'touch DNA evidence' (generally perceived to be the result of DNA obtained from shed skin cells transferred from donor to an object or person during physical contact). Although a genetic profile from trace biological evidence is routinely obtained, the tissue source of the profile is rarely known. This merely perpetuates the 'mystery' of the nature of touch DNA evidence allowing the significance or meaningfulness of genetic profiles obtained from these samples to be challenged.

2. Numerous reports state that the tissue source of origin of touch DNA evidence cannot be determined due to the small amount of biological material present, while many others conclude that the DNA profiles are obtained from shed skin cells as opposed to, say, saliva traces without any scientific basis for this assertion. Proper identification of the biological material present might be crucial to the investigation and prosecution of a criminal offense and a misrepresentation of the nature of the evidence can have undue influence on the perception of the circumstance of the crime.

3. The current method of recovery of trace DNA employs cotton swabs or adhesive tape to sample an area of interest. While of practical utility such a 'blind-swabbing' approach will necessarily co-sample cellular material from the different individuals whose cells are present on the item, even if the individuals' cells are located in geographically distinct locations on the item. Thus some of the DNA mixtures encountered in such touch DNA samples are artificially created by the swabbing itself. Secondly it is not possible to definitively identify whether the DNA profiles originate from skin cells or other epithelial cells due to the lack of appropriate biomarker assays for these cell types.

4. The current work sought to provide more clarity to so-called touch or trace DNA through an evaluation of the nature and origin of the biological material in trace evidence samples as well as the development of enhanced strategies for the analysis of the biological material present in these samples.

5. We firstly sought to microscopically characterize the structural nature and quantity of biological material present in touch or contact items often recovered as evidence. Optimized collection and removal strategies were developed to recover the biological material. Secondly, the current work sought to develop RNA/DNA co-isolation strategies using novel micro-volume molecular analysis workflows that may be more ideally suited for the recovery of nucleic acids from trace biological material. An identification of the skin versus non-skin tissue source of origin of recovered biological material was accomplished through the use of RNA (mRNA) profiling techniques. Additionally, enhanced amplification strategies were used to also obtain an STR profile of the donor of the biological material.

6. We have collected over 3,000 images of the biological (and non-biological material) present in various clothing items, household items and touched objects: shirt sleeve, shirt collar, shirt back, shirt front, pants (upper thigh), pants (leg), sock (ankle), sock (bottom), underwear (front), underwear (back), pillow case (both sides of pillow), sheets, towel, hat and couch, cell phones, computer mouse, door handles, car steering wheels, water bottles and fingerprints. Through the collection of these images, we have developed the first touch DNA micro-particle atlas, which could serve as a useful reference material for operational crime laboratories in support of their analysis of touch DNA evidence. We are attempting to make the atlas available to the forensic community via the World Wide Web.

7. The images contained in the micro-particle atlas permitted an evaluation of the morphological features of bio-particles in touch DNA evidence and allowed us to assess the type (e.g. single vs "clumps") and quantity of cellular material present. On each image we classified bio-particles as single bio-particles or "clumps". Most bio-particles, whether single or clumped, appeared to have a "dehydrated appearance" (e.g. flat, textured). There were no significant bio-particle morphological differences between items/objects. We did notice on some items, like towels or sheets, some of the bio-particles appeared more "rolled" or "squished" which is probably a result of the type of contact with these items. Additionally, for items like socks and hats, we noticed more background material which can be possibly due to dirt or sweat which would be more common on these types of images. We also observed more partial or degraded bio-particle material which again can be a result of the different type (or extent) of contact with skin surfaces for these items.

8. We were able to look at average length and width measurements for the bio-particles we identified, both singles and clumps. For the single bio-particles the average length and width was 33 and 34 μm, respectively, consistent with the diameter of single epithelial cells. For the "clumps", the average length and width was larger at 65 and 64 μm, respectively.

9. In order to avoid the challenges of 'blind swabbing' strategies, we developed a collection strategy for the recovery of bio-particles from 'touch DNA' which involves the following: 1) transfer of the biological material from touched objects and surfaces to a low-retention adhesive gel-film, 2) microscopic examination of the recovered biological material, and 3) recovery of single or few bio-particles or cells (identified as cells at this stage only if a nucleus can be identified) from the gel-film using a water soluble adhesive. The use of the water soluble adhesive permits the transfer of the recovered particles to a lysis buffer or directly into a PCR amplification reaction since the adhesive will dissolve thus releasing the particles into solution. The entire transfer process can be visualized under the stereo-microscope ensuring successful transfer of the recovered bio-particles for further analysis. All subsequent reactions (e.g. STR profiling) are performed in the same reaction tube thereby eliminating potential sample loss from additional manipulations or sample transfers.

10. We had originally planned to utilize the Advalytix AmpliGrid system for the micro-volume reactions. The Advaltyix AmpliGrid system is specifically designed for single cell molecular analysis workflows. However, during the course of this study we were informed that the instrument and consumables were no longer manufactured. Since this was no longer a long-term viable option for casework use, we discontinued all studies using the Advalytix system. We

continued to study micro-volume PCR reactions using standard equipment (ABI 9700 thermal cyclers). Development of these amplifications on standard equipment would provide a possible protocol for operational crime laboratories to use without the requirement to purchase additional equipment.

11. We developed a lower-cost, 'smart analysis' method that results in enhanced genetic analysis of touch DNA evidence (e.g. worn clothing items, touched/handled objects, skin/skin mixtures). We developed a one-step 5µl micro-volume lysis/STR amplification reaction (Identifiler Plus, 34 cycles) that permits the recovery of full or probative STR profiles of the donor of single (or few) bio-particles recovered from touch DNA evidence (e.g. worn clothing items and other household items, touched/handled objects and surfaces, skin/skin mixtures). The use of individual or few (i.e. "clumps") of bio-particles results in the ability to obtain with a high degree of probability single source (as opposed to mixture) profiles.

12. Importantly, even with the collection of clumped bio-particles, admixed DNA profiles were infrequently observed. Additionally, we did not see significant confounding results from "cell-free" DNA.

13. We demonstrated the successful use of our developed "smart" analysis methods for touch DNA evidence with the ability to recover probative single source profiles from single and "clumped" bio-particles from various touched objects and clothing items. With the feasibility of this method demonstrated, we then used this approach for the detection of male donor DNA (single source) in simulated physical contact/assault mixture samples (e.g. perpetrator grabbing a victim's wrist, neck or clothing, or contact with victim's bedding as in sexual assaults). Probative profiles were obtained from 33% and 40% of the single and clumped bio-particles tested and a single source male donor profile was identified in every mixture sample tested.

14. We have identified novel highly specific mRNA biomarkers for the identification of skin. Five mRNA markers were identified that demonstrated a high degree of specificity for skin. Using these markers, we were able to successfully detect and identify skin using as little as 5-25 pg of input total RNA from skin. Significantly, LCE1C was detectable in swabs of human skin and various touched objects. One of the markers, LCE1C, is particularly highly sensitive and was detected in the majority of skin samples tested including touched objects.

15. We developed enhanced RNA profiling strategies suitable for use with few isolated bio-particles and cells. We developed micro-volume cell lysis/reverse transcription reactions, combined with subsequent RNA analysis using capillary electrophoresis (CE) or high resolution melt analysis (HRM) body fluid identification assays, to provide the ability to identify the body fluid origin of individual or few isolated bio-particles.

16. We developed a simple pre-amplification step prior to RNA product detection to improve sensitivity. The pre-amplification method included the use of a triplex pre-amplification step to permit the simultaneous amplification of epithelial cell biomarkers for the identification of skin, vaginal secretions and saliva. This would permit an identification of the tissue source of origin of any isolated epithelial cells.

17. Uniquely, we have also developed an initial micro-volume DNA/RNA co-isolation strategy to permit tissue source identification and STR profiling from small numbers of bio-particles and cells from touch DNA evidence.

18. The results of the current work provide a 'smart' comprehensive molecular based approach to the characterization, analysis and interpretation of trace biological material recovered from touch and contact samples.

---

## I. INTRODUCTION

### A. Statement of the Problem

In forensic casework analysis it is sometimes necessary to obtain genetic profiles from increasingly smaller amounts of biological material left behind by persons involved in criminal offenses. The ability to obtain profiles from trace biological evidence is routinely demonstrated with so-called 'touch DNA evidence' (generally perceived to be the result of DNA obtained from shed skin cells transferred from donor to an object or person during physical contact). Although a genetic profile from trace biological evidence is routinely obtained, the tissue source of the profile is rarely known. This merely perpetuates the 'mystery' of the nature of touch DNA evidence allowing the significance or meaningfulness of genetic profiles obtained from these samples to be challenged. Numerous reports state that the tissue source of origin of touch DNA evidence cannot be determined due to the small amount of biological material present [1-4], while many others conclude that the DNA profiles are obtained from shed skin cells as opposed to, say, saliva traces without any scientific basis for this assertion [5-10]. Proper identification of the biological material present might be crucial to the investigation and prosecution of a criminal offense and a misrepresentation of the nature of the evidence can have undue influence on the perception of the circumstance of the crime. While it is not the primary responsibility of crime laboratory personnel to determine the significance of the biological evidence in relation to the circumstances of the crime, there is a responsibility to provide accurate molecular based characterizations of the evidence being presented. The current method of recovery of trace DNA employs cotton swabs or adhesive tape to sample an area of interest. While of practical utility such a 'blind-swabbing' approach will necessarily co-sample cellular material from the different individuals whose cells are present on the item, even if the individuals' cells are located in geographically distinct locations on the item. Thus some of the DNA mixtures encountered in such touch DNA samples are artificially created by the swabbing itself. Secondly it is not possible to definitively identify whether the DNA profiles originate from skin cells or other epithelial cells due to the lack of appropriate biomarker assays for these cell types. Therefore, the current work sought to provide more clarity to so-called touch or trace DNA through an evaluation of the nature and origin of the biological material in trace evidence samples as well as the development of enhanced strategies for the analysis of the biological material present in these samples.

## B. Literature Review

Crime laboratories are faced with processing a large volume of cases every year and, due to the ever-increasing importance of DNA profiling to the criminal justice system, there is increased pressure to produce results in a timely manner. In the past, standard practice in forensic casework analysis typically included a preliminary screening of evidentiary items recovered during the investigation of criminal offenses in order to identify the presence, and possible tissue origin, of biological material. However, it is often not possible to positively confirm the presence of some of the commonly found biological fluids and tissues due to the lack of specific methods. Additionally, evidentiary items frequently contain limited quantities of biological material and may thus be below the sensitivity limits of the few definitive conventional methods for body fluid identification that exist. Most laboratories will use, depending upon the case, a small number of non-specific but presumptive screening tests for blood, semen and saliva. It is somewhat understandable, therefore, that positive confirmation of the tissue source of origin of sample is often not carried out prior to processing the samples via the DNA analysis pipeline. This is particularly the case with so-called 'touch DNA evidence.'

'Touch DNA' evidence is generally perceived to be the result of DNA obtained from shed skin cells transferred from donor to an object or person during physical contact (i.e. Locard's exchange principle 'every contact leaves a trace') [5-10]. Therefore, it is often assumed that if DNA is recovered from an object that is typically handled, for example a knife, then the DNA originated from shed skin cells from the handler. However, in the absence of any supporting scientific data, this could be a precarious assumption. Numerous published reports demonstrate the ability to obtain DNA profiles from a variety of objects that have been in direct contact with a person including items such as paper and documents [11,12], keyboards [13], bedding and fabrics, shoe insoles [5], firearms and fired cartridges cases [14], drinking containers, pens and briefcase handles [15]. These studies involved the collection of biological material through swabbing, tape lifts or removal of portions of the material and do not include an assessment of the type of biological material present. Additionally, these studies typically involved simulated studies where there is prior knowledge of direct contact with skin and these same assumptions cannot be applied to forensic casework analysis where the precise circumstances and events of a crime are not typically known. While in many cases it may in fact be the direct result of skin contact, the presence of trace amounts of saliva from incidental (not surface to surface) contact cannot be excluded [16]. In one study the simple act of talking resulted in the production of 112-6720 saliva droplets with a mean diameter of 16μm [17]. A misrepresentation of the source of biological evidence could place undue weight to a given piece of evidence. Consider the example where a business man is found in his office strangled with his own neck tie. DNA recovered from the tie is linked to his business partner with whom the victim was seen arguing over financial matters a few hours before the body was discovered. If the DNA from the business partner suspect came from skin cells transferred onto the victim's tie during strangulation then this would be strong supportive evidence against the suspect. However, if it was determined that trace levels of saliva (i.e. buccal epithelial cells) were present then it could be argued that the DNA was from incidental contact with saliva from the business partner during the prior argument. In this scenario, the finding of saliva rather than skin could significantly affect the impact of the evidence. While it is not the responsibility of forensic casework analysts to interpret the circumstances or events of a crime, there is a responsibility to report scientific findings, not assumptions, in service of the law. It could be considered negligent to report that DNA is from shed skin cells if there is no scientific basis for such a conclusion and could

ultimately have serious repercussions for justice if such misrepresentations, even if inadvertently implied, are presented in court.

Very few studies involving the analysis of touch DNA samples have included an examination of the nature of the biological material present in these samples [18,19]. In the study conducted by Kita et al., microscopic examinations of human neck skin using histological and immunological staining provided a representation of the characteristics of outer epidermal skin layers (flattened cells with condensed nuclei which has lost their typical shape) [19]. Using antibodies to single stranded DNA they were able to identify fragmented DNA in stripped nuclei on the skin surface and uppermost layers [19]. However, when a fingerprint on a glass slide was examined, stripped nuclei were rarely detected and trace DNA was prevalent [19]. Therefore, this study demonstrates that assumptions made about the surface of some areas of skin may not directly apply to the material that is transferred to objects through touch or contact. Additionally, swabs of human skin surface were examined and the presence of degraded DNA was observed after the electrophoretic analysis of extracted DNA [19]. However, this study ignores the possibility that the degraded DNA observed was actually due to the presence of low molecular weight bacterial DNA from the microbiota known to be present on the surface of human skin. Allesandrini et al. performed experiments involving fingerprints on glass slides and found a considerable level of inter-individual variability in the number of nucleated cells (0 – 9 depending on the donor) and "stripped nuclei" (0 - 8 depending on donor) [18]. While these studies provide an indication that potential skin cellular material is present, they do not provide any methodologies to conclusively identify a skin source of origin. Additionally, the authors did not demonstrate that the recovered DNA profiles came directly from the identified cellular material. It is possible that the source of DNA did not originate from the shed skin cells but from 'naked DNA' from other body secretions such as sweat or sebaceous fluid.

The necessary ambiguity of the tissue source of origin of touch DNA evidence is being used to challenge the admissibility of evidence at trial and as the subject for appeal of adjudicated decisions. The issue is exemplified in the recent court case in which Terence and David Reed were convicted of the murder of Peter Hoe in North Yorkshire, UK (2006) [20]. A small amount of DNA matching the suspects (one with a profile attributed to Terence Reed and the other containing a mixture of David Reed and the victim) was recovered from two small pieces of plastic (presumed to be pieces of a knife handle) found near the victim. Initial appeals were made based on "the general reliability of DNA evidence using the LCN process including its lack of validation, the limited research, the absence of protocols, disputes over interpretation and the scope of evaluation in the case were made on the basis of low copy number profiling strategies that were used to obtain the profiles from a small amount of DNA" [20]. However, the appeals based on these notions were rejected after a determination that the profiles were interpretable [20]. Additional appeals were then made on the basis that "it was simply not possible to say how the material had been transferred; it could not be assumed that the material was skin cells as there was a real possibility of epithelial cells being transferred from saliva, whether by primary or secondary transfer; it was not possible to tell whether they had been transferred by primary, secondary or tertiary transfer or when this had happened" [20]. A recent article in one of the pre-eminent scientific journals, Nature, quotes ex-FBI chief scientist Dr Bruce Budowle as saying "with [low copy number typing] you can't say what the tissue source is" in support of an argument against the use of LTDNA analysis [2]. With some court decisions supporting the admissibility of profiles obtained through the use of low copy number profiling

techniques [21], challenges will likely continue to be made as to the issue of the uncertainty of the tissue source of the trace biological material from which a DNA profile is obtained.

An oft-quoted description of the purpose of a forensic investigation is:

> "Wherever he steps whatever he touched, whatever he leaves, even unconsciously, will serve as a silent witness against him, not only his fingerprints or his footprints, but his hair, the fibers from his clothes, the glass he breaks, the tool marks he leaves, the paint he scratches, the blood or semen he deposits or collects. All of these and more bear mute witness against him….It is factual evidence. Physical evidence cannot be wrong, it cannot perjure itself, it cannot be wholly absent. Only human failure to find it, study and understand it, can diminish its values." (*Harris vs. United States*. 331 U.S. 145 1947).

In regards to touch DNA evidence, appropriate research has led to the ability to "find it" and begin to "study it", but has largely failed in a complete ability to "understand it" allowing for its value to be diminished in criminal investigations. It is, therefore, essential that critical research be done to characterize the biological material recovered from touched objects, obtain a skin vs. non-skin tissue source identification method and to conclusively demonstrate a direct link between the biological material and recovery of DNA profiles from it. The current study sought to provide some of the necessary clarity to so-called touch DNA evidence.

### C. Statement of Hypothesis or Rationale for the Research

The current study sought to provide more clarity to so-called touch or trace DNA. A thorough evaluation of the nature and origin of biological material in trace evidence samples was proposed as was the development of strategies to provide definitive identification of the tissue source of origin of such evidence. We firstly sought to microscopically characterize the structural nature and quantity of biological material present in touch or contact items often recovered as evidence. Optimized collection and removal strategies were developed to recover the biological material. Secondly, the current work sought to develop RNA/DNA co-isolation strategies using novel micro-volume molecular analysis workflows that may be more ideally suited for the recovery of nucleic acids from trace biological material. An identification of the skin versus non-skin tissue source of origin of recovered biological material was accomplished through the use of RNA (mRNA) profiling techniques. Additionally, enhanced amplification strategies were used to also obtain an STR profile of the donor of the biological material. The current work therefore sought to provide a comprehensive molecular based approach to the characterization, analysis and interpretation of trace biological material recovered from touch and contact samples.

## II. METHODS

## DNA

*Sample collection*

'Touch DNA' and buccal 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. Single source touch samples were collected from worn clothing items and touched/handled objects (e.g. inside shirt collar, pant leg, coffee cup; one donor for each item). Several mixture samples were created in order to simulate forensic casework scenarios. Skin/skin contact mixture samples included simulation choking/strangulation (donor 1's hands on donor 2's neck) or physical assault (donor 1 grabbing donor 2's wrist). Skin/clothing contact mixtures were created to simulate physical altercations (donor 1 grabbing donor 2's shirt sleeve) or contact with bedding as in a sexual assault. For each mixture type, three donor sets were evaluated.

To recover biological material from the touched objects and surfaces, WF Gel-Film® x8 retention level (Gel-Pak®, Hayward, CA), was cut to a size appropriate for subsequent attachment to a glass microscope slide support (3" x 1" x 1mm, Fisher Scientific, Suwanee, GA). Using sterile tweezers, the back protective covering was removed to expose the adhesive back and the Gel-Film® was placed onto a clean glass microscope slide. The top protective plastic film layer was then removed using re-sterilized tweezers. The Gel-Film® surface was then repeatedly touched to the sample area (direct skin, clothing or object surface) several times to ensure sufficient transfer of biological material. Samples were stained with Trypan Blue (0.4%) (Sigma-Aldrich, St. Louis, MO) for 1 minute, then washed briefly by gentle flooding with sterile Millipore water. Samples were then air-dried at room temperature prior to proceeding to sample collection. All samples were stored at room temperature in microscope slide boxes protected from light.

Comparison samples were collected using 'blind swabbing' from a representative donor set of each mixture scenarios (one of the three donor sets tested). The area of contact between the 2 donors (skin or clothing surface) was swabbed with a pre-moistened (sterile Millipore water) sterile swab. Buccal samples (for donor reference profiles) were collected from donors using sterile swabs by swabbing the inside of each donor's mouth Swabs were dried at room temperature prior to storage at -20°C until needed.

*Bio-Particle collection*

Individual single and 'clumped' bio-particles were viewed, imaged and collected using a Leica M205C stereomicroscope (Micro Optics of FL, Inc, Davie, FL). Imaging (250X) and measurements were performed using Leica Application Suite LAS v4.3 software. For each sample, 20 single and 20 aggregated or 'clumped' bio-particles were collected. A "0" cell or negative control sample was included with each collection (adhesive touched to a seemingly empty area of the gel-film (no obvious bio-particles or other material present, but near other bio-particles and particulates)). Bio-particles were collected from the Gel-Pak® surface using 3M™ water-soluble wave solder tape (5414 transparent) on the end of a tungsten needle. 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 under the stereomicroscope. The collected bio-

particle was then transferred into a sterile 0.2ml PCR flat-cap tube (Phenix Research, Candler, NC)) containing 3.5µl of AmpFISTR® Identifiler Plus Master Mix (Life Technologies, Grand Island, NY) which consisted of 2.2µl PCR mix, 1.1µl primer mix, 1U AmpliTaq Gold® DNA polymerase).

*One-step Combined Direct Lysis/Autosomal Short Tandem Repeat (STR) Amplification*

A lysis buffer solution was prepared (for 10µl: 2.1x buffer-blue, 10% forensicGEM® reagent, sterile water) (ZyGEM forensicGEM™ tissue kit (VWR, Suwanne, GA) and a 1.5µl aliquot of the prepared lysis buffer was added to each reaction. Samples were amplified using a modified AmpFISTR® Identifiler Plus protocol: 75°C 15 min (lysis); 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 an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A positive control (9947a) and negative control (sterile Millipore water) were included with each amplification.

*Reference and comparison samples*

*DNA isolation and quantitation*

DNA was extracted from sample swabs using a QIAamp DNA investigator kit (QIAGEN, Germantown, MD) according to the manufacturer's recommended conditions (manual and QIACube protocols) using a 60µl elution volume (buffer EB). An extraction blank was included with each extraction as a negative control. DNA was quantified with the Quantifiler® Human DNA Quantification kit according to the manufacturer's recommended conditions. All quantitations were performed using an ABI Prism 7000 or 7500 real time PCR instrument (Life Technologies).

*Autosomal STR amplification*

Amplification of the reference and comparison samples was performed using the AmpFISTR® Identifiler Plus kit (Life Technologies) according to the manufacturer's recommended conditions (25µl reactions, 28 amplification cycles). One nanogram of input DNA or up to 10 µl sample extract (if low or undetected DNA quantitation values obtained) was used. The amplification program was as follows: 95°C 11 min; 28 cycles: 94°C 20 sec, 59°C 3 min; final extension 60°C 10 min. All amplifications were performed using an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A positive control (9947a) and negative control (sterile Millipore water) were included with each amplification.

*PCR Product detection*

An aliquot (1µl) of the amplified product was added to 9.7µl Hi-Di™ formamide (Life Technologies) and 0.3µl of GeneScan™ 500 LIZ® size standard (Life Technologies). Samples were injected onto an ABI Prism 3130 Genetic Analyzer using Module G5 (16 sec injection, 15kV, 60°C) and analyzed with GeneMapper analysis software v4.0 (Life Technologies). A peak detection threshold of 50 RFUs was used for allele detection.

**RNA - Standard**

*Body fluid samples*

Body fluids were collected from volunteers using procedures approved by the University of Central Florida's (UCF) Institutional Review Board as well as the Institute of Legal Medicine, Forensic Genetics, Zurich, Switzerland. Informed written consent was obtained from each donor. Blood samples were collected by venipuncture into additive-free vacutainers and 10 - 50 µl aliquots were placed onto cotton cloth and dried at room temperature. Freshly ejaculated semen was provided in sealed plastic tubes and stored frozen until they were dried onto sterile cotton swabs (full swabs (i.e. liquid semen allowed to fully absorb the cotton swab) or 10 µl aliquots dried onto cotton swab). Buccal samples (saliva) were collected from donors using sterile swabs by swabbing the inside of the donor's mouth. Semen-free vaginal secretions and menstrual blood were collected using sterile cotton swabs. Human skin total RNA was obtained from commercial sources: Stratagene/Agilent Technologies (Basel, Switzerland or Santa Clara, CA), Biochain® (Hayward, CA), Zenbio (Research Triangle Park, NC), Zyagen (San Diego, CA), and AMS Biotechnology (Bioggio-Lugano, Switzerland). Human tissue total RNA (adipose, bladder, brain, cervix, colon, esophagus, heart, kidney, liver, lung, ovary, placenta, prostate, skeletal muscle, small intestine, spleen, testes, thymus, thyroid, and trachea) was also obtained from commercial sources (FirstChoice® Human Total RNA Survey Panel, Ambion by Life Technologies, Foster City, CA or Rotkreuz, Switzerland). Skin samples tested included scraped or swabbed skin from friction ridge skin and hairy skin, and mock casework samples (swabs from various surfaces and objects (e.g. door handle, keyboard, telephone receiver)). Swabs of these samples were collected by swabbing the skin or object surface with a pre-moistened (sterile Millipore water) sterile swab. All body fluid samples were dried prior to storage. All body fluid samples were stored at -20°C (UCF) or at room temperature protected from light (Zurich) until needed. Total RNA samples (skin and tissues) were stored at -80°C. A 10 - 50 µl stain (cotton cloth) or a single cotton swab was used for RNA isolation.

*RNA Isolation*

Total RNA was extracted from blood, semen, saliva, vaginal secretions and menstrual blood using various extraction types including a manual organic RNA extraction (guanidine isothiocyanate-phenol:chloroform) and commercially available extraction kits (RNeasy® Mini kit and AllPrep DNA/RNA Mini kit (QIAGEN®, Hombrechtikon, Switzerland)) as previously described [22-24].

*DNase I Digestion*

All extracts were treated with DNase to remove residual DNA using the Turbo DNA-*free*™ kit (Applied Biosystems by Life Technologies (AB)) according to the manufacturer's protocol or on-column with the RNase-free DNase set (QIAGEN®) according to the manufacturer's protocol.

*RNA Quantitation*

RNA extracts were quantitated with Quant-iT™ RiboGreen® RNA Kit (Invitrogen by Life Technologies, Carlsbad, CA) or the Quant-iT RNA Assay (Invitrogen by Life Technologies, Rotkreuz, Switzerland) as previously described [22-25]. Fluorescence was determined using a Synergy™ 2 Multi-Mode microplate reader (BioTek® Instruments, Inc., Winooski, VT) or a Qubit fluorometer (Invitrogen).

*Whole Transcriptome Sequencing (RNA-Seq)*

Total RNA was isolated from two vaginal swabs (26 yr old female; 30 yr old female) as described above. Two human skin total RNA samples were obtained from commercial sources (Biochain®; Zyagen). Transcriptome sequencing (RNA-seq) was performed by Ambry Genetics (Aliso Viejo, CA) using 54 bp paired-end sequencing on the Illumina Genome Analyzer IIx. All samples were sequenced in duplicate.

*cDNA Synthesis*

All samples were reversed transcribed using the High Capacity cDNA Reverse Transcription kit (AB) according to manufacturer's protocols or SuperScript III reagents (Invitrogen). The desired total RNA input was reverse transcribed in a 20 µl RT reaction volume. If no quantitative value was obtained or quantitation was not performed, an aliquot of the total RNA extract was used (up to 11 µl using Superscript III and 14.2 µl using the High Capacity cDNA Reverse Transcription kit). A reverse transcription negative reaction (containing total RNA and reaction buffer but no reverse transcriptase enzyme) was performed for each sample.

*Polymerase Chain Reaction*

*Primers* - PCR primer sequences were designed using Primer 3 v.0.4.0. Primers were custom synthesized by Invitrogen, Applied Biosystems, or Microsynth (St. Gallen, Switzerland). The forward primer of each primer set was labeled with a fluorescent phosphoramidite dye (AB or Invitrogen by Life Technologies). The reverse primer sequence for LCE1C was modified during the course of this work to try to eliminate the presence of a second unidentified amplification product. For any studies that utilized the original reverse primer (5'-ACACTTTGGGGGACACTTTG-3'), the results are designated as LCE1C*. The current LCE1C primers are provided in Table 1. A housekeeping gene (UBC, ubiquitin C) was also used in the evaluation of human skin and touched objects (Zurich laboratory) to ensure the presence of total RNA (UBC-F: 5'-GGGTCGCAGTTCTTGTTTGT-3', R: 5'-TCCAGCAAAGATCAGCCTCT-3').

*Singleplex Reactions (UCF)* – 2 µl of the RT-reaction was amplified in a total reaction volume of 25 µl. The reaction mixture contained 0.16 – 2.4 µM primers (LCE1C* - 1.6 µM, LCE1C – 0.32 µM, LCE1D (late cornified envelope 1D) – 2.4 µM, LCE2D (late cornified envelope 2D) – 1.2 µM, IL1F7 (interleukin 1 family, member 7) – 0.16 µM, CCL27 (chemokine (C-C motif) ligand 27) – 1.2 µM), 250 µM dNTPs, 1x PCR Buffer II (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3.25 mM $MgCl_2$, and 2 units AmpliTaq Gold® (AB). All amplifications were performed on a GeneAmp® 9700 PCR System (AB): (1) 95°C 11 min, (2) 35 cycles: 94°C 20 sec, 58°C 30 sec, 72°C 40 sec; and (3) final extension at 72°C for 45 min.

*Singleplex Reactions (Zurich)* - Minor modifications were made to the standard singleplex reactions for Zurich-amplified samples. For amplification of LCE1C*, LCE1D, LCE2D, and CCL27, the following conditions were used: 2 µl of the RT-reaction was amplified in a total reaction volume of 25 µl. The reaction mixture contained 0.8 µM primers, 1 mM dNTPs (AB), 1xPCR Buffer I (AB), and 1.25 units AmpliTaq® Gold (AB). All amplifications were performed on a GeneAmp® 9700 PCR System (AB): (1) 95°C 11 min, (2) 35 cycles: 94°C 20 sec, 55°C 30 sec, 72°C 40 sec; and (3) final extension at 72°C for 5 min. For amplification of IL1F7, the following conditions were used: 2 µl of the RT-reaction was amplified in a total reaction volume of 25 µl. The reaction mixture contained 0.8 µM primers, 1 mM dNTPs (AB), 1xPCR Buffer II (AB), 3.5 mM $MgCl_2$ (AB) and 1.75 units AmpliTaq Gold® (AB). All

amplifications were performed on a GeneAmp® 9700 PCR System (AB): (1) 95°C 11 min, (2) 35 cycles: 94°C 20 sec, 58°C 30 sec, 72°C 40 sec; and (3) final extension at 72°C for 60 min.

*Tetraplex System* – 2 µl of the RT-reaction was amplified in a total reaction volume of 25 µl. The reaction mixture contained buffer, 0.16 – 0.8 µM primers (LCE1D – 0.8 µM, LCE2D – 0.8 µM, IL1F7 – 0.16 µM, CCL27 – 0.8 µM), 250 µM dNTPs (AB), 1x Advantage® HD Buffer, and 0.625 units Advantage® HD Polymerase (Clontech, Mountain View, CA). All amplifications were performed on a GeneAmp® 9700 PCR System (AB): (1) 98°C 2 min, (2) 32 cycles: 98°C 10 sec, 58°C 10 sec, 72°C 20 sec; and (3) final extension at 72°C for 5 min.

*Pentaplex System* – 2 µl of the RT-reaction was amplified in a total reaction volume of 25 µl using the QIAGEN® Multiplex PCR kit. The reaction mixture contained a primer mix of 0.08 µM (LCE1C) and 0.2 µM (LCE1D, LCE2D, IL1F7 and CCL27) primers, 1x QIAGEN Multiplex PCR Master Mix (HotStar Taq® DNA Polymerase, multiplex PCR buffer with 6 mM $MgCl_2$, dNTP mix) and 1x Q-solution. All amplifications were performed on a GeneAmp® 9700 PCR System (AB): (1) 95°C 15 min, (2) 35 cycles: 94°C 30 sec, 58°C 90 sec, 72°C 45 sec; and (3) final extension at 72°C for 30 min.

*PCR Product Detection - Capillary Electrophoresis*

Amplified fragments were detected with the ABI Prism 3130 or 3130xl Genetic Analyzer capillary electrophoresis system (AB). Using the 3130 Genetic Analyzer, a 1.0 µL aliquot of the amplified product was added to 9.7 µL of Hi-Di™ formamide (AB) and 0.3 µL of GeneScan™ 500 LIZ® size standard (AB) using the following electrophoresis conditions: 16 sec injection time, 1.2 kV injection voltage, 15 kV run voltage, 60°C, 20 min run time, dye set G5. Using the 3130xl Genetic Analyzer, a 1.0 µl aliquot of the amplified product was added to 12.75 µl Hi-Di™ Formamide (AB) and 0.25 µl of GeneScan™ 500 LIZ® size standard (AB) using the following electrophoresis conditions: 18 sec injection time, 1.2 kV injection voltage, 15 kV run voltage, 60°C, 20 min run time, dye set G5. All samples were analyzed with GeneMapper® Software v4.0 or ID-X v1.1.1 (peak detection thresholds of 25 RFUs for the 3130 and 100 RFUs for the 3130xl).

*DNA-extraction, amplification, detection*

DNA was co-extracted with the AllPrep DNA/RNA Mini Kit (QIAGEN) and eluted in 80-100 µl EB buffer (supplied in the AllPrep kit). Five microliters of the DNA extract were amplified with the AmpFlSTR® SEfiler Plus™ PCR amplification kit (AB) in a total reaction volume of 25 µl on a GeneAmp®9700 PCR System (AB) according to the manufacturer's protocol. PCR products were detected with a 3130xl Genetic Analyzer (AB). One microliter of the amplified sample was added to 12.75 µl Hi-Di™ formamide and 0.25 µl of GeneScan™500 LIZ® size standard (all from AB). The following electrophoresis conditions were used for the 3130xl Genetic Analyzer: 10 sec injection time, 3 kV injection voltage, 15 kV run voltage, 60°C, 25 min run time, Dye Set G5. Raw data were analyzed with the GeneMapper® Software (AB) with a peak detection threshold of 50 RFUs.

## RNA – Enhanced

*Body fluid samples*

Single source touch samples were collected from worn clothing items, direct skin samples and touched/handled objects (e.g. inside shirt collar, fingertips, car steering wheel). To recover biological material from the touched objects and surfaces, WF Gel-Film® x8 retention level (Gel-Pak®, Hayward, CA), was cut to a size appropriate for subsequent attachment to a glass microscope slide support (3" x 1" x 1mm, Fisher Scientific, Suwanee, GA). Using sterile tweezers, the back protective covering was removed to expose the adhesive back and the Gel-Film® was placed onto a clean glass microscope slide. The top protective plastic film layer was then removed using re-sterilized tweezers. The Gel-Film® surface was then repeatedly touched to the sample area (direct skin, clothing or object surface) several times to ensure sufficient transfer of biological material. Saliva and semen-free vaginal secretions were collected using sterile cotton-tipped swabs from volunteers. The swab was then rolled onto WF Gel-Film® x8 retention level that was adhered to a glass microscope slide support. Saliva and vaginal secretions Gel-Film® samples were air-dried at room temperature. All samples were stained with Trypan Blue (0.4%) (Sigma-Aldrich) for 1 minute, then washed briefly by gentle flooding with sterile Millipore water. Samples were then air-dried at room temperature prior to proceeding to sample collection. All samples were stored at room temperature in microscope slide boxes protected from light.

For positive control samples for saliva and vaginal secretions, total RNA was extracted from sterile cotton-tipped swabs with guanidine isothiocyanate-phenol:chloroform and precipitated with isopropanol as described above. Human Skin total RNA (positive control) was obtained from commercial sources as listed above.

*Biological material collection and cell lysis*

Bio-particles and saliva/vaginal epithelial cells were collected from the Gel-Pak® surface using 3M™ water-soluble wave solder tape (5414 transparent) on the end of a tungsten needle. 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 under the stereomicroscope. The collected bio-particle/cell was then transferred into a sterile 0.2 ml PCR flat-cap tube (Phenix Research) containing 3.55 µl of a lysis buffer solution (Zygem RNAgem™ tissue kit (VWR). The solution was prepared for 10 µl: 1x buffer-silver, 5% RNAgem™ and nuclease free water. A 3.55µl aliquot was used for each reaction. Samples were lysed for 5 minutes at 75°C using an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies).

*cDNA synthesis*

A reverse transcription reaction was performed using the High Capacity Reverse Transcriptase reaction kit (Life Technologies). The 5 µl reaction consisted of the following: 3.55 µl cell lysate (see above), 0.5 µl RT buffer, 0.2 µl dNTPs, 0.5 µl random primers, 0.25 µl Multiscribe reverse transcriptase. The reverse transcription program was as follows: 25°C 10 min; 37°C 120 min, 85°C 5 min. All reverse transcription reactions were performed using an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A positive control (1 ng total RNA extract for each body fluid) and negative control (reverse transcription blank, nuclease free water in place of sample) were included with each reaction.

*mRNA biomarker pre-amplification*

cDNA samples (5 µl) were pre-amplified with unlabeled primers specific to body fluid specific biomarkers: touch bio-particles - LCE1C; saliva - HTN3 [24]; vaginal secretions -

MYOZ1 [26], IL19 [26], CYP2A6/7 and NOX01. The 25 µl pre-amplification reaction consisted of the following: 12.5 µl Multiplex PCR kit master mix (QIAGEN), 2.5 µl primer mix (1 µM), 5 µl cDNA (see above) and 5 µl nuclease free water. For the triplex pre-amplification, 10uM primer concentrations were used. The amplification program was as follows: 95°C 5 min; 14 cycles: 94°C 15 sec, 60°C 2 min. All amplifications were performed using an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A negative control (nuclease free water) was included with each reaction.

*Product Detection*

Amplified fragments were detected using High resolution melt (HRM) analysis. The 25 µl reaction consisted of the following: 12.5 µl Type-iT HRM master mix (QIAGEN), 1.75 µl unlabeled primer mix, 5 µl pre-amplified cDNA (see above) and 5 µl nuclease free water. For the singleplex HRM assays a 10 µM primer mix was used for HTN3, MYOZ1, IL19, CYP2A6/7, NOXO1 and a 40 µM primer mix was used for LCE1C. For the triplex HRM assay, the primer mix was prepared as follows: HTN3 10 µM (5 parts), LCE1C 40 µM (8 parts), NOXO1 10 µM (8 parts). All samples were analyzed on a Rotor-Gene Q real-time PCR cycler (QIAGEN). The amplification program was as follows: 95°C 5 min; 45 cycles: 95°C 10 sec, 57°C 40 sec, 72°C 20 sec; HRM 73°-90-98°C (higher Tm value used in initial testing and then reduced once the range of Tm values in the assay was determined). A positive control (3µl 25 ng body fluid cDNA (saliva, vaginal or skin)) and negative control (nuclease free water) were included with each reaction.

*DNA/RNA Co-Isolation (Cells/Bio-Particles)*

Bio-particles (skin) and saliva/vaginal epithelial cells were collected from the Gel-Pak® surface using 3M™ water-soluble wave solder tape (5414 transparent) on the end of a tungsten needle. 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 under the stereomicroscope. The collected bio-particle/cell was then transferred into a sterile 0.2 ml PCR flat-cap tube (Phenix Research) containing 5.1 µl of a lysis buffer solution (Zygem RNAgem™ tissue kit , VWR). The lysis buffer solution (10 µl) comprised : 1x buffer-silver, 5% RNAgem™ and nuclease free water, from which a 5.1 µl aliquot was used for each reaction. The solution was prepared for 10 µl: 1x buffer-silver, 5% RNAgem™ and nuclease free water. A 5.1 µl aliquot was used for each reaction. Samples were lysed for 5 minutes at 75°C using an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A 1.5µl aliquot of lysate was amplified using AmpFISTR® 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 an Applied Biosystems 9700 GeneAmp® PCR system thermal cycler (Life Technologies). A positive control (9947a) and negative control (sterile Millipore water) were included with each amplification. The remaining 3.55 µl of cell lysate was reverse transcribed as described above. All pre-amplification and product detection (HRM) reactions were performed as described above.

## III. RESULTS

### A. Statement of Results

#### *1. Project Goals (Specific Aims)*

**Aim 1. Characterization of biological material from touched objects**
*Aim 1A. Microscopic analysis of cellular micro-particles*
*Aim 1B.Characterization of non-particulate biological material on touched objects*
*Aim 1C. Development of optimized recovery strategies*

**Aim 2. Genetic profiling of recovered biological materials from touched objects**
*Aim 2A. Development of efficient DNA and RNA co-isolation methods*
*Aim 2B. RNA Profiling for the identification of tissue source origin*
Aim 2B (i). Identification of RNA biomarkers for the identification of skin
Aim 2B (ii). Standard reverse transcription and amplification strategies
Aim 2B (iii). Enhanced reverse transcription and amplification strategies
*Aim 2C. STR profiling to identify the donor(s) of biological material*
Aim 2C (i). Standard amplification strategies
Aim 2C (ii). Enhanced amplification strategies

The specific aims are provided here as an overview for the original project plan. The results section will cover the results obtained from all aims but are re-organized into various sections in better accord with scientific and experimental logic.

#### *2. Characterization of biological material from touch objects*

Frequently in the analysis of 'touch DNA' evidence, a preliminary examination of the evidence in order to identify the types and quantity of biological material present is not performed. This often results in the inability to determine the source of the recovered biological material and therefore the context or nature of the recovered evidence can often not be deduced. A major objective of the current work was to perform a comprehensive characterization of the biological cellular material present on touched objects which, in many cases, will presumably be skin. Epidermal desquamation is an active developmental regulatory process that requires neither friction nor grooming to produce the estimated 1000 cells/cm$^2$/hour (or $10^8$ cells per day per individual) that are shed [27]. The shed cells (otherwise known as squames or scales) will comprise a range of masses from single cells to sheets of agglomerated cells. Thus touch DNA evidence would be expected to comprise cells in various states of agglomeration. Additionally, a majority of the cells found in the outer epidermal layer of skin are dead or dying keratinocytes (i.e. corneocytes) [28]. During terminal differentiation of these cells, there is a degradation of the cell nucleus and possible nuclear material [28]. Therefore, the cellular material recovered from touched objects may contain non-nucleated cells. Without the presence of a nucleus, it will be difficult to determine if the recovered material is in fact cellular biological material using a

microscopic examination. Therefore we have referred to the potential biological material in the touch samples as "bio-particles" for this study rather than "cells".

Our examinations permitted the development of a "micro-particle atlas" that contains thousands of images of bio-particles recovered from various clothing items, household items and touched objects. These images permitted an evaluation of the morphological features of bio-particles in touch DNA evidence and allowed us to assess the type (e.g. single vs "clumps") and quantity of cellular material present. We are currently attempting to make this atlas available online as this could be a useful reference material for operational crime laboratories in support of their analysis of touch DNA evidence.

For the atlas, we obtained sample collection sets (Gel-film®, see section 3 for Gel-film® description and procedures) from 15 donors (9 females and 6 males). The collection sets included samples of: shirt sleeve, shirt collar, shirt back, shirt front, pants (upper thigh), pants (leg), sock (ankle), sock (bottom), underwear (front), underwear (back), pillow case (both sides of pillow), sheets, towel, hat and couch (some items not available for individual donor sets). All samples were collected from areas in direct contact with skin (e.g. inside surface of clothing items). For the items with multiple sampling locations (e.g. shirts, pants), we wanted to evaluate different areas of these items in order to determine if differences in bio-particle quantity would be observed. This may provide valuable information for investigators if these items are encountered in criminal investigations. It could provide an indication as to which garment location may result in the highest bio-particle recovery and therefore greater potential for STR profiling success (although this is not solely dependent on the number of particles present, but also by the quality of the bio-particles). From each of these samples, we viewed and imaged 10 random field of views (FOV). Collectively, this resulted in a total of 2,260 images. On each image, any likely or easily identifiable "bio-particles" were measured (length vs width, in μm). Total counts of single bio-particles or "clumps" or agglomerated cells were recorded for each sample. We also expanded the atlas to include samples from frequently used items that may be found at crime scenes, etc. These items included cell phones (front and back, 11 donors – 8 females and 3 males), computer mouse (10 – each from a different computer; some single primary users, some multiple primary users), door handles (10 locations – both business and residential), car steering wheels (10 – 8 female and 2 male primary users) and water bottles (10 – 8 female and 2 male donors; rim and side of bottle sampled). We also gave special attention to fingerprints and collected images from index fingers and thumbs from 10 donors (8 females and 2 males). Overall, the atlas contains over 3,000 images of the material recovered from touched objects, clothing items and fingerprints.

Figures 1-3 contain representative images from the various samples in the atlas (each panels shows one FOV for each item). Each labeled (length and width) item in the image was identified as a "bio-particle". On each image we also noted any potential or possible bio-particles, which looked like cellular material but were maybe much smaller or partial particles and therefore could not be more definitively identified as biological material. We also noted any non-biological material that was present. In general, the non-biological material consisted of fibers and hairs. We also classified bio-particles as single bio-particles or "clumps". It was not possible to determine how many bio-particles were present in the "clumps". Most bio-particles, whether single or clumped, seemed to have a "dehydrated appearance" (e.g. flat, textured). There were no significant bio-particle morphological differences between items/objects. We did notice on some items, like towels or sheets, some of the bio-particles appeared more "rolled" or "squished" which is probably a result of the type of contact with these items. A towel is rubbed

along skin surfaces and can therefore create some additional friction or force on bio-particles to cause changes to the morphology. Additionally, for items like socks and hats, we noticed more background material which can be possibly due to dirt or sweat which would be more common on these types of images. We also observed more partial or degraded bio-particle material which again can be a result of the different type (or extent) of contact with skin surfaces for these items. The images in Figures 1 – 3 were selected to provide a consolidated overview of the bio-particles observed in the range of atlas items/objects.

Since each bio-particle was measured, we were able to look at average length and width measurements for the bio-particles we identified, both singles and clumps. Figure 4 compares the average length and width of all bio-particles amongst all item/object types in the atlas. For the single bio-particles the average length and width was 33 and 34 μm, respectively. For the "clumps", the average length and width was larger at 65 and 64 μm, respectively. It was interesting to note that the average length and width measurements for each bio-particle class did not vary, indicating a somewhat symmetrical morphology which is not unexpected for cellular material. Figures 5, 6 and 7 show the average sizes for the individual items/objects in the database grouped into clothing (Figure 5), household (Figure 6) and touched object (Figure 7) sections. From these graphs, it is easy to see the somewhat similar size averages amongst the various items for both single bio-particles and clumps. As expected, greater variation can be observed for the clumped samples compared to the single bio-particles (larger standard error).

From the atlas images, we were also able to evaluate the number of bio-particles per 10 FOVs. Each FOV is approximately 450 μm (width) by 340 μm (height) which is an approximately 0.15 $mm^2$ area of Gel-film®. The full Gel-film® size that was used for sample collection is ~ 35 mm (length) by ~23 mm (width). Therefore the 10 FOVs that were sampled represent only 0.2% (or $1/500^{th}$) of the overall sample area. We utilized such a large piece of Gel-film® so that we would have plenty of biological material to work with in subsequent profiling experiments. However this area was too large to be sampled in its entirety which is why the 10 FOVs were used. These FOVs were randomly selected to not introduce any bias by selecting areas of higher concentration. The total bio-particle counts do not necessarily provide an accurate representation of the number of bio-particles per 0.15 $mm^2$ of the garment or object however since the Gel-film® surface was placed on the item surface multiple times (although each sampling was collected within very close proximity to one another and the number of contacts made with the item surface never exceed 3-4).

The total number of bio-particles (single and "clumps") for the ten FOVs were calculated and the average total number of bio-particles (N = 10 – 15 donors) per object or item type are shown in Figures 8 (clothing items), Figure 9 (household items) and Figure 10 (touched objects). For all items in the atlas, there were more single bio-particles observed compared to clumped bio-particles. The clothing items generally had higher average total bio-particle totals which are not unexpected as they are in contact with skin throughout the day whereas the touched objects would be in less frequent contact with skin. Overall, towel samples had the lowest average total bio-particle amounts.

For several items, multiple locations within the item were sampled so that comparisons could be made in terms of the total number of bio-particles observed. For example, we sampled the sleeve, front, back and collar area of a shirt. As can be seen from Figure 8, shirt back and collar had slightly higher average total bio-particles (single). However, overall a significant difference between the different areas of the shirt was not observed. For pants, we sampled the upper thigh area as well as the lower leg. Some pants are looser at the lower leg portions and we

thought there might be a difference in the number of bio-particles present compared to the upper thigh region which is typically in closer contact to skin. However, the opposite was observed with the higher average total bio-particles observed for the pant leg sample. Socks, ankle and bottom, had relatively similar bio-particle amounts as well underwear front and back samples. For cell phones, we sampled both the front and back areas. There is considerable variation in the way in which cell phones are handled by users which would affect the amount of bio-particles recovered from both sides. Cell phones fronts can be held up to a user's face which would transfer material and would also be the surface for dialing or texting which would also result in transfer of bio-particles. Although many cell phone users do not always make direct contact with their face, most users will make contact with the back of the cell phone as it would be held in their hand while speaking. Cell phones can be cleaned or placed into pockets which could potentially remove bio-particles from all surfaces. As can be seen from Figure 10, the back of the cell phone had ~2-fold increase in the number of single bio-particles compared to the cell phone front.

Overall, the micro-particle atlas has been in a valuable tool in our analysis of touch DNA evidence and the type and quantity of biological material present. We hope to make the atlas available online to serve as a useful reference material for those interested in the analysis of touch DNA evidence.

---

## ***3. Development of optimized recovery strategies***

A critical factor in the analysis of touch DNA evidence is the successful recovery of the trace biological material present. Collection of touch DNA evidence typically involves swabbing of the suspected area with a cotton swab. However, with certain types of objects swabbing may fail to recover biological material contained in grooves and crevices. When biological material is recovered with a swab, successful analysis of samples recovered in this manner will, depending upon whether used wet or dry, be influenced by the absorptivity and adsorptivity of the swab used and also the efficiency of release of the biological material. The potential for loss of sample is increased during the physical manipulations required to remove the absorbed or adsorbed material from the collection swab. Additionally, the use of generalized swabbing techniques often result in the recovery of a non-resolvable or challenging admixed DNA profile as a result of the failure to conduct a prior inspection of biological material that is present to determine if separate recovery of the particles could be achieved. If a mixture was present due to multiple-contributor handling of the object, the contact between the object and second donor may result in a significant loss of biological material from the first donor. If the small amount of material remaining from the original donor was not selectively isolated and is instead recovered in a generalized sampling of biological material, standard extraction and analysis techniques may fail to detect that minor profile.

In order to avoid the above described challenges of 'blind swabbing' strategies, our experimental schema for the recovery of bio-particles from 'touch DNA' involves the following [[29], adapted from [30] and personal communications with McCrone Associates, Inc. Westmont, IL]: 1) transfer of the biological material from touched objects and surfaces to a low-retention adhesive material, 2) microscopic examination of the recovered biological material, and 3) isolation of single or few bio-particles or cells (identified as cells at this stage only if a nucleus can be identified) using a water soluble adhesive. The use of the water soluble adhesive permits

the transfer of the recovered particles to a lysis buffer or directly into a PCR amplification reaction since the adhesive will dissolve thus releasing the particles into solution. The entire transfer process can be visualized under the microscope ensuring successful transfer of the recovered bio-particles for further analysis. All subsequent reactions (e.g. STR profiling) are performed in the same reaction tube thereby eliminating potential sample loss from additional manipulations or sample transfers. These reactions will be described in sections 4-7 below.

The low-retention adhesive material that we utilized is WF Gel-Film® (Gel-Pak®). According to the manufacturer, the "gel" is a highly cross-linked proprietary polymer material. It immobilizes objects onto its surface once contacted based on surface tension. The degree of holding force is intrinsic to the tackiness of the "gel" material, which is referred to as the retention level. There are various levels of retention levels available for the Gel-film® (X0, X4, X8). We originally began by using Gel-film® with X0 retention level in order to ensure successful removal of the collected particles for subsequent analysis. However, in order to ensure that we were achieving maximum particle recovery, we also evaluated the X4 and X8 Gel-film®. Using the X0 film, there were sufficient amounts of particles collected. However, there would often be entire fields of view which did not contain any particles. We started to see an increase in the amount of particles recovered using the X4 gel-film. We saw the greatest amount of particle recovery with the X8 gel-film. There was rarely a field of view that did not contain a significant number of particles.

With the selection of the proper retention level for the Gel-film®, the optimized protocol that we developed for sample collection using the Gel-film® was as follows:

*Without initial glass slide support (for use with curved objects or difficult surfaces)*[29]

1. Cut the Gel-film® to a desired size, ensuring that the size is appropriate for subsequent attachment to the glass microscope slide support.
2. Prior to removing the top protective plastic film layer from the Gel-film® surface, make a shallow cut along the Gel-film® to create an area for handling separate from the sample collection area.
3. Remove the top protective plastic layer from the sample collection portion of the Gel-film®. From this point on, only handle the Gel-film® in the designated handling area in order to avoid contamination of the sample collection area.
4. Place the Gel-film® surface in contact with the touched object of interest. Apply a small amount of pressure to ensure efficient collection of bio-particles. Multiple samplings from the same area of the touched item can be collected on the same piece of Gel-film® if desired. Avoid using a rubbing motion during collection as this may disturb bio-particles on the Gel-film®.
5. Once collection is complete, the Gel-film® should then be attached to a glass microscope slide support. Holding the Gel-film® only in the designated handling area, remove the back protective covering to expose the adhesive backing. Carefully place the Gel-film® onto a clean glass microscope slide and press down on the edges and corners with sterile tweezers to secure the Gel-film® on the glass slide.

*With initial glass slide support*[29]

1. Cut the Gel-film® to the desired size, ensuring that the size is appropriate for subsequent attachment to the glass microscope slide support.

2. Remove the back protective covering of the Gel-film® to expose the adhesive backing. Place the Gel-film® onto a clean glass microscope slide and press to secure the Gel-film® on the glass slide. The Gel-film® contains a top protective layer, so the entire surface of the Gel-film® can be pressed down onto the slide.
3. Once the Gel-film® is secure on the glass slide support, the top protective layer can be removed using sterile tweezers.
4. Place the Gel-film® surface in contact with the touched object of interest. Apply a small amount of pressure to ensure efficient collection of bio-particles. If too much pressure is applied, the glass slide could break. Multiple samplings from the same area of the touched item can be collected on the same piece of Gel-film® if desired. Avoid using a rubbing motion during collection as this may disturb bio-particles on the Gel-film®.

*Optional cell staining*[29]

1. Place the sample (Gel-film® containing recovered bio-particles from touched object on the glass slide support) onto a slide staining rack over a sink.
2. Cover the entire sample surface of the Gel-film® with trypan blue stain using a disposable transfer pipet.
3. Incubate at room temperature for 1 min.
4. Remove excess stain. Gentle flooding with sterile water can be used if needed.
5. Allow the samples to air dry before proceeding to sample preparation.

Once samples were transferred to Gel-film® (with or without staining), they were viewed using a high powered stereomicroscope (Leica M205C which has reportedly one of the highest resolutions available (1280x) for such a microscope and a high zoom ratio (20.5:1)). We originally were using epi-illumination on the stereomicroscope. However, we had some difficulty in visualizing the particles due to the greyish color of the Gel-film®. We therefore switch to a transmitted light base for the stereomicroscope. With the transmitted light base, we were better able to visualize the bio-particles. Using the Leica M205C stereomicroscope, we generally view the bio-particles using ~120x microscope magnification (which is reported as ~200x for images captured using the Leica Application Suite LAS v4.3 software).

In order to collect or isolate bio-particles from Gel-film® samples, the following procedure was used [29] (Figure 11):

1. Remove the appropriate number of 0.2 mL PCR tubes from their container and place them on a rack. Label the tubes appropriately.
2. Add appropriate lysis buffer or amplification mix to the 0.2mL PCR tube (development of our methods will be described in section 4). Cap each tube loosely or tightly if desired.
3. Place a piece of double-sided tape onto a clean glass microscope slide or directly on the glass block. This will be used to hold the 0.2 mL PCR tube in place during sample preparation. This slide should be prepared fresh for each collection.
4. Place a piece of double-side tape (or two side-by-side depending on the width of the tape) onto a clean glass microscope slide. Place a piece of the 3M™ water soluble wave solder tape on top of the double sided tape. This slide can be stored for future use. Place the first 0.2 mL PCR tube flat on its side and place on the double sided tape. Use gentle force to secure the tube on the double sided tape. Set this tube aside until the sample is collected.
5. Place the 3M™ water soluble wave solder tape slide on a glass block and view under the microscope (low magnification). Using the tip of the tungsten needle, lightly scrape the

surface of the tape in order to collect a small amount ("ball") of the adhesive material on the tip of the tungsten needle. The size of the "ball" can be increased if a larger number of bio-particles will be collected or multiple collections can be placed into the same collection tube.

6. Carefully remove the tungsten needle with adhesive from under the microscope. The needle can be placed in a rack (tip side up) if needed.
7. Place the sample (Gel-film® containing recovered bio-particles from touched object on the glass slide support) on a glass block and place on microscope stage. Adjust the focus and magnification until the bio-particles can be easily viewed. Determine which bio-particles will be collected (our recommendations for bio-particle selection will be provided later in the report).
8. Retrieve the tungsten needle with adhesive and bring over surface of the Gel-film® where the targeted bio-particles are located.
9. Place the tungsten needle with adhesive over the bio-particles of interest and press down so the adhesive is in contact with the bio-particles. Lift the needle up to ensure that the bio-particle has been removed from the Gel-film® surface. Repeat this process until the desired number of bio-particles has been collected.
10. Once the desired number of bio-particles has been collected, keep the tungsten needed in hand and replace the sample with the prepared 0.2 mL PCR tube on the microscope stage. Lower the magnification on the microscope so the bottom of the 0.2 mL tube containing the lysis buffer is in focus.
11. Carefully insert the tungsten needle into the 0.2 mL PCR tube, avoiding contact with the tube walls, until the needle is placed into the lysis buffer. Hold the needle in the lysis buffer until the adhesive dissolves and the bio-particles are released into solution.
12. Remove the 0.2 mL PCR tube from the double sided tape and place upright. Close the lid to the tube tightly. If necessary, briefly centrifuge the tube in a mini-centrifuge to collect all liquid at the bottom of the tube.
13. Clean the tungsten needle between samples.
14. Repeat steps 6- 12 until all samples have been collected.

This protocol has been highly successful throughout the study. The Gel-film® permits efficient bio-particle collection and bio-particles are easily removed (even with the X8 retention level Gel-film®) for subsequent analysis. However, during the course of this work we also evaluated an alternative adhesive material for bio-particle recovery – DIFF-Lift™ tape (Lynn Peavy Company). Diff-Lift™ tape is flexible so it can be molded to a variety of surfaces and is extremely adhesive. When a fingertip is placed directly onto the surface tape, it uniquely permits an evaluation of the fingerprint including the distribution of micro-particles throughout the fingerprint. Examples of the types of images (with and without trypan blue staining) that are obtained with this film are shown in Figure 12. As can be seen from these images, the ridge detail is clearly visible along with the presence of micro-particles along the edges of these ridges. The placement of a fingerprint directly onto the surface of the highly adhesive DIFF-Lift™ tape, while useful for characterization studies, may not represent a realistic view of the amount of bio-particles transferred in typical touch DNA samples. Touch samples are likely to contain fewer particles than what is observed for the direct fingerprint samples. However, the use of this tape does provide an opportunity to evaluate the nature and distribution of micro-particles in fingerprints.

Since the DIFF-lift™ tape was successful in the recovery of bio-particles from finger surfaces, we were hopeful that the DIFF-lift™ tape could be used for bio-particle recovery from objects. While we were able to collect bio-particles using the tape, the high adhesive strength of was too strong and we were unable to successfully remove particles from the DIFF-Lift™ tape and in many cases the water soluble adhesive was pulled from the needle used for collection. Accordingly we did not proceed with this method for bio-particle recovery

---

## ***4. STR profiling of isolated bio-particles – method development***

3.5 µl One-Step Lysis/STR Amplification

When biological material is recovered with a swab, successful analysis of samples recovered in this manner will, depending upon whether used wet or dry, be influenced by the absorptivity and adsorptivity of the swab used and also the efficiency of release of the biological material. The potential for loss of sample is increased during the physical manipulations required to remove the absorbed or adsorbed material from the collection swab. Additionally, the use of generalized swabbing techniques often result in the recovery of a non-resolvable or challenging admixed DNA profile. If a mixture was present due to multiple contributor handling of the object, the contact between the object and second donor may result in a significant loss of biological material from the first donor. If the small amount of material remaining from the original donor was not selectively isolated and is instead recovered in a generalized sampling of biological material, standard extraction and analysis techniques may fail to recover that minor profile. Therefore, a main aim of the current work was to develop more selective and efficient removal strategies for collection of cellular micro-particles present on touched objects.

We had originally planned to utilize the Advalytix AmpliGrid system for the micro-volume reactions. The Advaltyix AmpliGrid system is specifically designed for single cell molecular analysis workflows. The AmpliGrid slides contain 48 separate hydrophilic reaction sites surrounded by a hydrophobic ring which allows for reagents to remain in the proper position. Individual or cell "clusters" isolated using the micro-manipulation could be placed directly onto the AmpliGrid slides. The slides are then placed into a specially designed 'AmpliSpeed thermocycler' for PCR analysis. The flexibility of the AmpliGrid system allowed for the use of any lysis reagent in micro-volume quantities. Using this system we had initial success in developing a 1 µl STR amplification reaction (Identifiler, Life Technologies) using standard DNA extracts. However, we started obtaining inconsistent results with assays performed on this instrument using our previously successful amplification conditions. During a repair of the instrument, we were informed that the instrument and consumables were no longer manufactured. Since this was no longer a long-term viable option for casework use, we discontinued all studies using the Advalytix system.

Since the Advalytix system was no longer a feasible option, we wanted to continue to evaluate alternative micro-volume strategies for the analysis of bio-particles isolated from touch DNA evidence. We continued to study micro-volume PCR reactions using standard equipment (ABI 9700 thermal cyclers). Development of these amplifications on standard equipment would provide a possible protocol for operational crime laboratories to use without the requirement to purchase additional equipment. To begin our development of low volume PCR reactions, we initially amplified a small number of DNA samples (1ng input) using decreasing PCR reaction

volumes. We evaluated the use of 10 µl, 5µl, 2.5µl and 1µl reaction volumes for the Identifiler®, Identifiler® Plus and Yfiler® amplification kits. We utilized the amplification programs, including cycle number, recommended by the manufacturer (28 cycles for Identifiler®, 29 cycles for Identifiler® Plus and 30 cycles for Yfiler®) since we were using 1ng of input. We were able to obtain successful results using the 2.5 – 10µl reaction volumes. However, we had variable success with the 1µl Identifiler® amplifications. Initial experiments were performed using just the 1µl reactions without any kind of sealing solution over the reaction. We observed complete sample evaporation for several samples. The use of 1µl reaction volumes in standard thermal cyclers was therefore not ideal as it required the use of mineral oil to prevent sample evaporation which then also resulted in the need for post-PCR purification which ended up diluting the amplified product since it could not be eluted into the same 1µl volume. Therefore, after much optimization of these reactions, we determined that it was not suitable for use.

We next evaluated the use of additional micro-volume PCR reaction volumes (2.5 – 5µl) using 5 pg of input DNA (single cell equivalents) using both standard (28) and increased (36) cycle number. We evaluated profile recovery using each reaction volume, but also evaluated evaporation rates (volume remaining after amplification compared to starting volume) and average RFU values. The results from this experiment are summarized in Figure 13. Surprisingly, similar profile recovery values were obtained using standard and increased cycle numbers, indicating the potential improvement in sensitivity simply from using a micro-volume PCR reaction rather than having to increase cycle numbers. As can be seen from Figure 13, even with the increase to 2.5 µl reaction volumes the sample evaporation rate was high. We ultimately decided on the use of 3.5 µl reaction volumes based on profile recovery success rates, low evaporation rates and high RFU values.

We next needed to determine if these reactions would be suitable for use with isolated cells and bio-particles. Before using the reduced volume amplifications with bio-particles isolated from touch samples, we first evaluated their use with buccal epithelial cells (isolated using micro-manipulation). Unlike the reactions with extracted DNA, for use with cells we also had to consider the use of a direct PCR amplification reaction or whether a lysis step is needed prior to amplification. The cells can be placed directly in the amplification solution without the need for a prior lysis step or additional sample or tube manipulation. The 95°C hot start is likely sufficient to lyse cells. However, we also realized that the use of the lysis step may result in more efficient cell lysis. Our existing cell lysis protocol involved the use of the Zygem forensicGEM™ product. We have extensive experience with this lysis method and we have used it routinely for the analysis of micro-dissected epithelial cells with great success. In an attempt to simplify analysis but still include the lysis step, we realized that it might be possible to combine the lysis and amplification reactions into a single reaction. The current lysis protocol involves a 75°C step for 15 min followed by inactivation at 95°C. The Identifiler Plus amplification already contains a 95°C hot start, which could also be used to inactivate our lysis enzyme. Therefore, all we would need is an extra 75°C 15 min step in the beginning of the amplification in order to allow the lysis to be performed prior to amplification but without the need to stop and add the amplification reaction mix after completion. Taq Gold polymerase would not be active during this initial 75°C step since it is only activated with the 95°C hot start. We therefore evaluated the use of adding the lysis into the amplification protocol. We initially performed this combined reaction using standard reaction volumes to ensure that it would work before applying it to micro-volume reactions. We collected 1, 2, 5 and 10 buccal cells (0 cell sample used as control) and evaluated profile recovery using a direct PCR amplification (no lysis) and the combined lysis

and amplification reaction (Identifiler Plus, 34 cycles). The use of the combined lysis and amplification reaction resulted in significantly higher profile recovery rates (data not shown). We therefore continued to evaluate the use of this combined lysis/amplification reaction using micro-volume PCR reactions.

We evaluated the use of reduced volume combined lysis/amplification reactions. We evaluated 12.5, 6.25 and 3.5µl amplification reactions. We simply scaled down the lysis and amplification reactions to keep the same ratio of reagents for each reduced volume reaction. We again used 1, 2, 5 and 10 cells for analysis. The highest allele recovery percentage across all cell numbers was when the 3.5µl reaction volume was used (supporting the notion that reducing the sample reaction volume improves sensitivity and profile recovery) (Figure 14). There was some allele drop-in for the 0 cell sample using the 3.5µl reaction which is possible if there is cell-free DNA on the Gel-Pak surface where the "0 cell" sample is taken from. Therefore, at this stage in the analysis we were not concerned about the minor detection in the 0 cell sample. Based on the results of these initial experiments, we were reasonably confident that we had developed a robust and highly efficient reduced volume amplification reaction.

We confirmed the reproducibility of the 3.5µl combined lysis/amplification reaction. We collected 1, 2, 5 and 10 cell samples from four additional donors (total of five donors). We obtained successful (~25 – 100%) profile recovery from all five donors with an improvement in profile recovery for most cell numbers (see Figure 15). We then evaluated the use of the developed method with 1 cell samples since our ultimate goal is the application of this method to single or few bio-particles isolated from touch samples. We collected ten separate 1-buccal cell samples from different donors (4 male and 4 female donors) and analyzed them using the 3.5µl combined lysis/amplification reaction. The results are provided in Figure 16 and, in most cases (except for the M4 male donor), an improvement in profile recovery was observed when the 3.5µl amplification is used compared to the 25µl reaction. Profile recovery rates ranged from ~20-65%, which we considered successful results considering we were analyzing single buccal cells. In addition to the improvement in overall profile recovery, we also evaluated the average RFU values for the 1-cell samples using both a 3.5 and 5µl reaction volume. A significant increase in average RFU value was observed when the 3.5µl amplification was used (~4-fold increase, data not shown).

We used the 3.5µl combined lysis/amplification method to analyze bio-particles collected from touch samples. Since we were collecting bio-particles from touch samples, we also included an evaluation of individual particles as well as "clumps" to determine which particle type would result in the most successful profile recovery. Therefore, from each sample we examined 20 single particles as well as 20 clump samples (one clump collected per tube). We collected these particle sets from various worn clothing items including a sweater sleeve, a pant leg, a shirt collar, the back of a shirt (inside) and the waistband of a pair of shorts (elastic). We selected these items to have a range of fabric types and to evaluate profile recovery from various clothing items to determine if better results would be obtained with any of the clothing items. We also took images of each of the collected particles so that we could evaluate cell morphology of any of the samples from which profiles were obtained. For the individual particles, we made every attempt to identify and collect nucleated bio-particles as these would most likely result in the highest profile recovery. DNA profiles (mainly partial profiles) were recovered from only ~2-3 of the 20 cells/clumps for all of the items tested except for the shirt collar. A significant increase in the number of replicates in which profiles were obtained was observed for the shirt collar sample. For the shirt collar sample, DNA profiles (range of ~15–70% profile recovery)

were obtained for 9/20 individual particles (45%) and 4/20 clump particles (20%). The allele recovery results from the shirt collar sample are provided in Figure 17. Figure 18 shows an example of an individual particle (image of the collected particle is shown on the left) and the partial DNA profile obtained from it.

5 $\mu l^+$ One-Step Lysis/STR Amplification

The results of the experiments described above demonstrate the successful application of the developed 3.5µl combined lysis/amplification reaction to the analysis of bio-particles recovered from touch samples. However, we wanted to continue to optimize the developed method to still further improve profile recovery. When we were reviewing the obtained DNA profiles, we started to notice an increased amount of split peaks and often out of bin alleles that appeared to be resulting from an inefficient non-template addition (Taq (?) polymerase in the Identifiler Plus kit). This is not entirely unexpected since we are not only using increased amplification cycles, but we are using an extremely small amount of amplification reaction mix (only 1.4µl of amplification mix which contains the enzyme). We therefore decided to include an additional unit (U) of TaqGold to the amplification mix (3.5$\mu l^+$). We originally included the additional TaqGold to improve the efficiency of non-template addition, but we also thought that this may help improve profile recovery as well. We noticed a significant improvement in the number of individual and clump bio-particles from which a DNA profile (mainly partial profiles) were obtained as well as in the percent of profile recovery. We also decided to try a 5µl reaction volume so that slightly more amplification reaction mix could be used (additional kit enzyme) in addition to the extra TaqGold that was added to the reaction. We evaluated the use of the three reaction mixes with bio-particles (20 individual and 20 clumps) collected from the shirt collar. The best results were obtained using the modified 5µl (5$\mu l^+$) combined lysis/amplification reaction (modified = addition of 1U of Taq Gold) (from ~10% to 40% profile recovery, data not shown). We also compared the 5$\mu l^+$ lysis/amplification to standard reaction volume (25µl). There was a ~2-fold increase in profile recovery for single bio-particles and ~4-fold increase in profile recovery for clumps using the 5$\mu l^+$ lysis/amplification.

Since these initial comparison studies evaluated bio-particles from only one source (shirt collar), we evaluated the use of the 5$\mu l^+$ lysis/amplification with additional samples. We collected 20 single bio-particles and 20 clumps from samples collected from the elastic waistband of shorts, a shirt back and a sweater sleeve. This expanded the study to 80 single bio-particle samples and 80 clumps. We evaluated the original 3.5µl, 3.5$\mu l^+$ and 5$\mu l^+$ reactions. A comparison of the average bio-particle recovery amongst the three reactions is provided in Figure 19. With the additional testing, we continued to see an improvement in profile recovery with the 5$\mu l^+$ reaction. For individual particles there was an increase in profile recovery from 6% to 10% (highly probative profiles) using the 5$\mu l^+$ reaction. For clumped particles there was an increase in highly probative profiles from 7% to 15%, with an increase in the amount of full profiles obtained (now 10%, as compared to 1% with previous methods). Therefore, we were confident that the modified 5µl reaction was optimal for use with isolated bio-particles. In addition to the improvement in profile recovery, the use of the 5µl+ reaction also reduces the cost of analysis compared to standard methods (which even with the additional Taq Gold the cost is significantly less than standard reaction volume). Throughout our current work, our experiment design has been to collect 20 individual and 20 clumps from each item samples. This would not be possible with standard reaction volumes due to the high reagent cost.

## ***5. STR profiling of isolated bio-particles – application to touched/contacted objects***

With the successful development of the 5µl+ one-step lysis/amplification protocol, we proceeded to further evaluate our ability to obtain DNA profiles from bio-particles collected from a variety of sources. Our previous experiments were largely focused on bio-particles recovered from worn clothing items. Our next set of experiments focused on the following: 1) evaluation of representative examples of touched objects (chair armrest, car steering wheel, cell phone and coffee cup, cigarettes, pen); 2) evaluation of bio-particles collected directly from skin surface; 3) stained vs. unstained bio-particles; 4) evaluation of potential "cell-free" DNA and 5) evaluation of "fluid-like material".

Touched objects

We evaluated profile recovery from several touched objects including the armrest of a chair (located in a single occupant office), a coffee cup (middle section of the cup where it would be held by the donor), a cell phone (front) and a car steering wheel. For each item, we collected 20 single or individual bio-particle samples (1 bio-particle per tube/reaction x 20) and 20 "clumps" (total of ~160 samples in the study, 80 individual bio-particles and 80 clumps). The allele recovery for all of the samples for each object is provided in Figure 20 and the profile quality distributions are shown in Figure 21. The coffee cup and steering wheel samples had the highest number of individual samples with allele recovery (at least 10/20 replicates with allele recovery for both individual and clumps) as well as the highest number of probative and full profiles. While no profile was recovered for a majority of the chair armrest samples, both single and clumps, a full STR profile was obtained from one of the cell clump samples (Figure 22) and therefore the profile of donor could still be determined. Figure 22 shows the "clump" that was collected as well as the full profile obtained from it (profile accuracy confirmed by comparison to reference profile from sole habitual chair occupant). Even for the cell phone sample in which the least amount of profile recovery was observed, a highly probative profile with 25/30 alleles was obtained for one of the individual bio-particles. Therefore, overall a donor profile was obtained from all objects after cell capture and analysis.

Even though we developed this approach for use with touch DNA evidence, it is still expected to be a highly optimized method for the potential analysis of single or few human cells of any kind (e.g. buccal, vaginal, etc). Touch DNA evidence may also contain cellular material from other non-skin fluids and tissues and therefore any method designed for the analysis of touch bio-particles needs to also provide results for other cell types. The next two item types that we tested were specifically selected since they would have areas of likely buccal and touch contact: a cigarette and a pen (with evidence of "chewing" on the pen cap).

For the cigarette sample, we prepared a sample on Gel-film® from the end of the cigarette that would have been in contact with the mouth and also a separate one from the area that would likely have been contacted by the donor's fingers. During sample collection, we protected the area of the cigarette butt that we did not want to sample with paper to ensure that only material from the target area was sampled. We collected 20 single bio-particles and 20 clumps from both regions of the cigarette butt and the STR profiling results are shown in Figure 23. The mouth-contact region of the cigarette butt had a higher number of samples in which DNA profiles (partial or full) were obtained. More than 15 alleles were obtained in 10 out of the 20 clump samples and full profiles were obtained for 5 of the 20 clump samples. For the touch-contact

portion of the cigarette butt, the profile recovery was not as extensive (the number of bio-particles was greatly reduced for the overall sample in comparison to the mouth-contact area). However two of the individual bio-particle samples contained more than 20 alleles and would be highly probative profiles in terms of the random match probability. The ability to obtain this quality of profile from an individual cell or bio-particle further demonstrates the success of the developed collection and profiling strategies. A representative bio-particle and STR profile from each of the regions of the cigarette are shown in Figure 24.

Similar results were observed for the pen sample with a significant increase in profile recovery for the mouth-contact area (chewed pen cap) versus the touch-contact area (body of the pen). While it is expected that many of the "bio-particles" in the chewed pen cap sample will be buccal cells, the success of this experiment demonstrates the ability to obtain high quality and reproducible profiles from single and few cells or bio-particles. For the pen cap area, 75% of the single bio-particles or cells resulted in allele recovery (9 out of 20 with 20-30 alleles) and 100% of the clump samples resulted in allele recovery (17 out of 20 with full profiles). Figure 25 shows images of all 20 of the individual bio-particles/cells collected and the percentage of allele recovery obtained from each. As can be seen from these images, defined nuclei were present for many of the samples which is not typical (although occasionally observed) for touch/contact bio-particle samples. For the body of the pen sample, very little allele recovery was observed (1/20 individual bio-particles and 3/20 clumps, with 6 or less alleles observed in all samples). Profile recovery from touch samples will be variable and therefore not all samples will provide a probative profile. In these instances, additional individual or clump samples can be recovered and analyzed.

Direct Skin

We evaluated direct skin samples (i.e. Gel-film® touched direct to the skin surface). We sampled fingerprints from the thumb and index finger as well as samples from the forearm from six donors (three males and three females, although each donor provided only one of the direct skin samples). For each sample, we collected 20 single or individual bio-particles (1 bio-particle per tube, 20 samples) and 20 clumps (1 clump per tube, 20 samples). The results from this study are summarized in Figure 26. Overall, a greater number of profiles, including highly probative and full profiles, were obtained from the male samples in this small sample set. For the profiles obtained from single bio-particles, 15% were highly probative (21-29 alleles) with 9% exhibiting full profiles. For the profiles obtained from clumps, 27% were highly probative (5% full profiles). In comparison, for the female samples, partial profiles (1-20 alleles) only were obtained for 12% of the single bio-particles with none of the profiles possessing > 6 alleles. For the female clump samples, 8% of samples resulted in highly probative or full profiles (2% full profiles). For the female donors, the least amount of profile recovery was observed from the forearm sample. For the male donors, the highest number of full or highly probative samples was obtained for the thumb sample (50% highly probative, 15% full profiles). The index finger resulted in 10% highly probative profiles (5% full profiles) and the forearm sample resulted in 20% highly probative profiles (15% full profiles).

The increased success with profile recovery from the male samples might not be entirely unexpected as there are typically different personal grooming practices between the genders. Additionally, there is typically more body hair on males which may also help retain some of the shed skin cells. These are only speculative generalizations and no definitive conclusions regarding differences between males and females can be made without more extensive studies.

It is interesting to note that the bio-particles observed in direct skin samples tend to look different than those recovered from worn clothing or touched objects. In general for the clothing or touched objects, more individual or single bio-particles are observed. As can be seen from Figure 27, the direct skin samples, particularly the forearm sample, have more "sheets" or "skin rafts" than other samples.

Stained vs unstained

As mentioned above, larger "sheets" of bio-particles ("skin rafts") were observed from the direct skin samples, particularly for the forearm samples. A majority of the skin rafts did not appear to be stained by Trypan blue. Trypan blue is used to selectively stain 'dead' cells which in the case of touch samples indicates perforations, tears or breaks in the cell membrane to allow the stain to enter the cell. This of course also could mean that any nuclear material has "leaked" out of the cell. Therefore, since we noticed a lot of unstained material from the direct skin samples, we wanted to evaluate whether the unstained cells would result in higher allele recovery than the stained cells. For this study, we utilized a male thumb print sample (forearm was not used as the thumb print would be more representative of the type of skin surface that would be involved in the deposition of touch DNA evidence). Twenty stained and twenty unstained clumped bio-particles (1 clump per sample) were collected. Partial or probative DNA profiles were obtained in 85% the stained clump bio-particle samples (50% highly probative profiles, 15% full profiles) whereas partial profiles were obtained from only 35% of the unstained clump bio-particle samples with a majority of these profiles not highly probative (6/7 samples with 5 alleles or less). While this was only a single study, it did not appear that unstained cells would provide a greater chance of successful profile recovery. Therefore, we continued to focus on the collection of stained bio-particles throughout the rest of the study.

"Cell-free" DNA

While we demonstrated the presence of cellular material in touched object and clothing samples, there is a possibility that naked or non-cellular DNA is also present in associated secretions and fluids deposited during contact or from perforated or burst cells/bio-particles in which nuclear material will have "leaked" out. In blind-swabbing approaches this material would be collected without knowledge of whether it was from intact cells or cell-free DNA. In all experiments performed in this study, we have collected a "0" cell sample. A "0" cell sample means that the water soluble adhesive is touched to apparently empty or blank area of Gel-film® (i.e. does not contain any obvious cellular material). Throughout testing of the 5µl+ one-step lysis/amplification protocol, we had thirty-three "0" cell samples and evaluated these samples for possible detection of cell-free DNA. Amongst the 33 "0" cell samples, no alleles were observed for 27 of them (~82%). For five of the remaining six samples, only one allele was observed which is possibly due to allelic drop in (which was not frequently observed throughout the study, but was observed occasionally). The remaining sample had three alleles. This initial study did not suggest an abundance of cell free DNA present on the Gel-film® surface.

Since only one "0" cell sample was collected during each of these experiments, we wanted to perform further experiments with "0" cell samples to ensure that multiple samplings of blank areas from the same Gel-film® would not result in an increased number of alleles. We collected 20 "0" cell samples (to be consistent with the number of single and clump bio-particles we typically collect) from worn clothing samples: shirt back, sweater sleeve, shirt collar and shorts waistband (total N = 80). Seventy-eight percent (62/80) samples had no alleles detected.

For the remaining 18 samples, 14 of the 18 (78%) had only one allele present which again suggests allelic drop in rather than the presence of cell-free DNA. Three of the four remaining samples had three alleles present and the fourth sample had seven alleles (none of which matched the donor profile).

Collectively, 89 out of 113 (79%) "0" cell or blank samples had no alleles present. Due to the small number of alleles observed in the other 24 samples, the presence of cell free genomic DNA (at least in any significant quantity) was not indicated and therefore no further studies on cell free DNA were performed.

"Fluid-like" Material

A goal of this work was to determine if we could identify morphological or other bio-particle features that would result in a greater chance for the recovery of a probative DNA probative. Our primary collection tool was of course to look for nucleated cells first which would be expected to contain DNA in the nucleus. However, in many samples nucleated cells could not be identified. Failure to identify a defined nucleus does not necessarily mean that a bio-particle did not contain DNA. During the cell death process, the nuclear membrane will start to break up and a defined nucleus may not be visible. If nucleated bio-particles were not observed or if only observed in small number, we attempted to collect bio-particles that had a more structured appearance (appeared to have texture and depth) and did not appear completely flat and "deflated". However, throughout the course of the study, we noticed areas of material that appeared "fluid-like" (Figure 28). It is unclear what this material actually is, but it generally appears to look like an area of cellular material in which the outer cell membrane has broken down and the cell contents are still present in this "fluid-like" material. There will be varying stages of cell breakdown observed in these samples and we hypothesize that these areas are an intermediate stage after the cell membrane has broken down, but before the material is fully dispersed. Since these areas were not the typical bio-particles we had typically been seeing throughout our work, we decided to collect samples of the "fluid-like" material to determine if profiles would be obtained from it.

We collected "fluid-like" material from a male shirt collar, female fingertip, female forearm and a male neck sample. We collected ten samples from each item. The results from this study are provided in Figure 29. As can be seen from this graph, a significant number of samples resulted in profile recovery including full or highly probative profiles. For the male shirt collar sample, full profiles were obtained for 7 of the 10 samples collected. A representative "fluid-like" material sample (image and obtained profiles) from the shirt collar is provided in Figure 30. With the successful recovery of profiles, we included "fluid-like" material regions as an area of interest during sample collection as well.

***6. STR profiling of isolated bio-particles – identification of male donor in simulated physical assault mixtures***

All of the above described experiments involved sampling from individual objects with generally one primary user or donor. However, an advantage of the developed "smart" approach is to collect single bio-particles and reduce or eliminate the potential recovery of admixed DNA profiles. We therefore wanted to evaluate the use of our approach with simulated casework mixture samples. We selected three physical assault mixture scenarios with the ultimate goal of identifying male bio-particles (transferred to the victim during the assault) amongst an overwhelming amount of female (victim) cells. The three simulated physical assault mixture scenarios were: A) a male donor grabbing (forcefully) the shirtsleeve of a female donor (physical assault or restraint; skin-clothing contact); B) a male donor grabbing a female donor's wrist (physical assault or restraint; skin-skin contact); and C) a male donor grabbing a female's neck (choking/strangulation; skin-skin contact).

Prior to starting an analysis of these mixture samples with our developed approach, we wanted to initially evaluate the expected mixtures using standard analysis. For two of the mixture types (wrist grab and shirt sleeve grab), we had three sets of donors simulate the physical contact and then collected samples from known areas of contact between the two donors using standard swabbing of the contact areas using sterile distilled water cotton swabs. DNA was isolated from these samples using the QIAmp DNA Investigator kit (QIAGEN) and amplified (1 ng target) using Identifiler Plus (standard (28) cycle number). For the wrist grab samples, the following results were obtained: donor set 1 – only 7 alleles were observed (all less than 200 RFUs) with a majority belonging to the female donor; donor set 2 – only 3 alleles were observed (all less than 200 RFUs) and all belonged to the female donor; donor set 3 – a partial profile of the male donor was observed (16/30 alleles), although all alleles except for one were well below 100 RFUs and therefore would not likely be above the decision threshold for most laboratories. Overall then, the male donor was only identified in one of the three wrist grab samples using standard analysis and this profile would likely not be usable by most laboratories due to the low signal intensity. For the shirt sleeve samples, the following results were obtained: donor set 1 – admixed DNA profile (interpretable mixture), donor set 2 – only 4 alleles observed (~200 RFUs or below) (one from the male, one from the female and two shared) and donor set 3 – no profile obtained. Overall, amongst the six samples tested only one of them resulted in recovery of a reportable male donor profile and this was in an admixed DNA profile with significant loss of information content compared to a single source. We therefore next evaluated whether our developed approach (sample collection on Gel-film®, micro-manipulation to collect bio-particles and analysis with the one-step lysis/amplification protocol) would result in recovery of single source male DNA ("assailant") profiles.

Using our developed approach, we collected 20 single and 20 clumped bio-particles from each sample and typed them using our 5µl$^{+}$ reduced volume Identifiler Plus system (15 STR loci, 30 alleles possible). An overall summary of the number of bio-particles in which a DNA profile (partial or full) was obtained is provided in Figure 31 (A – single bio-particles, B – clumped bio-particles). For each mixture type, the three donor sets are shown and the number of bio-particles in which a profile of the male or female donor, an inconclusive profile (too few alleles to determine from which donor it originated) or no profile was obtained is displayed. As can be seen from this general overview, the male donor was successfully detected in at least one of the

bio-particles recovered from each of the mixture scenarios tested. For donor set 2 of the choking (neck grab) experiment, the male donor was detected in 13 and 15 of the 20 recovered samples (single and clumped, respectively). An admixed DNA profile was only observed for one sample (one clumped bio-particle sample from donor set 2 choking experiment) out of the 360 samples tested in this study. Each mixture scenario will be described in more detail below. This is extremely significant since one of the main goals for the developed methods is to be able to recover single source donor profiles by "smart" selection of individual and clumped bio-particles. Throughout the entire study, very few mixtures were observed even with clumped bio-particles. Therefore collection of single or clumped bio-particles should result in the recovery of single source DNA profiles for a majority of samples.

Skin-Clothing Contact (Shirt sleeve grab)

Allele recovery results from the skin-clothing mixture (male donor forcefully grabbing a female shirt sleeve; three donor sets) are provided in Figure 32. Interestingly, a reference-like profile (full or highly probative (> 25 alleles)) for the female wearer in each donor set was obtained. For donor set 1, a highly probative profile (23/30 alleles) of the male donor was obtained from a single bio-particle sample. Profiles that could be sourced to the male donor were detected in 3 of the 40 samples (20 single/20 clumps). For donor set 2, a probative profile of the male donor was obtained for one of the clumped bio-particle samples. Profiles that could be sourced to the male donor in the experiment were identified in 6 of the 40 samples. For donor set 3, highly probative profiles were obtained for three of the clumped bio-particle samples, including two full profiles. Profiles that could be sourced to the male donor in the experiment were obtained for 9 of the 40 samples. The male profile obtained from a single bio-particle from donor set 1 is shown in Figure 33 (image of the bio-particle collected is also shown). Even though a full profile was not obtained for this sample (23/30 alleles), it is clear from this profile that it would be highly probative and is of good quality considering it is from a profile obtained from a single bio-particle. Thus, the results of this experiment demonstrate the ability to identify single source bio-particles from the male assailant in this simulated casework experiment. No significant mixtures were obtained from the clump (or the single cell) samples.

Skin-Skin Contact (Wrist Grab)

For the skin-skin contact experiment (male donor grabbing a female's wrist), we had 3 donor sets (3 males and 3 females; different individuals, no overlapping donors between sets). Figure 34 shows the distribution of the donor source of the evaluated bio-particles (20 singles and 20 clumps) and the number of alleles obtained for each sample. For the single bio-particles collected (20 from each donor set, 60 total), 17% were identified as originating from the female donors, and 6% matched the male donors. For the clumped bio-particles, the male donor was identified in 15% of the samples and the female donor identified in 10% of the samples. For both the single and clumped samples, ~5% of the samples contained insufficient alleles for a source determination with only 1 or 2 alleles recovered. For donor set 1, a full male profile was obtained from a single bio-particle and a clumped bio-particle. A second clumped bio-particle had only one allele drop out and was nearly a full male profile (29/30 alleles). For donor set 2, a highly probative male profile (23/30 alleles) was obtained for one of the clumped bio-particles. For donor set 3, a full and a highly probative (29/30 alleles) profile was obtained from two of the clumped bio-particle samples. Therefore, we were again successful in obtaining a male donor profile from each of the wrist grab mixtures.

Skin-Skin Contact (Neck grab/Choking)

For an additional skin-skin contact mixture (neck grab, simulated choking/strangulation), we had three donor sets (3 male 'assailants' and 3 females; no overlapping donors between sets). Figure 35 shows the distribution of the donor source of the evaluated bio-particles (20 singles and 20 clumps) and the number of alleles obtained for each sample. For the single bio-particles collected (20 from each donor set, 60 total), 8% were identified as originating from the female donors (only identified in donor set 1), and 27% matched the male donors. For the clumped bio-particles, the male donor was identified in 27% of the samples and the female donor identified in 15% of the samples. For the single and clumped samples, ~5% and ~3%, respectively, of the samples contained insufficient alleles for a source determination with 5 or less alleles recovered. An admixed profile was obtained for one of the clumped bio-particle samples for donor 2. Male donor 2 transferred significantly more bio-particles than the other two donors. Thirteen out of 40 bio-particles gave single source male profiles with >20 alleles. Male donor 1 produced a male donor profile from a single cell with 16 alleles. With the exception of a single source partial male profile with 6 alleles, the third donor's DNA profile was not obtained from the simulated choking experiment. A representative male profile obtained from one of single bio-particle from donor set 2 is shown in Figure 36 (image of the bio-particle collected is also shown).

Skin-Bedding Contact (Simulated Sexual Assault Scenario)

We evaluated another type of mixture sample that possibly could be encountered in sexual assault cases which involved sampling of bedding (i.e. sheets) after a sexual assault. To simulate this mixture type, female volunteers slept on freshly washed bedding for one night and then a male donor was asked to make brief contact with the sheets (i.e. rolling around for a short period of time on the surface of the sheets) as would occur during a sexual assault. Gel-film® samples were then collected from the sheets. Twenty single and 20 clumped bio-particle samples were then collected (three donor sets; no overlapping donors between sets) in an attempt to again detect the male ("assailant") donor in this mixture type. The results of this experiment are summarized in Figure 37. For the single bio-particles collected (20 from each donor set, 60 total), 27% were identified as originating from the female donors, and 7% matched the male donors. For the clumped bio-particles, the male donor was identified in 35% of the samples and the female donor identified in 25% of the samples. For donor set 1 a full male profile was obtained from a single clumped bio-particle. For donor set 2, a probative 27 allele profile from the male donor was obtained. Donor set 3 was less successful although a 16 locus partial profile from the male donor was obtained from a single clumped bio-particle. For the single and clumped samples, ~3% and ~2%, respectively, of the samples contained insufficient alleles for a source determination with only ~1-2 alleles recovered. One mixture profile was observed for one of the single bio-particles for donor set 3 which is atypical since a single bio-particle was collected. However it is not possible to preclude the possibility that additional material was inadvertently collected. Admixed DNA profiles were obtained in only ~8% of the clump samples (5/60 samples). It is possible that a greater number of mixture samples (although still relatively small in comparison to the entire sample set) were obtained for this type of mixture due to additional movement around the bedding which may force bio-particles together into clumps. Even though a mixture was obtained in a few samples, a single source profile of the male donor was obtained for each mixture (whether by single or clumped bio-particle collection) demonstrating the successful use, and potential future application, of this method in forensic casework.

For all mixture scenarios, we collected 20 single and 20 clumped bio-particles. This was done for consistency between experiments. However, it is possible that rather than collecting this number of samples, one would simply collect as many samples as was desired with the potential to perform additional samplings as needed since there is typically a significant quantity of bio-particles on each Gel-film® sample.

---

## ***7. RNA Profiling for the identification of tissue source of origin of isolated bio-particles***

### a. Identification of RNA Biomarkers for the Identification of Skin[31]

*Identification of skin-specific gene candidates*

In order to identify potential skin-specific gene candidates, we performed literature searches and targeted 83 specific genes that are, or are likely to be, involved in skin development and/or regulation based upon an *a priori* understanding of skin physiological processes. The second approach to candidate identification was by means of deep sequencing of the skin cell transcriptome using a 'Next Generation Sequencing' platform (RNA-Seq). Whole transcriptome sequencing from another related external epithelial tissue type of forensic interest, namely vaginal secretions (VS), was also carried out for comparison purposes in an attempt to facilitate the finding of expressed genes in skin that are not present in VS. Fifty-four base pair (bp) paired-end sequencing was performed on total RNA isolated from vaginal secretions (two donors) and human skin (two individual donors). Sequencing data was obtained for all samples (average matched read counts of 17,000,000 for skin samples and 12,000,000 for vaginal samples). Sample data were mapped to the RefSeq human reference sequence assembly (GRCch37/hg19), which permitted the maximum count numbers for each of over 42,000 gene segments to be determined in each sample, thus indicating expression levels of each gene ('DGE' or digital gene expression). The maximum count values were then compared between the vaginal and skin samples in order to identify putative highly abundant and skin-specific candidates. In this manner 20 potential skin candidates were targeted for further investigation.

The 103 gene candidates identified using both approaches were screened by gel electrophoresis of singleplex end-point PCR products to provide an initial indication of transcript abundance and relative expression in a small panel of forensically relevant biological fluids and tissues (blood, semen, saliva, vaginal secretions, menstrual blood and skin) (data not shown). Candidates were rejected if: (i) no amplification products were obtained; or (ii) cross-reactivity with other non-skin body fluids was observed; or (iii) non-specific amplification products were present. Based on the results of this preliminary screening, five putative skin-specific candidates were identified including late cornified envelope genes 1C, 1D and 2D (LCE1C, LCE1D, LCE2D), interleukin 1 family member 7 (IL1F7, also known as IL37), and chemokine (c-c motif) ligand 27 (CCL27). Table 1 lists the primers, expected amplimer sizes, and the average RNA-Seq count values for each candidate in skin and vaginal samples. As can be seen from the average count values for each of the four samples (two vaginal, two skin), all candidates were found to be in higher abundance in skin, with significant lower count values observed in the vaginal samples. This digital gene expression data supported the initial screening results indicating a high degree of specificity for skin. The attributed biological functions of the five candidates are consistent with a high degree of skin specificity. Thus the late cornified envelope

(LCE) gene cluster is part of the epidermal differentiation complex and encodes for stratum-corneum proteins [32]. Studies have shown that the LCE cluster contains "groups" (1, 2 and 3) displaying differential expression with groups 1 and 2 dominant in skin (external epithelial) and down-regulated or undetectable in internal epithelial [32,33]. CCL27 is involved in the recruitment of CLA+ memory T cells to normal or inflamed skin. The most abundant expression of CCL27 is observed in keratinocytes of epidermal basal layers [34,35]. IL1F7 plays a predominant role in suppression of inflammatory responses and is synthesized by, inter alia, keratinocytes [36,37]. Multiple splice variants have been discovered for IL1F7, a-e [38]. Our primers were designed for isoform IL1F7b (also referred to as variant 1).

Subsequent to the initial screening, the five candidates were assayed using a laser-induced fluorescence-capillary electrophoresis detection platform, the latter of which was employed in all subsequent studies. Representative electropherograms from singleplex amplifications of each candidate are shown in Figure 38. During early testing of LCE1C, a product of the expected size (160 bp) was observed, but often a second co-amplification product (145 bp) was also obtained. The original primer set (designated LCE1C* in this report) was utilized for some studies before the primer set was re-designed in order to eliminate the appearance of this second product. Due to the large number of members in the LCE family, it is possible that an additional isoform was being amplified. A re-design of the reverse primer permitted an amplification of a significantly smaller sized product (58 bp), that was often accompanied by another product of unknown origin ~2 bases smaller (56 bp) than the expected product. The double peaks do not interfere with the interpretation of the results presented in this report but will be more fully characterized in subsequent work.

An initial preliminary evaluation of the skin specificity of the five biomarkers was carried out with a range of body fluids and skin. The results indicated that the biomarkers exhibited strong expression in skin samples and a high degree of specificity for skin. Varying amounts of input total RNA from each of the body fluids was used (~1 ng – 25 ng) along with some samples that had not been quantitated. No cross-reactivity with blood or semen was observed for any of the five candidates (4 – 10 donors tested per candidate). No cross-reactivity was observed for saliva or buccal swabs for LCE1C, LCE1D, LCE2D or CCL27 (10 - 13 donors tested per candidate). A minor IL1F7 peak (161 RFUs) was detected in one of eleven saliva/buccal samples tested. Cross-reactivity for a small number of samples was observed for vaginal secretions (LCE1C – 3/10 donors; LCE2D – 2/10 donors) and menstrual blood (LCE1C – 2/9 donors; LCE1D – 1/10 donors; LCE2D – 1/10 donors; IL1F7 – 1/6 donors). Despite the detection of some of the biomarkers in some non-skin samples, the intensity of the observed products was 10-45 times less than that of skin and many of these products were present in samples that had not had the extracted RNA quantitated.

*Sensitivity of skin biomarkers*

A forensically relevant assay should be evaluated for 'analytical sensitivity' using varying levels of input analyte to determine the assay's limit of detection (LOD) and dynamic range. In this paper we, like some others, refer to biomarker 'sensitivity' when really describing 'assay-specific sensitivity.' We are not measuring the absolute abundance of the biomarker in these sensitivity studies, since differently configured assays for the same biomarker may exhibit different LODs. The optimal input of total RNA for each of the five skin candidates was determined in singleplex assays using a range of input RNA (5 pg to 25 ng) and the results are shown in Table 2. The highest sensitivity was observed for LCE1C and CCL27 with detection

down to 0.005 ng (5 pg). While not all donors were detected at this input level, the gene product signal intensity for those that were detected (>500 RFUs for approximately half of the donors tested) was significantly above the signal to noise ratio. IL1F7 was also detected down to 5 pg of input total RNA but with slightly reduced signal intensities than that observed for LCE1C and CCL27 at this input level. LCE2D was detected in more than half of the 100 pg samples (8/13), with only sporadic detection of a few donors with less input. LCE1D proved to be the least sensitive marker, requiring 5 ng of input for detection in the majority of samples. Overall, the high sensitivity of most of the candidates indicated their potential suitability for use in the analysis of 'touch DNA' samples.

*Confirmation of the specificity of the skin biomarkers using optimal input amounts*

The sensitivity testing indicated that significantly higher input RNA amounts than what was optimal for each candidate was used in the preliminary specificity testing described above. Therefore, additional specificity testing was performed for each of the five candidates using a more optimal and realistic input amount, given their potential utility for touch samples (LCE1C - 25 pg; LCE2D, CCL27, IL1F7 - 250 pg; LCE1D - 5 ng). As can be seen from Table 3, a majority of all skin samples were detected at this input whereas no detection was observed in any of the other body fluid samples. This confirmed the high degree of skin specificity of each of the candidates and also further demonstrated the need to use appropriate input amounts of RNA in order to maintain this level of specificity. In addition to the body fluid samples, the expression of each candidate was evaluated in a panel of twenty human tissues (Table 3). Significantly, no cross-reactivity was observed for a majority of tissues. A minor weak product (52 RFUs) was observed in kidney tissue for IL1F7 and a stronger CCL27 product (769 RFUs) was observed in thyroid tissue. However, these tissues are unlikely to be encountered at crime scenes and therefore are not likely to interfere with the identification of skin in forensic analyses.

*Detection of skin biomarkers in swabs of human skin and touched objects*

The expression of the five skin markers was evaluated in a variety of extraneous human skin swabs or scrapings of human skin (e.g. forearm, knee, palm of hand, back of hand, face, sole of foot, leg, forehead, arm, wrist, finger, and neck) as well as touched objects from the indoor environment (e.g. mouse, telephone, coffee pot, keyboard, gloves, pencils, car keys and scissors). Each marker was detected in human skin samples and touched object samples (Figure 39), although not in all samples tested (~50% success rate) and expression of only one or two markers in each individual sample was often observed (Figure 39). Although co-expression of all five markers was not observed for any of the samples tested, some did express three or four of the markers. Significantly, the most sensitive marker (LCE1C) was detected in 100% of an additional set of samples tested (N = 12), which included swabs of human skin (e.g. cheek, arm, leg and forehead) as well as touched objects (e.g pencil, mouse, phone, door handle). The significantly higher sensitivity and rate of detection in touch samples with LCE1C was promising. However, it needed to be demonstrated that this ultra-high sensitivity did not inadvertently result in "false positive" results (i.e. cross reactivity with non-skin touched samples). Thus locations within our laboratory presumed to be negative for skin contact were swabbed and tested for LCE1C expression. Thirteen areas were tested with negative results except for two areas that, upon further investigation, could indeed have been touched (data not shown).

*Multiplex RT-PCR assays for skin identification*

Since not all of the skin biomarkers are detected in every 'touch DNA' sample, such samples will need to be analyzed using a panel of skin biomarkers. It will not be ideal, then, in terms of time or cost or in the interests of sample consumption, to use the singleplex assays described up to this point. Accordingly we developed two multiplex amplification systems that permit the simultaneous analysis of four (tetraplex, Figure 40A) or five skin markers (pentaplex, Figure 40B), respectively. The tetraplex incorporated the use of an alternative high fidelity polymerase (Advantage HD® polymerase (Clontech)). LCE1C could not be included in this multiplex system due to technical difficulties. A pentaplex system incorporating all five of the skin biomarkers was developed using a commercially available multiplex PCR kit (QIAGEN Multiplex PCR kit) (Figure 40B). The sensitivity of both multiplexes was evaluated using a range of input total skin RNA from different sources (Table 4). All four markers were detected by the tetraplex with 100 pg or more of RNA in a majority of samples, with all markers being strongly detected in all samples using 1 ng or more. While a smaller number of samples were tested for each input amount with the pentaplex, it is clear from Table 4 that sensitivity was improved since several markers were detected with as little as 25 pg. The more sensitive pentaplex system may be better suited for use with *bona fide* casework 'touch DNA' samples and will be the subject of a subsequent detailed validation study that will be reported elsewhere.

**b. Development of Optimized Reverse Transcription and Amplification Strategies**

A goal of this project was to be able to collect individual or small numbers of 'bio-particles' from touched objects, determine the tissue source of origin and obtain a genetic profile of the donor of the biological material. With the success of the micro-volume reactions developed for DNA, we also wanted to evaluate micro-volume RNA reactions to determine if the tissue source of origin could be obtained from isolated epithelial cells and bio-particles isolated from touch DNA samples. As described above, we identified a skin biomarker, LCE1C, which performed extremely well with touch DNA samples. Our initial experiments with the use of reduced volume reverse transcription and body fluid multiplex amplifications using total RNA (standard extracts) demonstrated an improvement in profiling success with the reduced volume amplifications when compared to standard reaction volumes (performed using total RNA extracts not cells; data not shown). This was evidenced by improved signal intensity and also sensitivity (biomarkers detected with lower total RNA input amounts) with the reduced volume reactions. After optimization of both reduced volume reactions, it was determined that the most suitable reactions were 5µl for both the reverse transcription reaction and the body fluid multiplex amplification.

However, despite the success of the micro-volume RNA reactions, our prior extensive experience with RNA, including RNA profiling from small numbers of epithelial cells, indicated that enhanced strategies for increased sensitivity to permit identification of the tissue source of origin of small numbers of cells would be needed. During our literature survey we came across a suggested pre-amplification protocol using the QIAGEN Multiplex PCR kit (from the manufacturer). While not specifically designed for use with isolated cells, we believed this would be an attractive approach to try with our isolated bio-particles. We therefore developed a short pre-amplification reaction using 14 amplification cycles and unlabeled LCE1C primers (the same primers that would be used in subsequent detection assays). We initially tested the success of this pre-amplification method using serially diluted touch particles (counted under the microscope using a hemacytometer and serially diluted) that had been extracted with standard

RNA extraction methods. Without prior pre-amplification, LCE1C was not detected in any of these samples. However, with the developed pre-amplification step, LCE1C was detected in all samples, which was very encouraging

With the development of a pre-amplification method, we employed this approach to bio-particles collected from touch samples using our developed micro-manipulation protocol. We collected 10, 25, 50, 100 and 0 bio-particle samples from various clothing items including a sweater sleeve, a sock, a shirt collar, a shirt (back side) and a lab coat sleeve. All of our previous work involved the use of extracted RNA. With the micro-manipulation approach we typically perform a direct lysis reaction or direct PCR reaction. We have had previous success with DNA recovery from bio-particles using direct PCR amplifications and therefore we decided to try a short lysis step prior to reverse transcription to quickly lyse the cells. This step needed to be short since we did not want to damage the RNA during the exposure to high temperatures for longer periods of time. The reverse transcription reaction includes a 3-minute incubation at 75$^{o}$C for standard RNA samples, so we decided to utilize this step for possible cell lysis. After this incubation the reduced volume RT reaction was performed. The entire 5µl RT product was then added to the pre-amplification reaction (25µl). A 5µl aliquot of the pre-amplification reaction was then used in the standard body fluid identification multiplex (the standard reaction volume was used to allow for an increased amount of the pre-amplification product to be used if needed). Using this approach we were able to successfully detect LCE1C in a few 10 and 25 bio-particle samples (Figure 41). However, LCE1C was also detected in a few of the 0 cell control samples. It is possible that bio-particles were inadvertently collected in these samples or that the pre-amplification reaction was too sensitive and would result in false positive results. Since not all bio-particle samples were positive, we did not believe that the assay was too sensitive and that false positive results would necessarily be a continued issue.

Although we wished to further evaluate the use of this approach with isolated bio-particles, we also wanted to try to improve RNA profiling success. Initial experiments involved the use of 10-100 individual bio-particles. For the next set of experiments, we decided to evaluate bio-particle "clumps" or larger masses of cells to determine if we could achieve greater profiling success since a larger number of bio-particles would be used. We frequently observe clumps of bio-particles and therefore we know they are available for collection for most samples. We collected 1, 5, 10 and 20 bio-particle "clumps" and repeated the above-described method involving the LCE1C pre-amplification. We collected these samples from a shirt collar. Using this approach, we were able to successfully detect LCE1C in all samples (1-20 clumps). This was the first time we had achieved 100% profiling success with bio-particles recovered from touch samples (no detection of LCE1C in the 0 cell sample). The electropherograms for each of the clump samples showing LCE1C detection are provided in Figure 42.

The analysis described thus far included the use of a CE-based body fluid multiplex for detection. However, in separate work we had begun to develop mRNA profiling with high resolution melt (HRM) assays for a determination of the body fluid or tissue source of origin. HRM is a rapid and relatively cheap bio-analytical method. As its name suggests, it is a technique that permits the identification of specific PCR products (i.e. amplicons) by their melting temperature ($T_m$). An amplicon's precise melting temperature is dependent upon its sequence, length and the ionic strength of its environment and can be measured post-PCR to within 0.1$^{o}$C with specialized software and hardware. HRM assays require only the use of unlabeled PCR primers and a single saturating intercalating fluorescent dye (e.g. Eva Green). After amplification the amplicon is melted slowly by increasing the temperature and a loss of

fluorescence occurs as the DNA strands separate and release the bound saturating dye into solution. By measuring the negative first derivative of fluorescence (F) with respect to temperature (T) (-dF/dT) a distinct and characteristic melt curve is obtained with the peak maximum representing the $T_m$. Importantly, due to the high resolution nature of the amplicon melt analysis, it is possible to perform multiplex analysis of several amplicons in one tube. With the successful developed of highly sensitive HRM assays for body fluid identification, we performed a singleplex LCE1C HRM assay using another aliquot of the pre-amplified clump samples. LCE1C was again detected in each of the clump samples (data not shown). Since these assays demonstrated suitable sensitivity and required only the use of unlabeled PCR primers, all further analysis was performed using HRM analysis. Additionally, in separate work we had found that the use of an RNA lysis buffer (*RNA*GEM lysis) improved analysis of buccal epithelial cells. We therefore further modified our existing protocol to include the use of the *RNA*GEM lysis buffer rather than a simple heat lysis step. Unlike the DNA micro-volume reactions in which the lysis and amplification could be performed in the same reaction (the STR amplification begins with a 95$^o$C step which simultaneously inactivates the lysis enzyme), the RNA lysis had to be performed in a separate step prior to the start of the micro-volume RT reaction. The RNA lysis step is a simple 5 min incubation at 75$^o$C. However, the RT enzyme is essentially inactivated at 85$^o$C and therefore we felt that combining the lysis and RT reactions would affect the efficiency of the RT enzyme. Bio-particles are collected into the lysis reaction (3.55 µl) and then once the 5 min step is completed, the RT reaction mix (1.45 µl) is added and the RT reaction is performed.

Although the initial experiment was successful with detection in all clumped bio-particles, we wanted to evaluate the sensitivity of the assay for the detection of LCE1C in single or few bio-particles. We made minor modifications to the cell lysis times as well as the number of pre-amplification cycles in order to improve sensitivity. We included clump bio-particles in all optimization experiments in order to ensure that any protocol modifications would work with both types of bio-particles (single versus clumped). Using the modified protocol, we were able to detect LCE1C (identification of the skin source of origin) in 25 and 50 single bio-particles (Figure 43) recovered from a shirt collar. LCE1C was also successfully detected in and 1, 5, 10, and 20 clumped bio-particles from the same shirt (Figure 43).

With the successful analysis of the bio-particles recovered from the shirt collar sample, we next wanted to analyze additional touch DNA samples to determine if the skin source of origin could be determined. We collected bio-particles (10, 25 and 50 single particles as well as 1, 5, 10 and 20 "clumps") from a chair armrest, a door handle, a coffee cup (side of the cup that would be gripped by the donor's hand) and a car steering wheel. All samples were collected in the RNA lysis buffer, lysed, reverse transcribed using the micro-volume RT reaction, pre-amplified using a singleplex LCE1C reaction and analyzed with the singleplex LCE1C HRM assay. Although not all samples were positive, we were able to successfully detect the skin source of origin for several samples from each of the touched objects (ex: coffee cup – 50 single bio-particles; chair arm rest – 10, 25 and 50 single bio-particles and 10 clumps; door handle – 25 and 50 single bio-particles, 10 clumps; steering wheel – 20 clumps) (Figure 44, armrest). While we fully recognize that further validation of this method will be required as these were preliminary experiments involving a smaller number of samples, the results demonstrate that we can successfully isolate RNA from bio-particles and utilize HRM assays for a determination of the source of origin of the bio-particles. Future work will focus on the number of bio-particles needed for successful analysis.

While we had successfully demonstrated in preliminary experiments that we could determine the skin source of origin of bio-particles in touched objects and clothing samples, skin may not be the only possible tissue/fluid source of origin of 'particles' recovered from these types of items. Therefore, it would be ideal to develop an assay that would permit the identification of the common epithelial cell containing fluids and tissues, namely skin, saliva and vaginal secretions. We have previously successfully developed multiplex HRM assays, including an epithelial cell triplex assay [39]. However, new biomarkers have been identified since the development of the initial triplex assay and therefore we decided to modify this assay to include some of the newer more specific biomarkers. Before we could begin the development of the triplex assay, we needed to ensure that individual saliva and vaginal biomarkers were suitable for use with isolated cells (previously tested with whole sample extracts not individual cells). For saliva, we evaluated HTN3 and STATH as potential saliva biomarkers using singleplex HRM assays. We collected 10, 25, 50 and 100 buccal cells and performed our developed micro-volume RT and pre-amplification reactions. HTN3 was successfully detected in the 25, 50 and 100 cell samples with an expected Tm of ~78ºC (data not shown). For the STATH singleplex, STATH was not detected in any of the cell samples (data not shown). A saliva RNA extract made using standard RNA extraction methods was used as a positive control (25ng) and had a peak at the expected Tm of 80.9ºC. The lack of detection of STATH may be due to a sensitivity limitation for this biomarker (i.e not highly abundant in cellular fractions).

For vaginal cells, we evaluated the use of vaginal biomarkers MYOZ1, IL19, CYP2A6/7 and NOX01 (CYP2A6/7 and NOXO1 are new vaginal biomarkers that we have identified in separate work; will be reported in a separate publication). We again collected cells using the same protocol described for touch bio-particles and saliva cells. Initial testing showed that the biomarkers MYOZ1, IL19 and NOX01 were detectable in cell preparations of 10, 25, 50 and 100, while CYP2A6/7 had irregularly shaped and low peaks for the 50 and 100 cell counts (data not shown). Subsequent testing with reduced cell counts (1, 2 and 5 cells) showed IL19 and NOX01 were detectable down to a single cell, but MYOZ1 was not observed in less than 10 cells (data not shown). The melt temperature of the IL19 amplicon product was close to that of LCE1C (~81.5ºC for IL19 and ~82 ºC for LCE1C) and therefore it would not be suitable to be incorporated into a multiplex reaction that contains LCE1C. We therefore decided to select NOX01 as the vaginal secretion cell biomarker for the HRM triplex assay. The triplex assay now contains biomarkers for the identification of skin (LCE1C), saliva (HTN3) and vaginal secretions (NOXO1). The triplex assay requires the use of a triplex pre-amplification step as well. We are still in the preliminary stages of evaluating the triplex assay (including the triplex pre-amplification) for use with isolated cells. Initial testing included the analysis of skin (10, 25 and 50 single bio-particles and 1, 5, 10 and 20 "clumps"), saliva (10, 25 and 50 buccal cells) and vaginal secretions (10, 25 and 50 cells). Using this assay, skin was correctly identified in the 50 single and 1, 5, 10 and 20 "clump" samples (5/7 samples positive). Saliva was only detected in the 10 cell sample. Vaginal secretions was detected in the 10, 25 and 50 cell samples. A representative melt plot for each of the body fluid types (vaginal – 10 cells, saliva – 10 cells, skin – 1 "clump) using this assay is shown in Figure 45. While work needs to be done to improve detection success for buccal cells (saliva) and resolve issues with HTN3 detection in 0 cell and negative control samples (data not shown), these initial results demonstrate the potential success in the identification of the fluid/tissue source of origin of isolated epithelial cells. We will continue to optimize and validate the triplex assay to improve typing success.

**c. DNA/RNA Co-Isolation from Bio-Particles and Epithelial Cells**

The above experiments describe the successful identification of the tissue source of origin from epithelial cells including, uniquely, an identification of skin as the tissue source of origin of bio-particles recovered from touch DNA evidence. However, the developed approach is limited to RNA analysis only and would not include the potential to simultaneously recover an STR profile of the cell donor. This is not ideal as separate sampling of bio-particles for DNA and RNA analysis would not definitely identify the source of origin of the same bio-particles used for STR profiling. Therefore, we ultimately wanted to evaluate potential co-extraction methods in order to obtain an STR profile of the bio-particle donor as well the tissue source of origin for the same sample. We performed some preliminary experiments with various methods for co-isolation of DNA and RNA from bio-particles recovered from touch DNA evidence. We had existing highly optimized co-extraction methods that were developed for use with forensic samples: in-house manual organic extraction and the QIAGEN AllPrep DNA/RNA Mini kit. We performed some initial testing using these methods; however these were with touch DNA evidence samples collected using a "blind-swabbing" approach which is not the "smart" approach we were aiming to use in this study. Therefore, we didn't feel that these methods would be the most suitable in the existing form. We also made attempts to develop strategies for cell type identification prior to bio-particle isolation using fluorescently labeled probes that bind along a target mRNA sequence in order to produce sufficient signal to be detected using fluorescence microscopy (data not shown). This is still an area that we are actively evaluating, but during the time frame of this project we were not able to successfully develop this method for touch DNA evidence. With the successful development of the individual DNA and RNA micro-volume reactions, we wanted to continue to evaluate these methods to determine if we could potentially develop a micro-volume co-extraction method. We envisioned the co-extraction to be a simple division of the micro-volume lysate into DNA and RNA analysis pipelines. The limitation with this approach would be the small amount of biological material going to each analysis pipeline. However, we observed suitable sensitivities of each individual method in initial testing and were therefore hopeful that a co-extraction method would be possible. The DNA and RNA lysis buffers were different and therefore we needed to evaluate the use of the DNA- or RNA-specific lysis buffers to see which was more compatible for the recovery of both DNA and RNA. After initial experiments to test the use of both lysis buffers (data not shown), it was decided that the *RNA*GEM lysis buffer would be suitable for to co-extraction. In the separate DNA and RNA profiling assays, 3.55 µl of lysate was used for RNA and 1.5 µl of lysate was used for the DNA assays. Since this was sufficient for the separate assays, we wanted to keep the same volumes of lysate for the co-extraction. Therefore, cells were collected in 5.1 µl of lysis buffer and following an initial lysis for 5 min at 75$^{o}$C, 3.55 µl of lysate was used for subsequent RNA profiling assays and 1.5 µl of lysate was used for subsequent DNA profiling. Since these starting volumes were the same, no further modifications to the existing DNA or RNA profiling methods were necessary. Since this was initial testing and optimization of the triplex epithelial cell HRM assay was not complete, we utilize singleplex HRM assays for tissue source identification (RNA). For the initial experiments, we evaluated 1, 2, 5, 10, 25 and 50 cells for saliva and vaginal secretions and for skin (male shirt collar sample) we evaluated 10, 25 and 50 single bio-particles and 1, 5, 10 and 20 clumps. The results from the initial experiments are shown in Figure 46. For saliva, DNA profiles were obtained for all samples tested, with probative profiles obtained for all samples except the 1 cell sample. Saliva was correctly identified in the 5, 25 and 50 cell samples. Therefore, while there was not 100%

success in terms of both DNA and RNA profiling for all samples, we were able to for the first time demonstrate the simultaneous ability to obtain a tissue source of origin and a probative (even full) profile of the cell donor! This was demonstrated with the 5, 25 and 50 cell samples. Similar results were obtained for the vaginal secretions samples. DNA profiles were obtained for all samples, including full profiles for the 25 and 50 cell samples. The 2 and 5 cell samples contained over 20 alleles (highly probative) and >10 alleles were recovered from the 1 cell sample. The presence of vaginal secretions was successfully identified in almost all samples, with the exception of the 1 and 5 cell samples. For the 2, 10, 25 and 50 cell samples, we again demonstrated the ability to determine the tissue source of origin and obtain an STR profile of the donor from the same sample. For the touch samples, the results were even more surprising. Highly probative STR profiles were obtained from all samples with no fewer than 15 alleles recovered, with full profiles or >20 alleles obtained for a majority of samples. LCE1C (i.e. skin) was correctly identified in 4 of the samples: 25 and 50 single bio-particles as well as 5 and 20 "clumps". Therefore, overall from small numbers of isolated buccal, vaginal and skin cells, we were able to successfully determine the source as well as the STR profile of the cell donor from an individual sample. While this was only a preliminary study, these results were extremely promising and accomplished a major goal of the current study. We were able to utilize a "smart" approach to the isolation of bio-particles (i.e. visual and carefully select the bio-particles for analysis) and then perform a micro-volume co-isolation to determine the tissue source of origin (RNA profiling) and the STR profile of the sample donor (DNA profiling) from the same bio-particle sample. Additionally, there is potential for increased sensitivity and profiling success with further optimization.

## B. TABLES

### Table 1. Skin-Specific Gene Characteristics

| Gene | Accession # | Primers (5'-3') | Size (bp) | RNA-seq – Max Count Values* | | | |
|---|---|---|---|---|---|---|---|
| | | | | Skin-1 | Skin-2 | Vag-1 | Vag-2 |
| LCE1C | NM_178351 | F: GCTGAAGGACCCTGTGCT<br>R: CAGGACATCTTGGTGGCG | 56/58 | 842 | 1703 | 8 | 51 |
| LCE1D | NM_178352 | F: CCTGTGCTGCCTGTGACT<br>R: GGCACTTAGGGGGACATTTA | 142 | 166 | 102 | 1 | 2 |
| LCE2D | NM_178430 | F: TCTGTGCTTTTGCATGTGAC<br>R: GGACCACAGCAGGAAGAGAC | 193 | 194 | 428 | 90 | 127 |
| CCL27 | NM_006664 | F: AGCACTGCCTGCTGTACTCA<br>R: TTCAGCCCATTTTCCTTAGC | 254 | 964 | 1897 | 0 | 7 |
| IL1F7 | NM_173203 | F: CCAGTGCTGCTTAGAAGACC<br>R: TCACCTTTGGACTTGTGTGAA | 92 | 93 | 421 | 0 | 2 |

*Max count values for each donor (1 and 2) are an average of two replicates

### Table 2. Skin biomarker sensitivity

Shading indicates the average RFUs obtained for each biomarker (dark grey >1000 RFUs; medium grey 500-999 RFUs; light grey 51-499 RFUs; white <50 RFUs). The number of samples in which the biomarker was detected out of the total number of samples tested is indicated. Average RFU values were determined using only the samples in which the marker was detected.

| RNA input (ng) | Average Signal Intensity (RFU) | | | | |
|---|---|---|---|---|---|
| | LCE1C | LCE1D | LCE2D | CCL27 | IL1F7 |
| 25 | NT | 7/8 | 6/6 | 6/6 | 6/6 |
| 10 | NT | 13/15 | 6/6 | 6/6 | 6/6 |
| 5 | NT | 12/15 | 6/6 | 6/6 | 6/6 |
| 1 | NT | 4/8 | 6/6 | 6/6 | 6/6 |
| 0.5 | NT | 2/8 | 6/6 | 6/6 | 6/6 |
| 0.25 | 10/11 | 1/8 | 10/13 | 6/6 | 13/13 |
| 0.1 | 9/11 | 1/8 | 8/13 | 12/13 | 12/13 |
| 0.05 | 9/11 | 0/8 | 1/13 | 6/13 | 12/13 |
| 0.025 | 10/11 | 0/8 | 2/13 | 5/13 | 11/13 |
| 0.015 | 5/7 | 0/8 | 0/13 | 5/13 | 10/13 |
| 0.005 | 4/7 | 0/8 | 1/13 | 7/13 | 7/13 |

NT = not tested

**Table 3. Skin biomarker specificity**

Shading indicates the average RFUs obtained for each biomarker (dark grey >1000 RFUs; medium grey 500-999 RFUs; light grey 51-499 RFUs; white <50 RFUs). The number of samples in which the biomarker was detected out of the total number of samples tested is indicated.

| Body Fluid | LCE1C | LCE1D | LCE2D | CCL27 | IL1F7 |
|---|---|---|---|---|---|
| | 25 pg | 5 ng | 250 pg | 250 pg | 250 pg |
| Skin | 10/11 | 12/15 | 10/13 | 6/6 | 13/13 |
| Blood | 0/4 | 0/5 | 0/4 | 0/4 | 0/4 |
| Semen | 0/4 | 0/5 | 0/4 | 0/4 | 0/4 |
| Saliva/Buccal | 0/4 | 0/5 | 0/4 | 0/4 | 0/4 |
| Vaginal Secretions | 0/4 | 0/5 | 0/4 | 0/4 | 0/4 |
| Menstrual Blood | 0/4 | 0/5 | 0/4 | 0/4 | 0/4 |
| Tissue | LCE1C | LCE1D | LCE2D | CCL27 | IL1F7 |
| | ~1 ng | ~1 ng | ~1 ng | ~1 ng | ~1 ng |
| Adipose | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Bladder | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Brain | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Cervix | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Colon | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Esophagus | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Heart | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Liver | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Lung | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Kidney | 0/1 | 0/1 | 0/1 | 0/1 | 1/1 |
| Placenta | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Prostate | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Ovary | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Skeletal Muscle | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Small Intestine | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Spleen | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Testes | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Thymus | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |
| Thyroid | 0/1 | 0/1 | 0/1 | 1/1 | 0/1 |
| Trachea | 0/1 | 0/1 | 0/1 | 0/1 | 0/1 |

**Table 4. Sensitivity of the tetraplex and pentaplex skin assays**

Shading indicates the average RFUs obtained for each biomarker in the multiplex system (dark grey >1000 RFUs; medium grey 500-999 RFUs; light grey 51-499 RFUs; white <50 RFUs). The number of RNA samples in which the biomarker was detected out of the total number of RNA samples tested (from different individuals) is indicated. Average RFU values were determined using only the samples in which the marker was detected.

| | Tetraplex | | | | Pentaplex | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Input (ng) | CCL27 | LCE2D | IL1F7 | LCE1D | LCE1C | IL1F7 | LCE1D | LCE2D | CCL27 |
| 25 | 4/4 | 4/4 | 4/4 | 4/4 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 10 | 4/4 | 4/4 | 4/4 | 4/4 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 5 | 4/4 | 4/4 | 4/4 | 4/4 | 2/2 | 2/2 | 2/2 | 2/2 | 2/2 |
| 1 | 4/4 | 4/4 | 4/4 | 4/4 | 5/5 | 5/5 | 4/5 | 5/5 | 4/5 |
| 0.5 | 4/4 | | | 3/4 | 5/5 | 4/5 | 5/5 | 5/5 | 4/5 |
| 0.25 | | | | 9/11 | 5/5 | | 4/5 | 5/5 | 4/5 |
| 0.1 | | | | 8/11 | 5/5 | 2/5 | | 3/5 | 3/5 |
| 0.05 | | | | 2/11 | 5/5 | | 0/5 | | 3/5 |
| 0.025 | | | 1/11 | 3/11 | 5/5 | 0/5 | 0/5 | | |
| 0.015 | 0/4 | 0/4 | 0/4 | 0/4 | NT | NT | NT | NT | NT |
| 0.005 | 0/4 | 0/4 | 0/4 | 0/4 | NT | NT | NT | NT | NT |

NT = not tested

## C. FIGURES

**Figure 1. Bio-Particle Images from Touch Atlas.** Bio-particles recovered from various worn clothing items, touched/handled objects and household items, and direct skin were imaged and characterized. Single and 'clumped' bio-particles were measured using a Leica M205C stereomicroscope to determine typical size and level of presence on different items. A) shirt collar, B) pant leg, C) steering wheel, D) cell phone (back), E) fingerprint (thumb) and F) fingerprint (index finger)

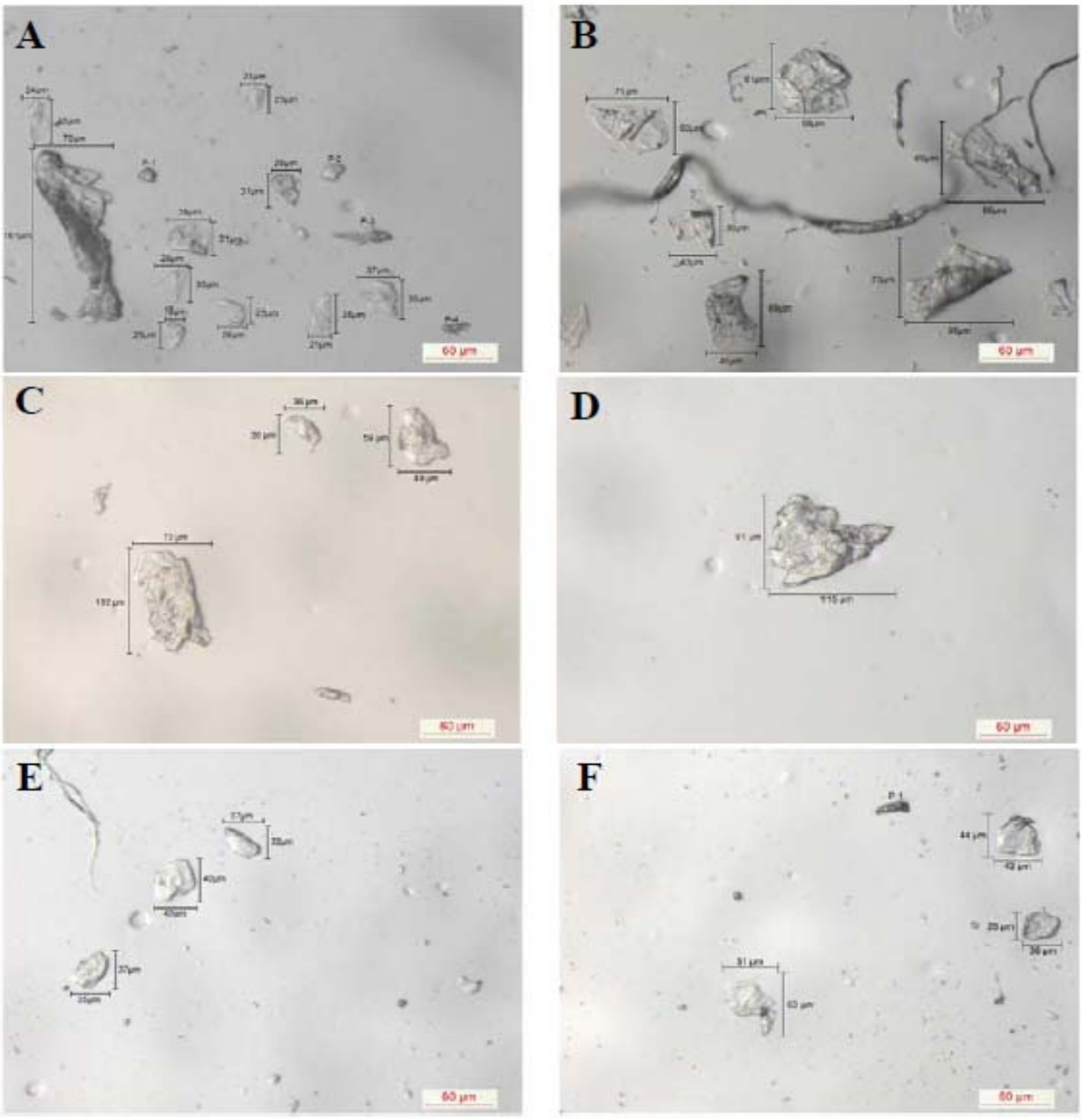


**Figure 2. Bio-Particle Images from Touch Atlas.** Bio-particles recovered from various worn clothing items, touched/handled objects and household items, and direct skin were imaged and characterized. Single and 'clumped' bio-particles were measured using a Leica M205C stereomicroscope to determine typical size and level of presence on different items. A) couch, B) pillow case, C) door handle, D) sock (ankle), E) underwear (back) and F) sheets.

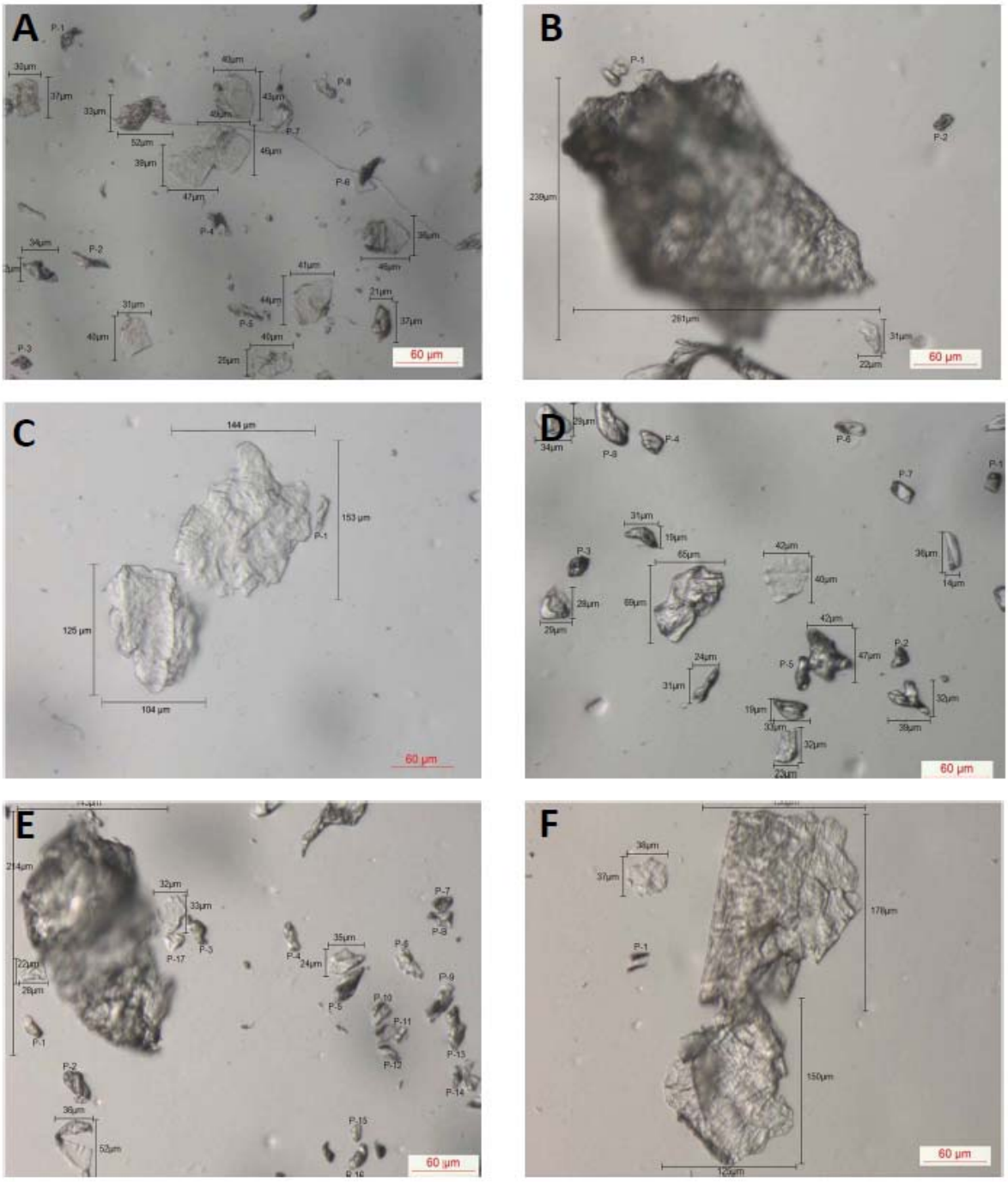


**Figure 3. Bio-Particle Images from Touch Atlas.** Bio-particles recovered from various worn clothing items, touched/handled objects and household items, and direct skin were imaged and characterized. Single and 'clumped' bio-particles were measured using a Leica M205C stereomicroscope to determine typical size and level of presence on different items. A) water bottle, B) computer mouse, C) towel and D) hat (rim).

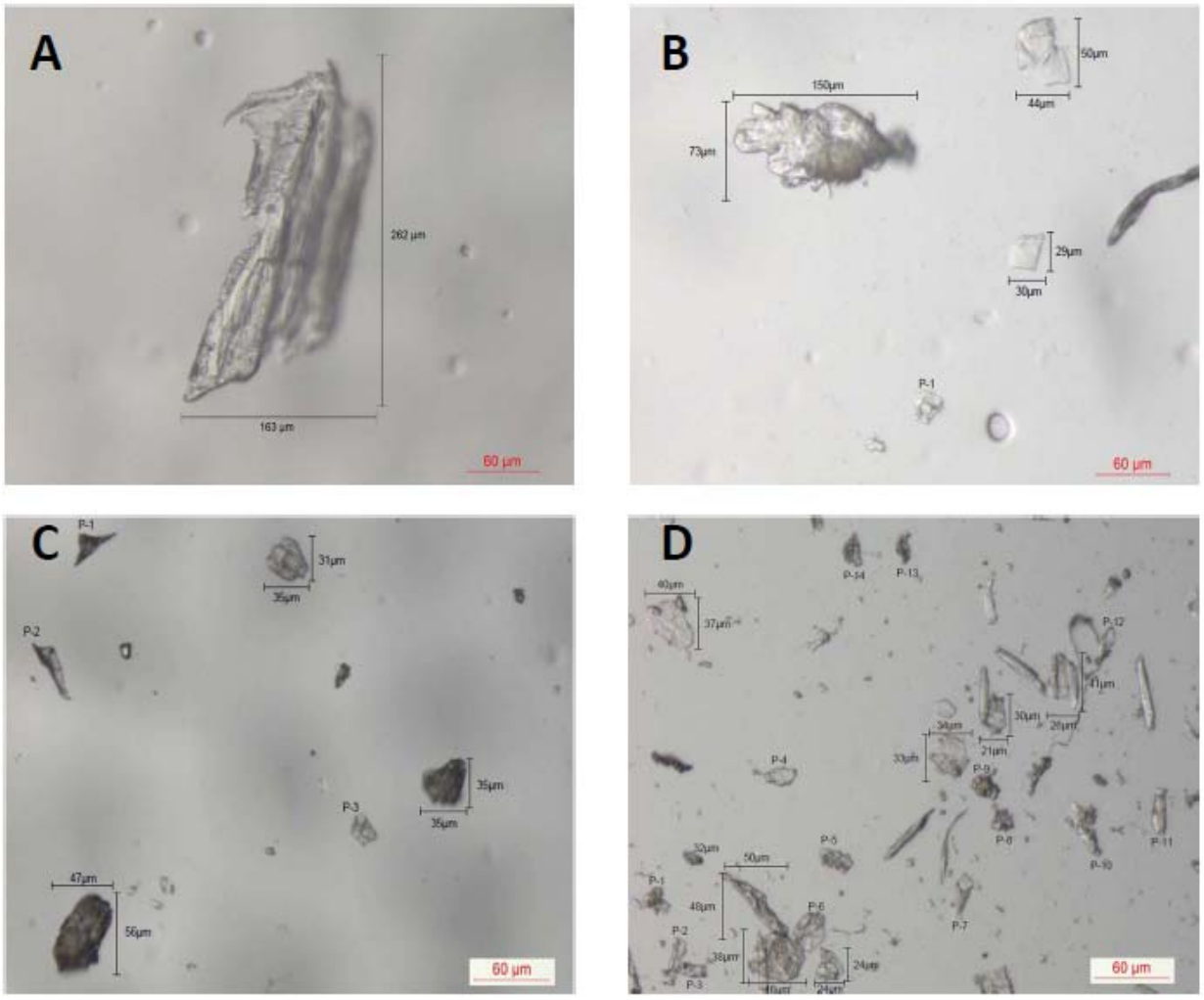


**Figure 4. Average Bio-Particle Size (Length/Width) for all Item/Object Types.** Each set of two bars represents length and width measurements. N = 25 (overall average of the average length/width from 25 different items (clothing, household and touched objects). Error bars represent standard error.

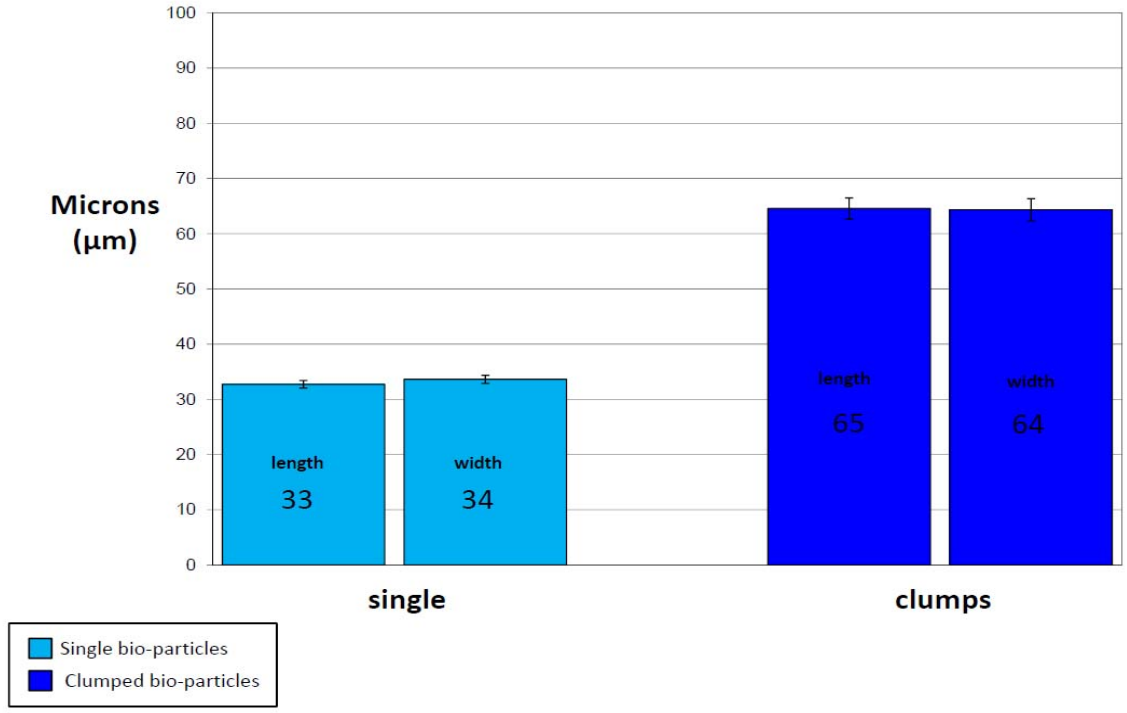
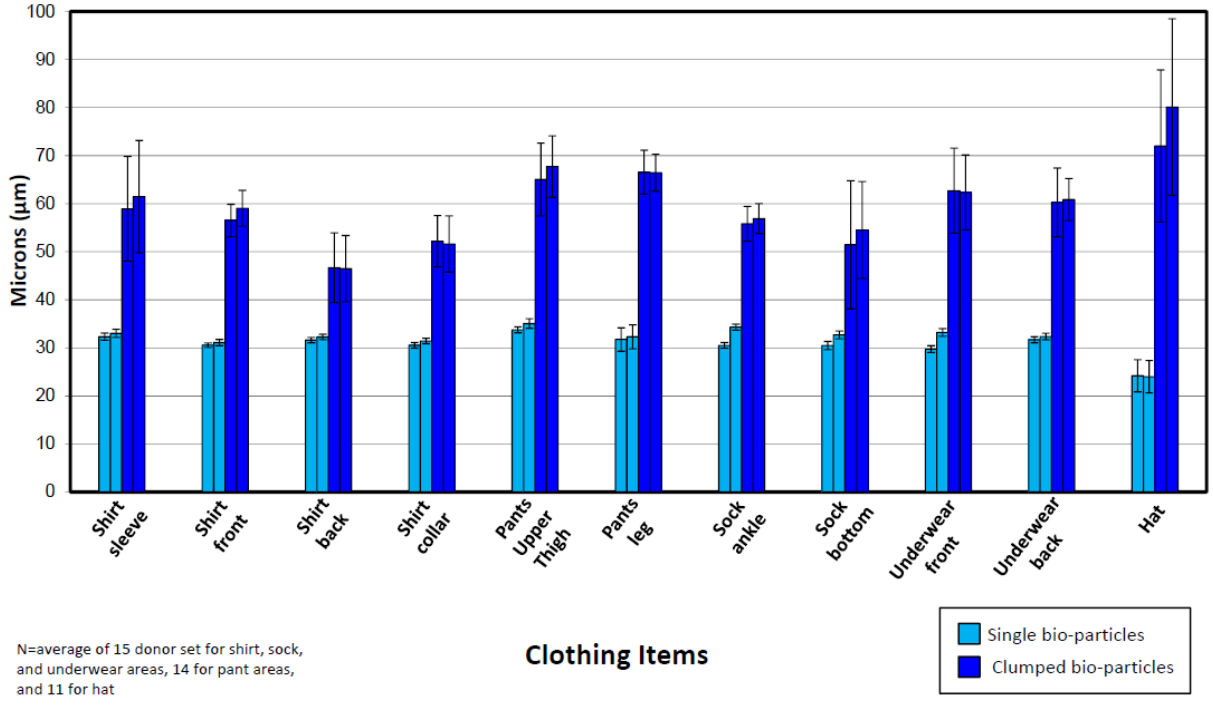



**Figure 5. Average Bio-Particle Size (Length/Width) for Clothing items.** Each set of two bars represents length and width measurements. Error bars represent standard error.






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 6. Average Bio-Particle Size (Length/Width) for all Household Items.** Each set of two bars represents length and width measurements. Error bars represent standard error.

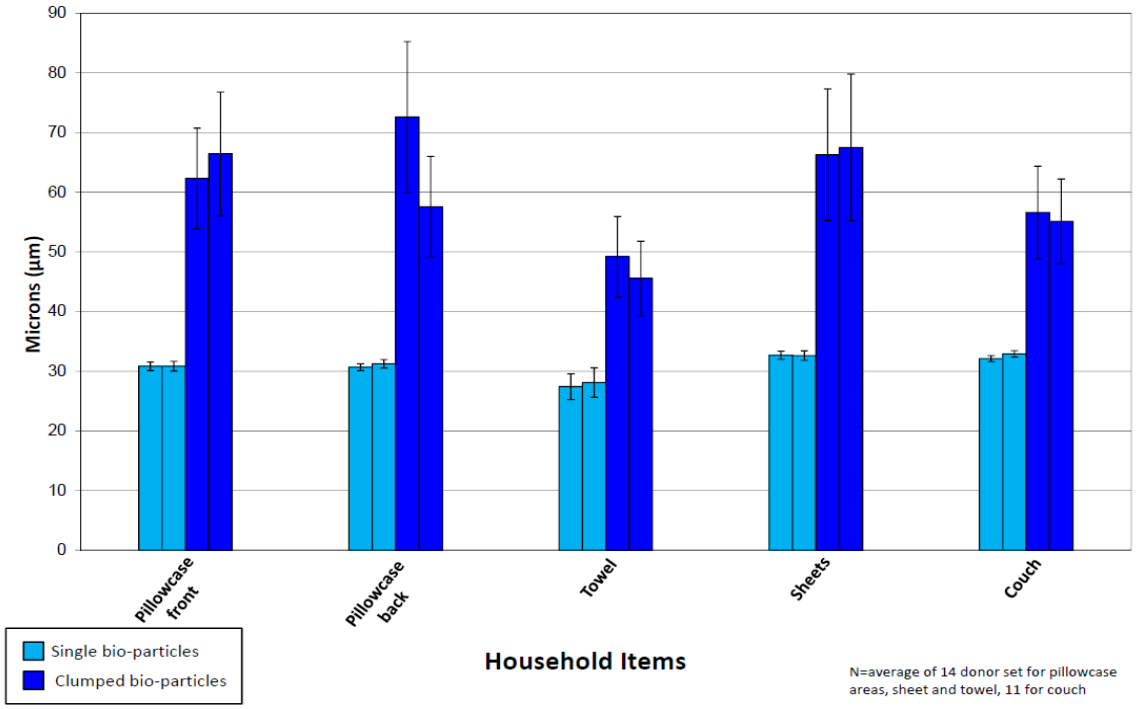
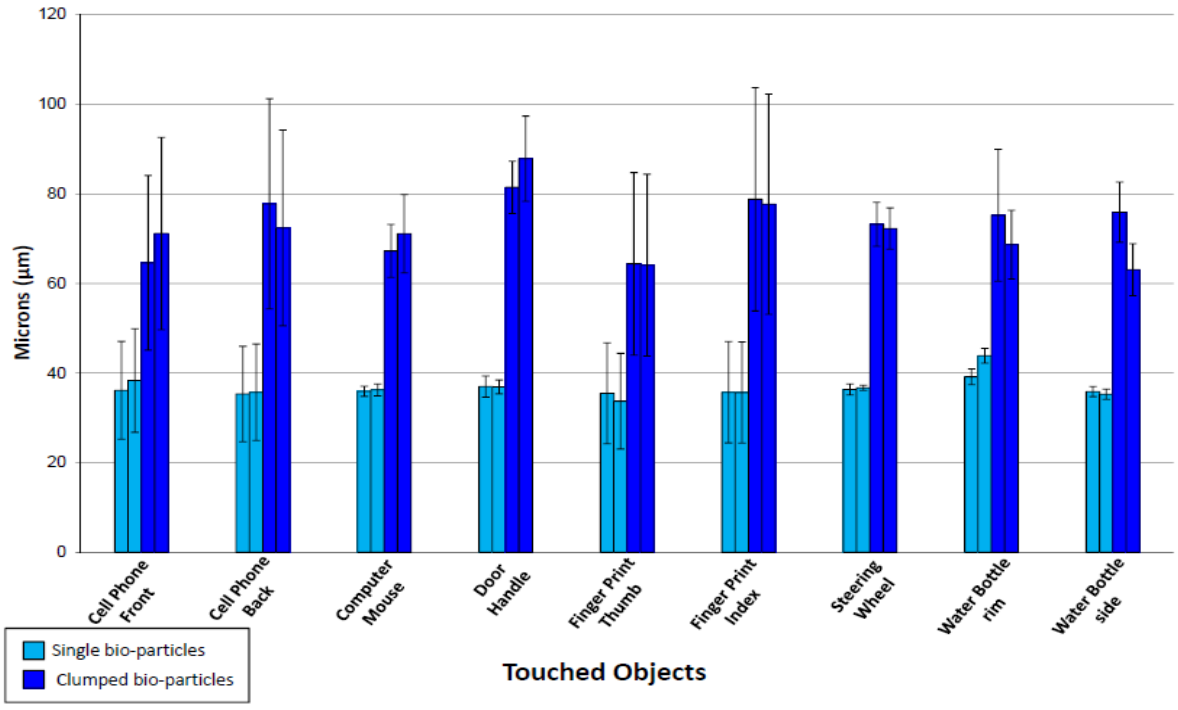



**Figure 7. Average Bio-Particle Size (Length/Width) for Touched Objects and Fingerprints.** Each set of two bars represents length and width measurements. Error bars represent standard error.






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 8. Average Number of Bio-particles for Clothing Items.**
Error bars represent standard error. N = 15 donor sets for shirt, sock and underwear areas; N = 14 donor sets for pants; N = 11 donor sets for hat

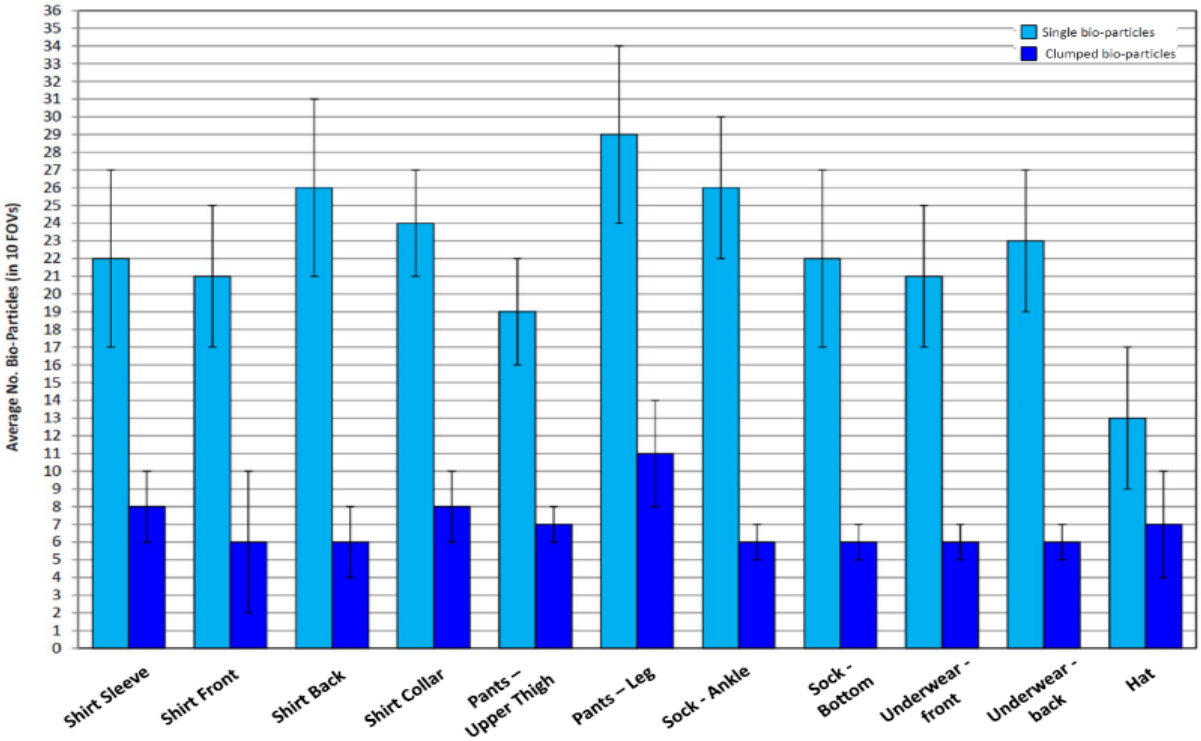
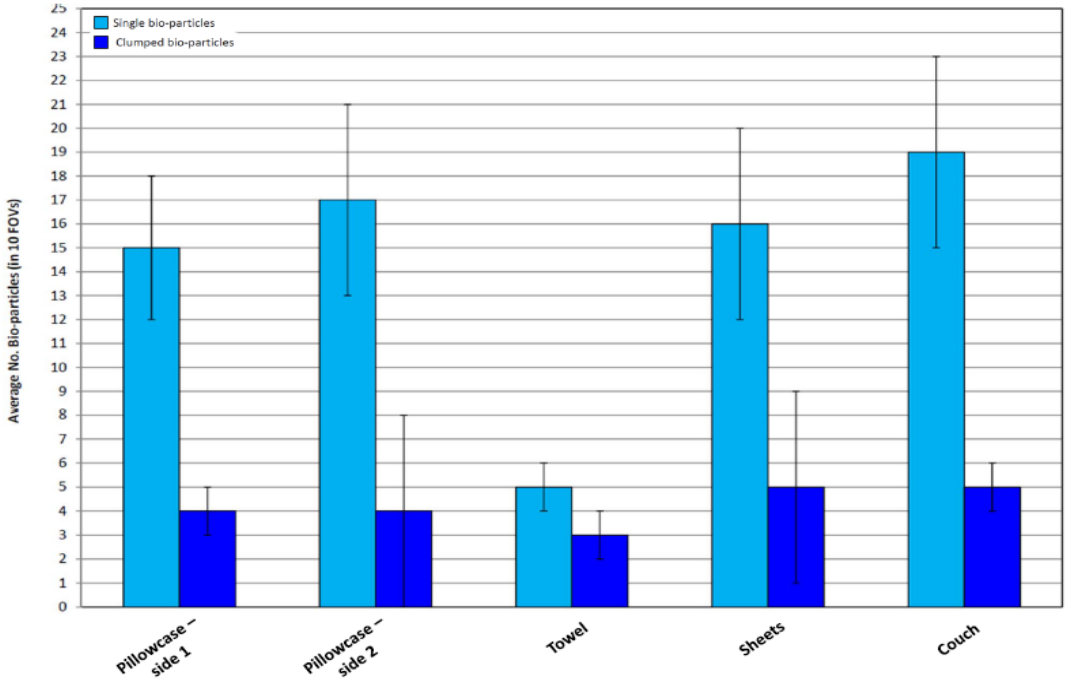



**Figure 9. Average Number of Bio-particles for Household Items.**
Error bars represent standard error. N = 14 donor sets for pillowcase, towels and sheets; N = 12 donor sets for couch






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 10. Average Number of Bio-particles for Household Items.**
Error bars represent standard error. N = 10 donor sets for all items except cell phone with N = 11 donor sets

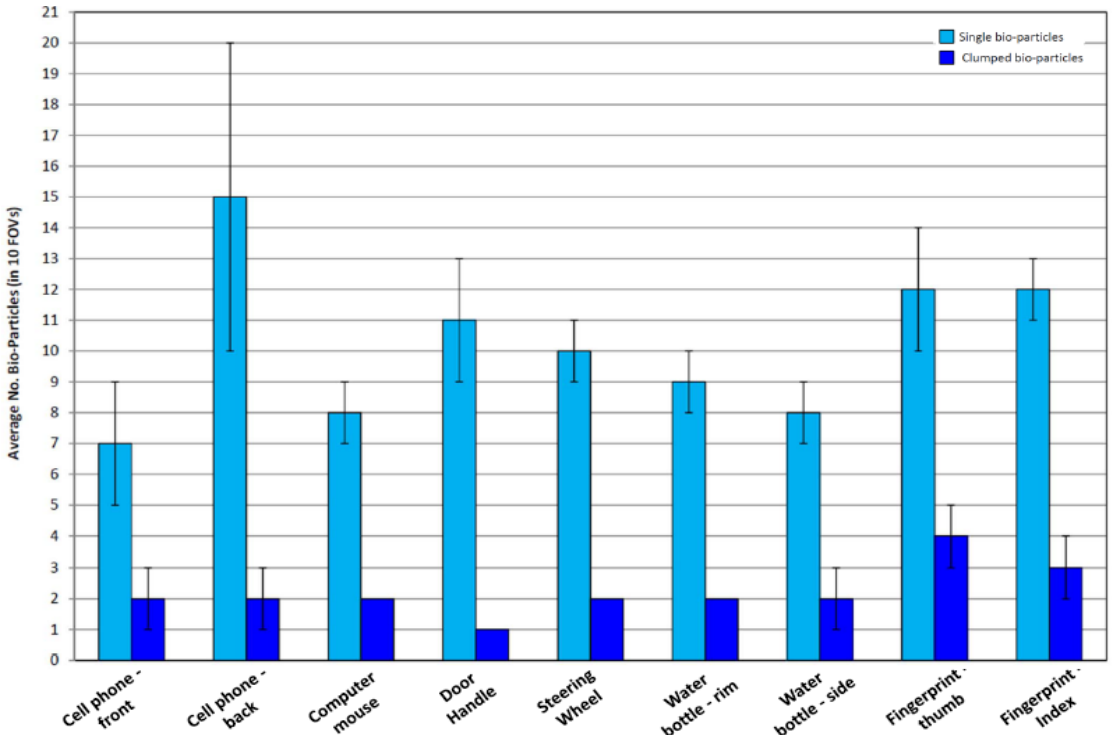
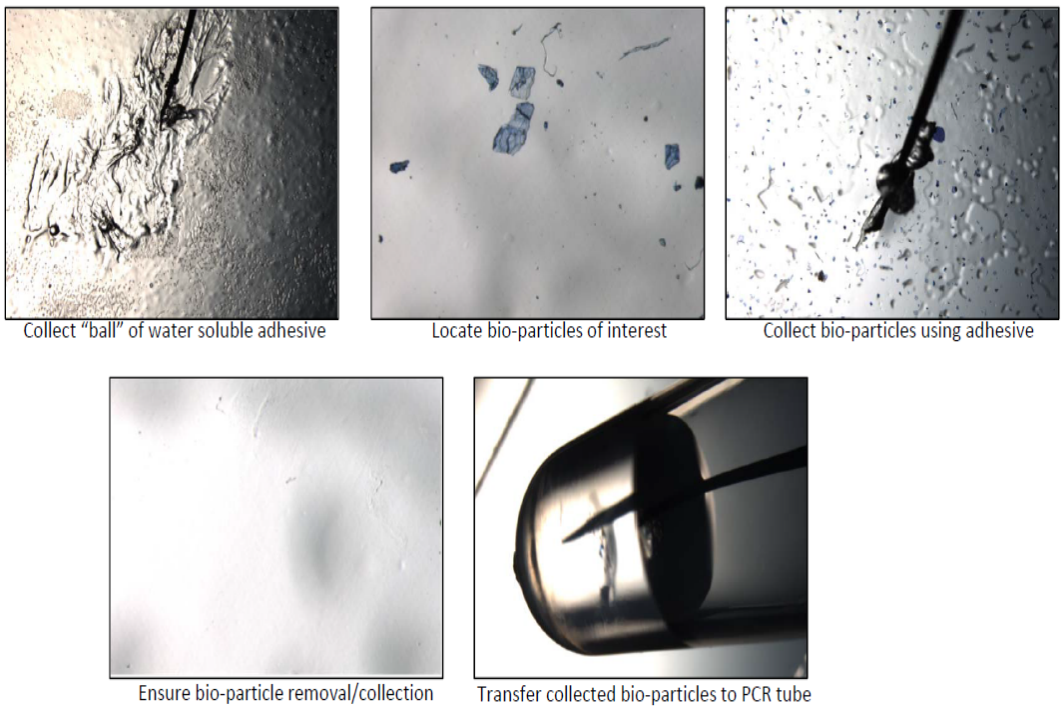


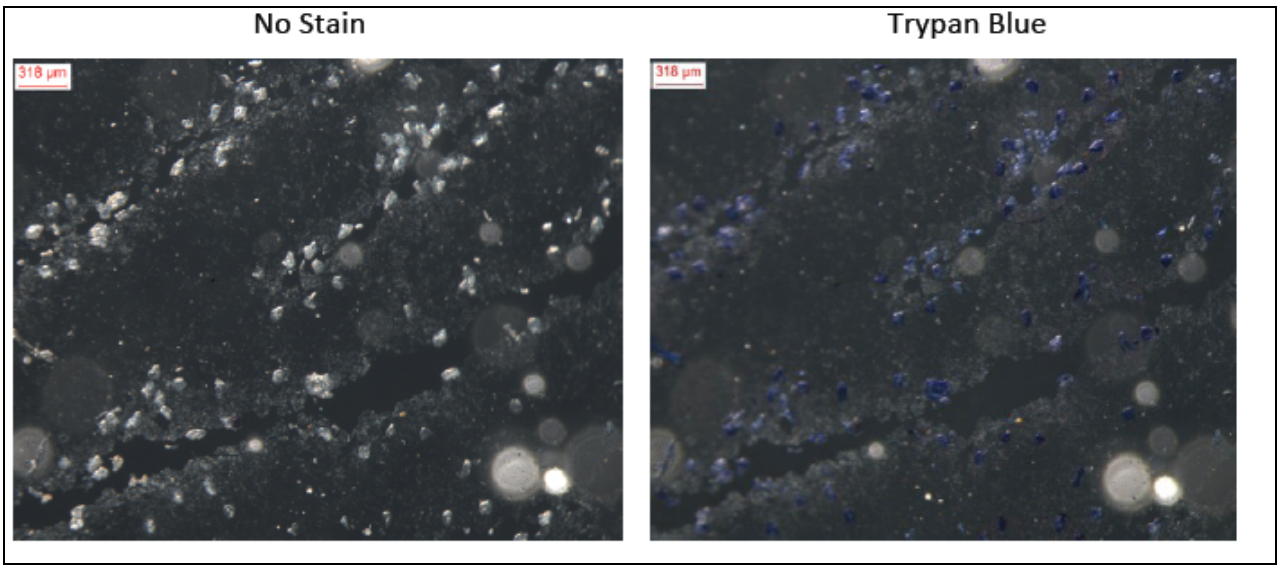

**Figure 11. Bio-Particle Collection From Gel-film® Surface Using Water Soluble Adhesive and Tungsten Needle**



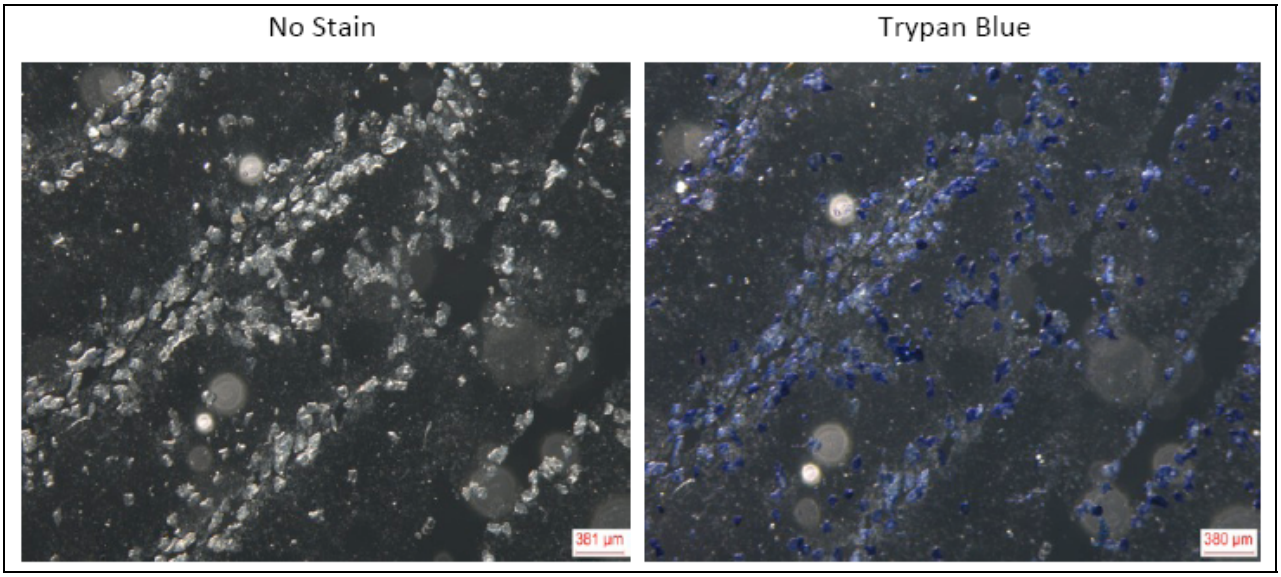



This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 12. Evaluation of Micro-Particle Distribution in Fingerprints (DIFF-lift™ tape)**

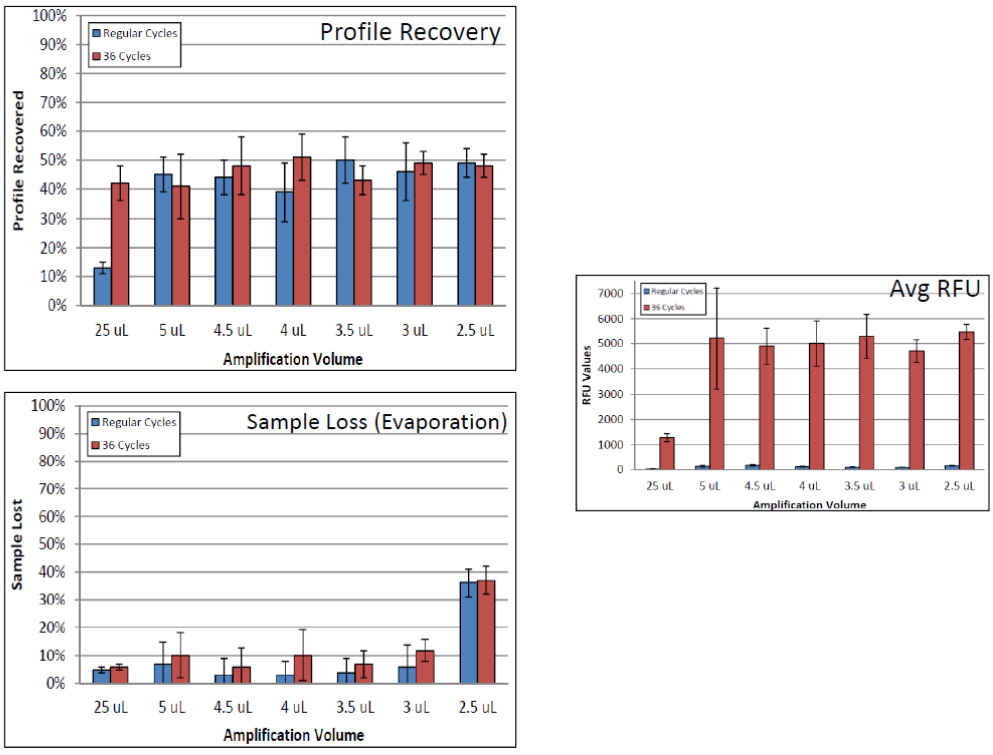



**Donor 1 – Middle finger**



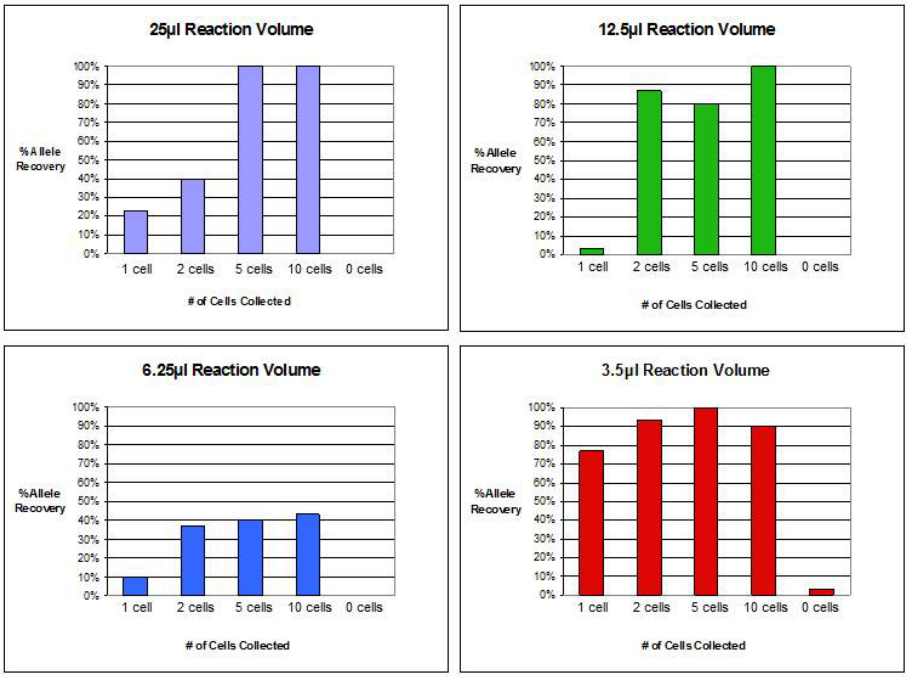

**Donor 2 – Ring finger**

**Figure 13. Comparison of profile recovery, evaporation rates and average RFU values for 5pg DNA inputs using micro-volume PCR reactions (28 vs 36 amplification cycles).**




N=1; 5 reps (3-5µl); 20 reps (2.5µl, 25µl)

**Figure 14. Comparison of Profile Recovery (Buccal cells, Identifiler Plus, 34 cycles) using Reduced Volume Combined Lysis/Amplification Reactions**






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 15. Reproducibility of Profile Recovery from Isolated Buccal Cells (Micro-manipulation) using the 3.5µl Combined Lysis/Amplification Reactions**

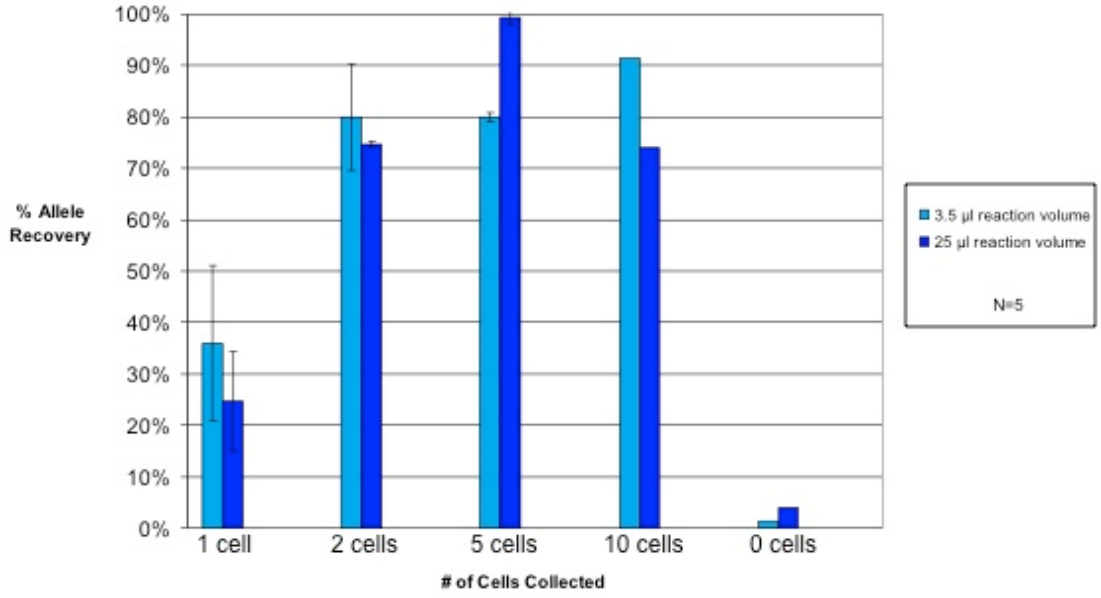



**Figure 16. Profile Recovery from Single Buccal Cells (Micro-manipulation) using the 3.5µl Combined Lysis/Amplification Reactions (25µl reactions included for comparison)**

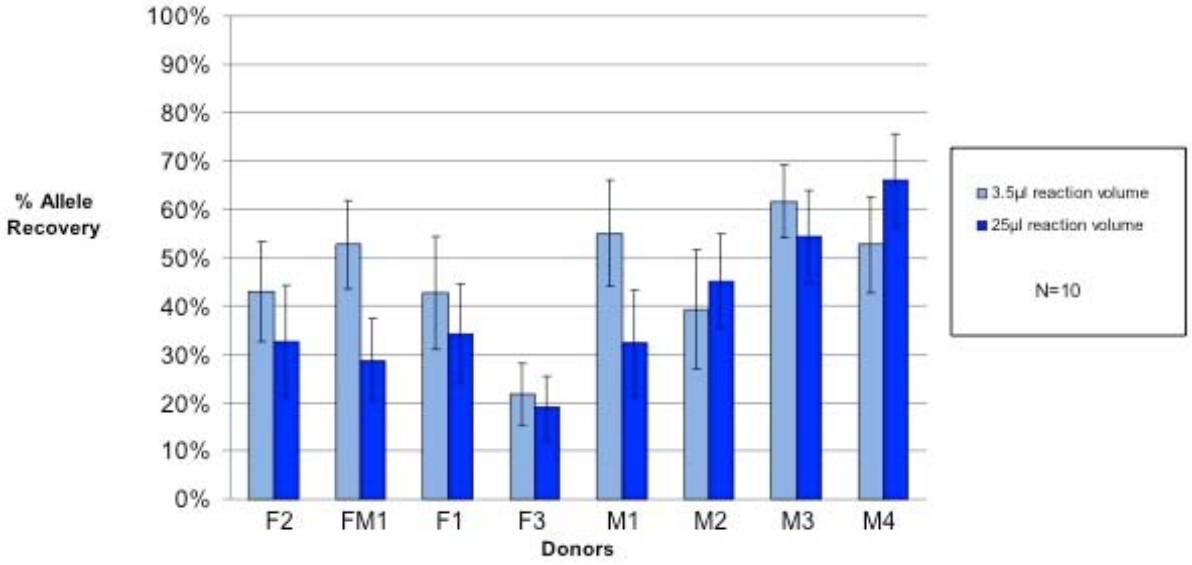


**Figure 17. Profile recovery From Individual Bio-Particles and Bio-Particle Clumps (20 replicates) Collected From a Shirt Collar**

***3.5µl Combined Lysis/Amplification, Identifiler Plus, 34 cycles**

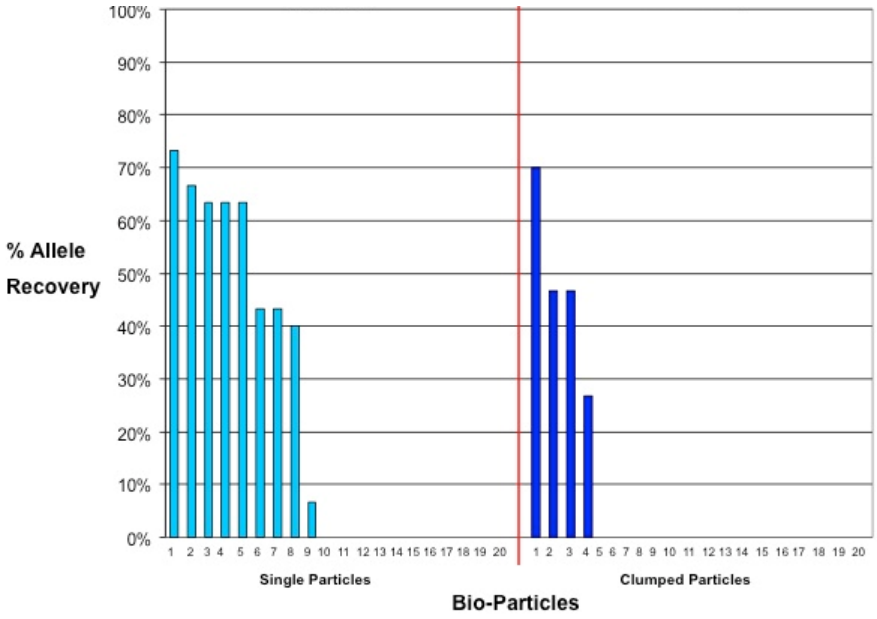
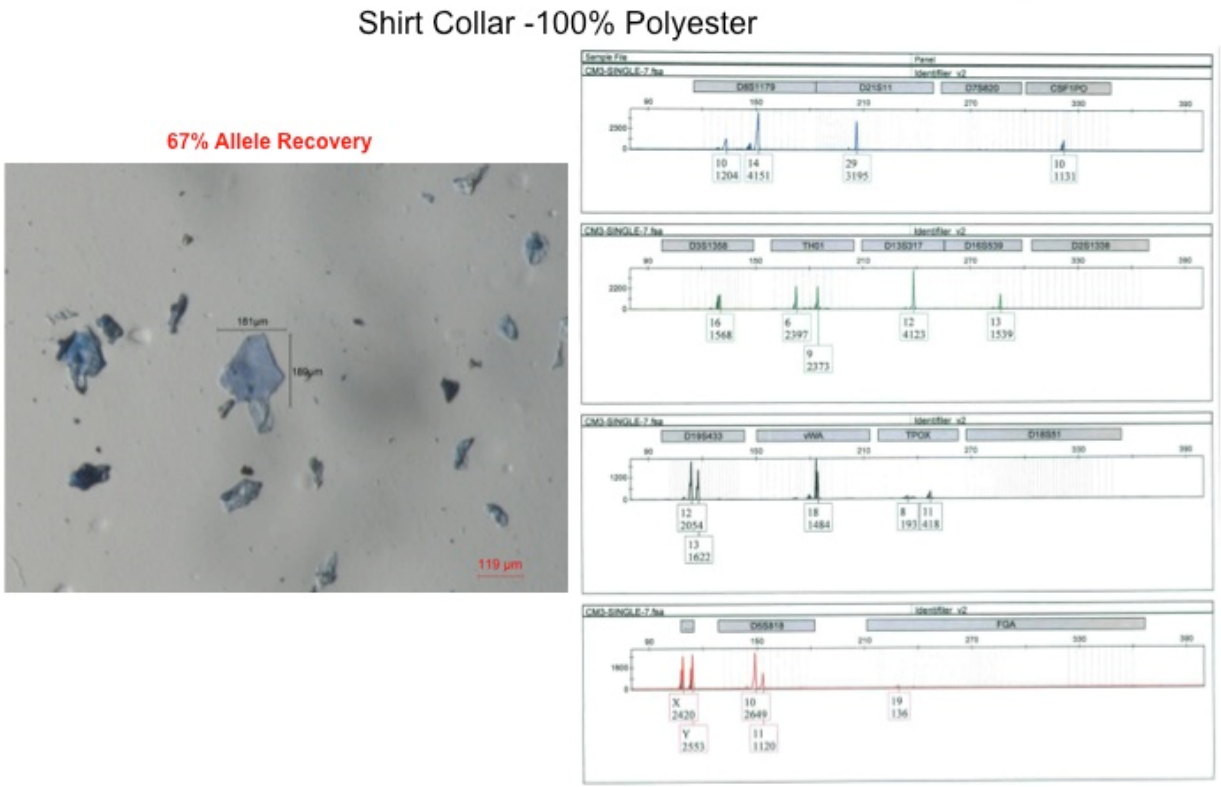



**Figure 18. Profile recovery From An Individual Shirt Collar Bio-Particle**

***3.5µl Combined Lyssi/Amplification, Identifiler Plus, 34 cycles**






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 19. Comparison of Profile Recovery Using the One-Step Lysis/Amplification reactions** (Identifiler Plus, 34 cycles)

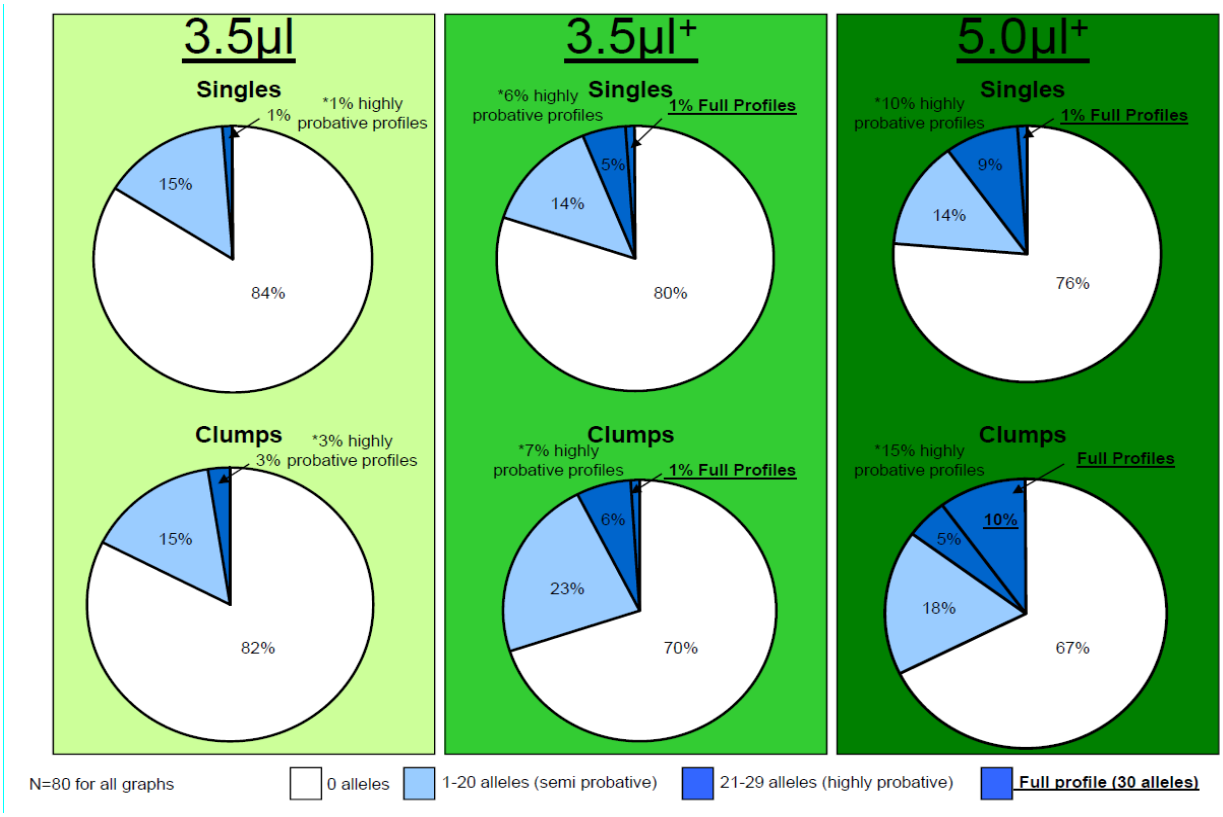


**Figure 20. Comparison of Profile Recovery (# of Alleles) From Bio-Particles Isolated from Touched Objects Using the 5µl+ One-Step Lysis/Amplification reactions**

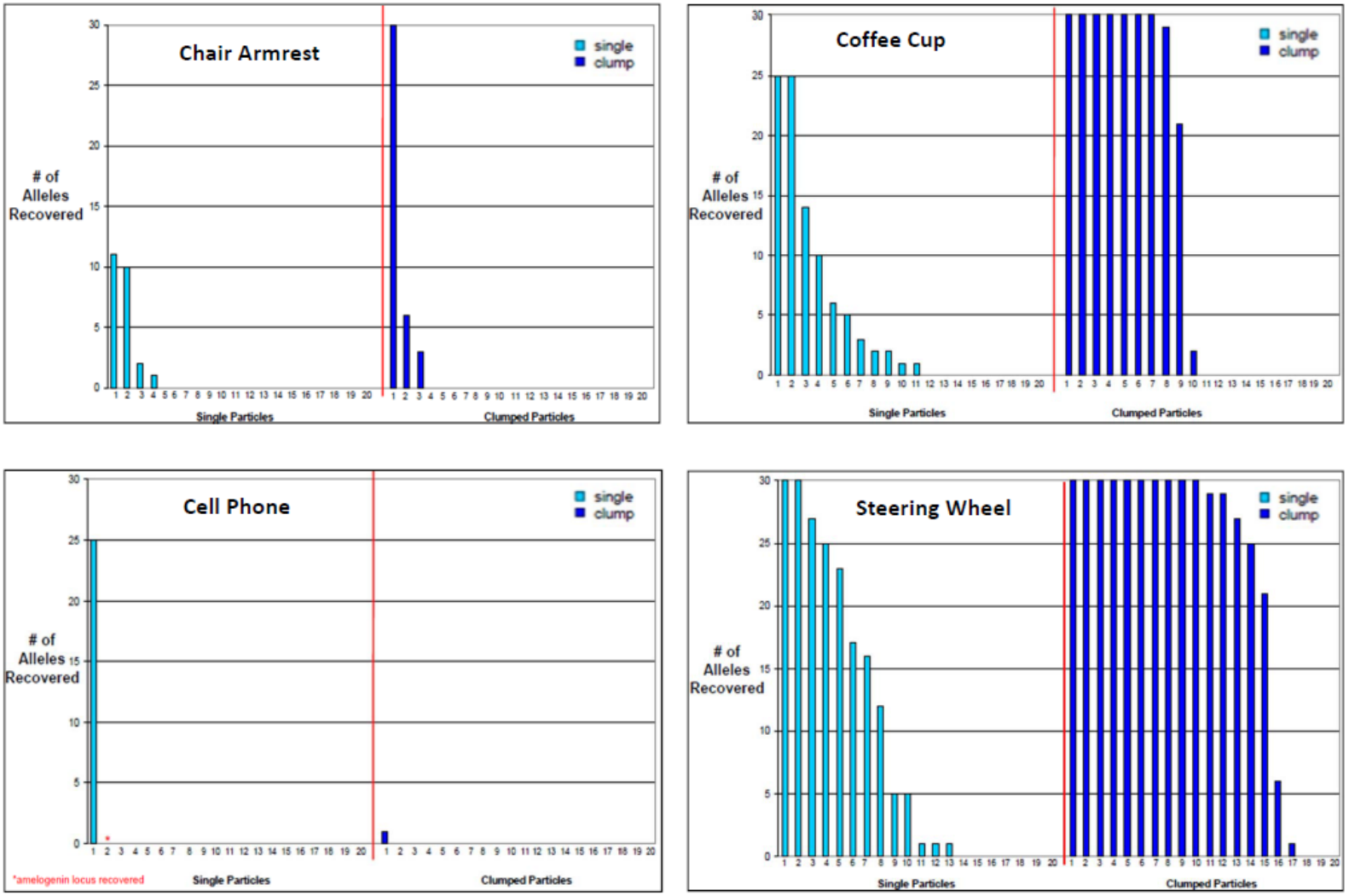


**Figure 21. Profile Quality Assessment From Bio-Particles Isolated from Touched Objects Using the 5µl$^{+}$ One-Step Lysis/Amplification reactions**

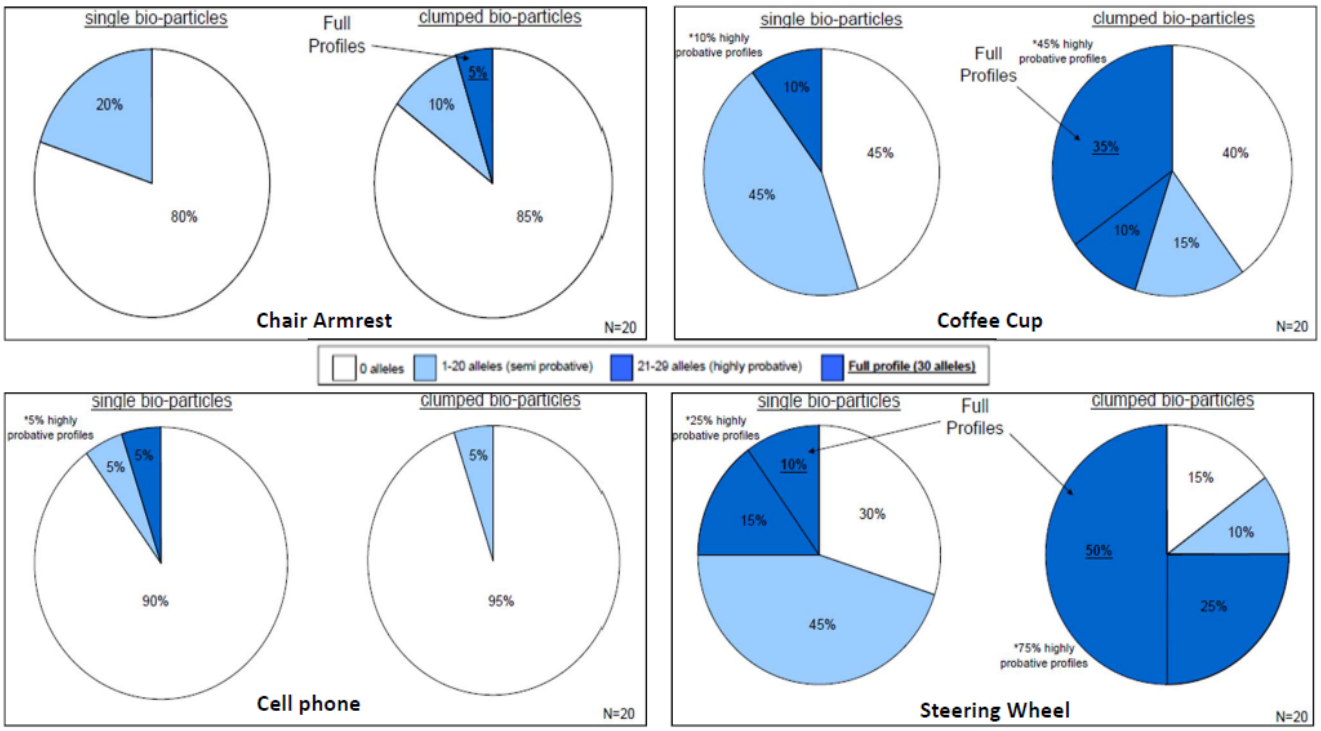
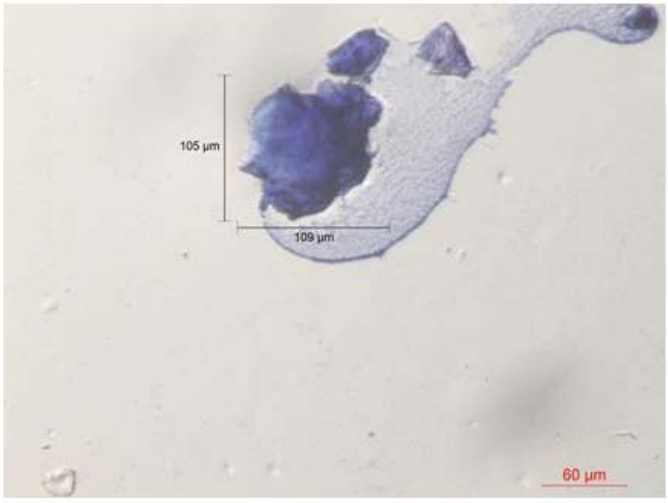
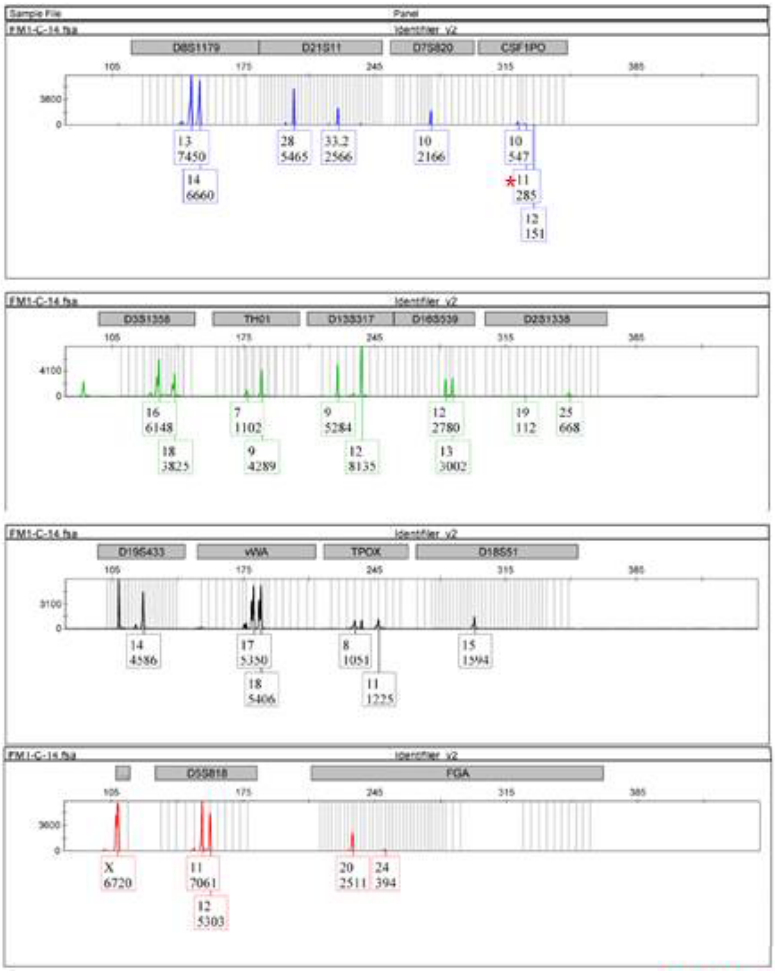


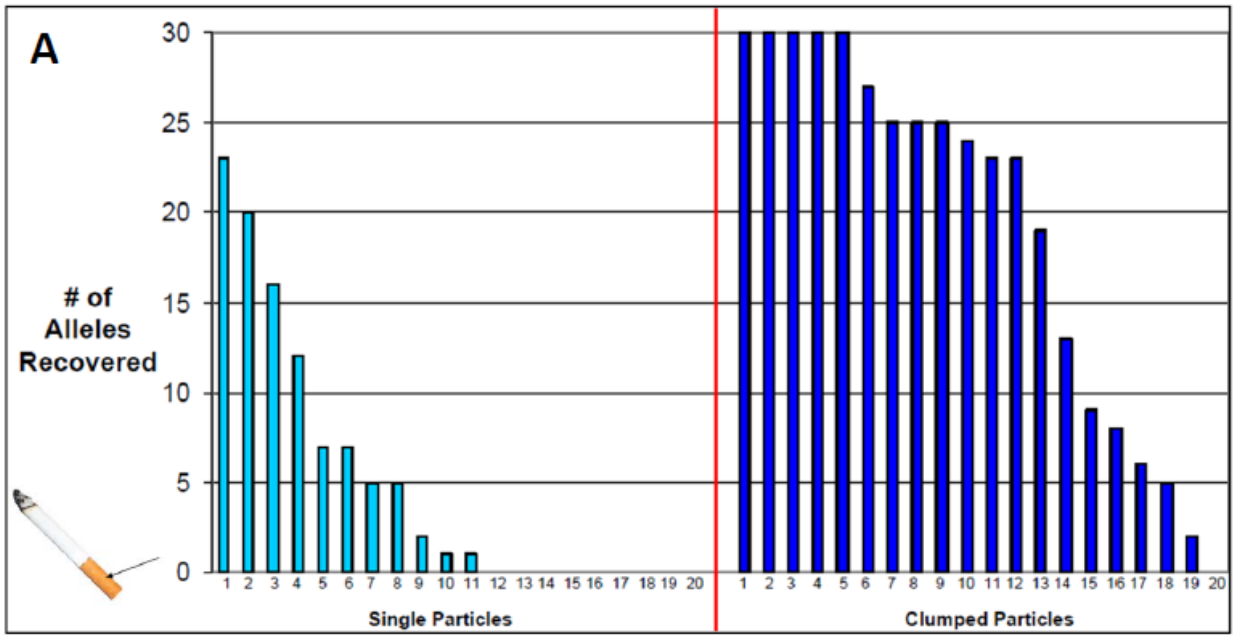
**Figure 22. Full STR Profile Obtained From a "Clump" Bio-Particle from a Chair Armrest**

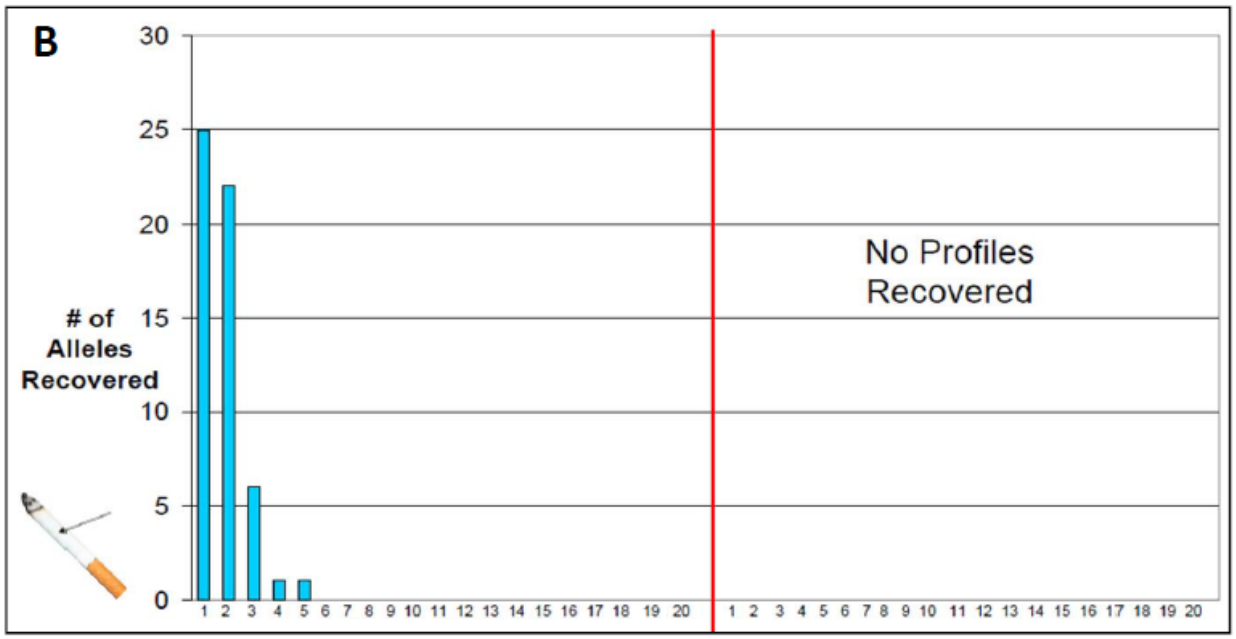



*** = possible increased stutter**

**Figure 23. Comparison of Profile Recovery between Two Areas of a Cigarette Butt**. A) STR profiling results from 20 single bio-particles (light blue) and 20 clumps (dark blue) from the area of the cigarette butt in contact with the donor's mouth; B) STR profiling results from 20 single bio-particles (light blue) and 20 clumps (dark blue) from the area of the cigarette butt contacted by the donor's fingers (touch).






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 24. STR Profiles From Bio-Particles Recovered from a Cigarette Butt**. A) STR profile (Identifiler Plus, 34 cycles) from a bio-particle "clump" collected from the mouth contact area of a cigarette butt (the clump collected is shown in the top panel); B) STR profile (Identifiler Plus, 34 cycles) from a single bio-particle collected from the finger contact area of a cigarette butt (the bio-particle collected is shown in the top panel).

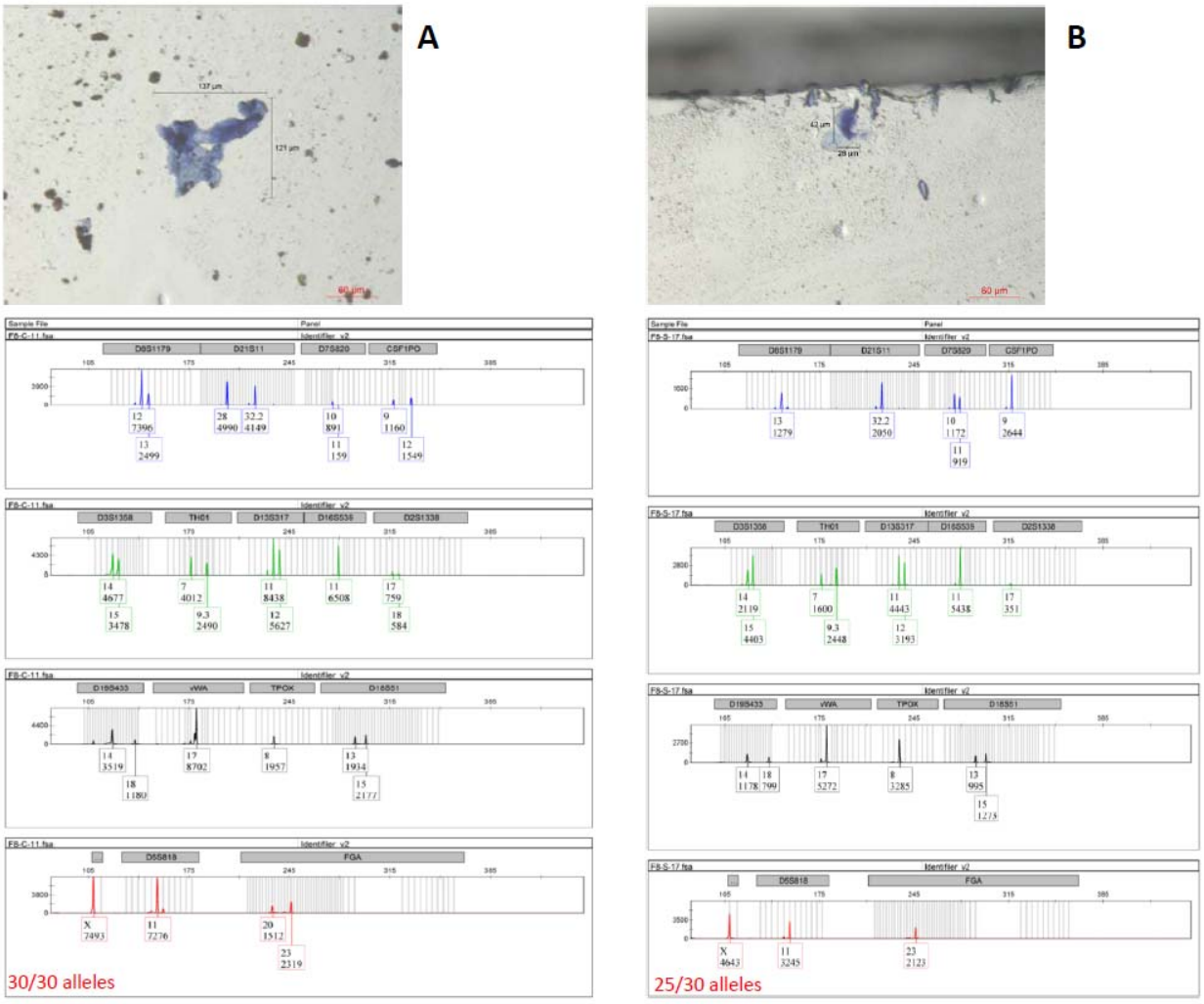


**Figure 25. Images of 20 Single Bio-Particles Collected From a Chewed Pen Cap**. STR profiling (% allele recovery) are listed for each of the collected single bio-particles.

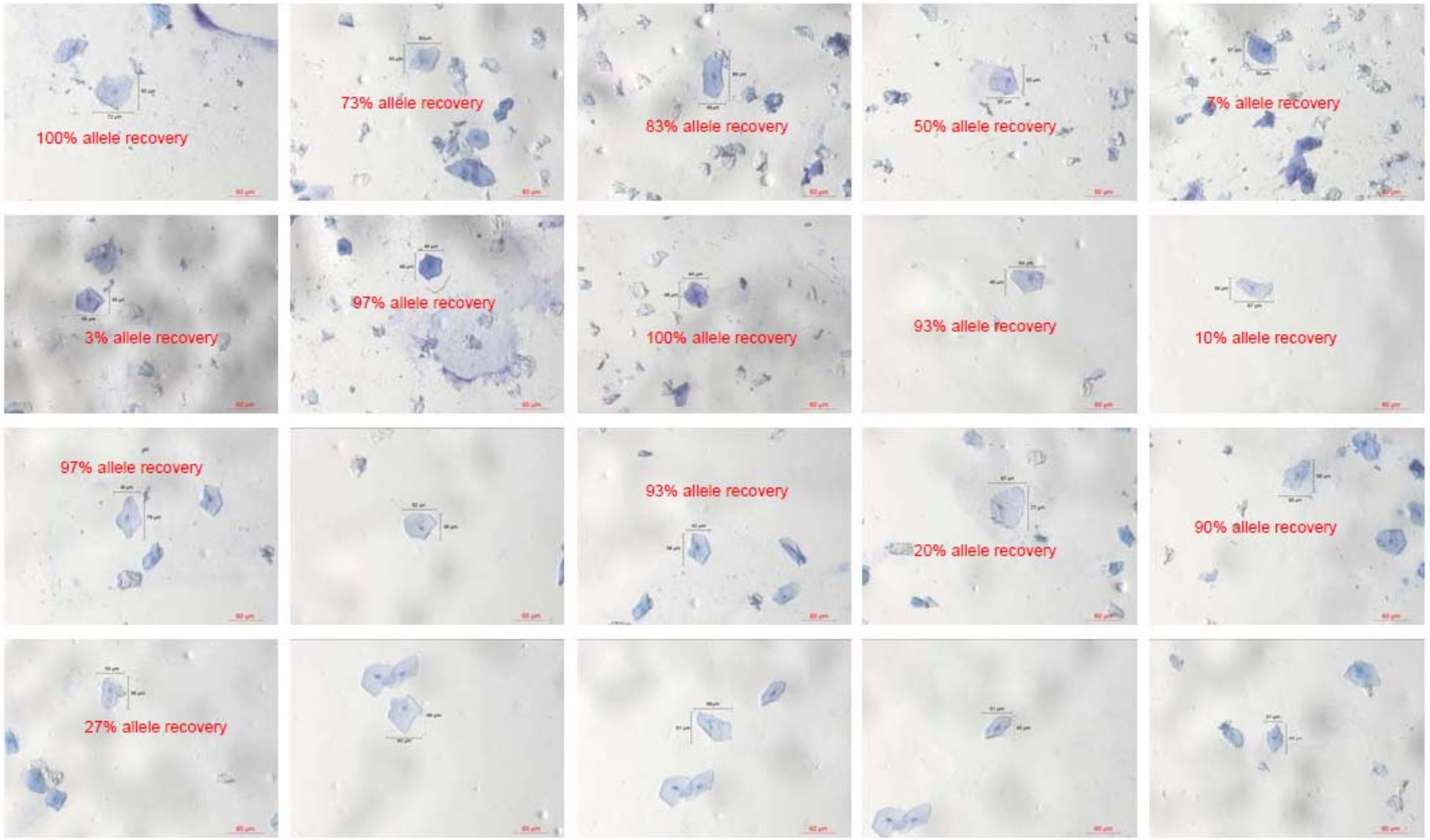


**Figure 26. Comparison of Profile Recovery between Direct Skin Samples from Male and Female Donors**. Direct skin samples (Gel-film® touched directly to the skin surface) from index finger, thumb and forearm were collected from male and female donors (one donor per skin surface, so overall N=6 (three male, three female samples). For each sample 20 single bio-particles and 20 clumps were collected. Therefore each graph represents the percent of profiles out of 60 samples.

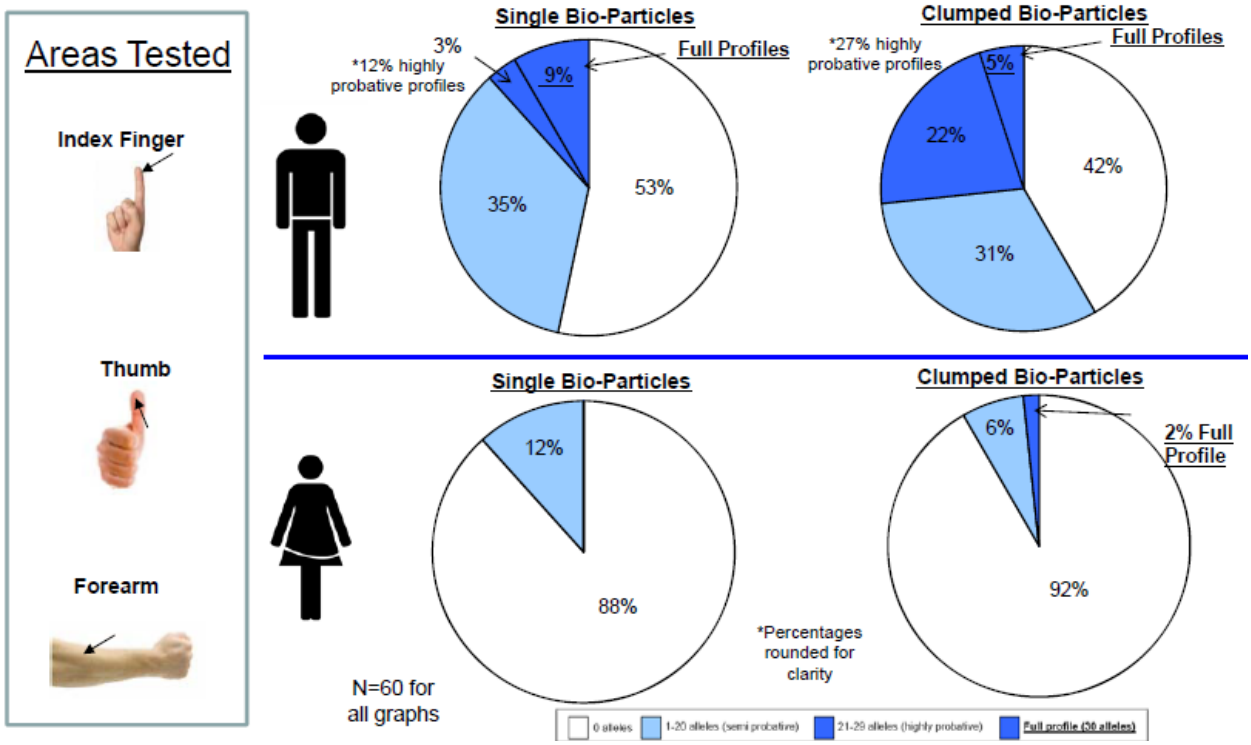
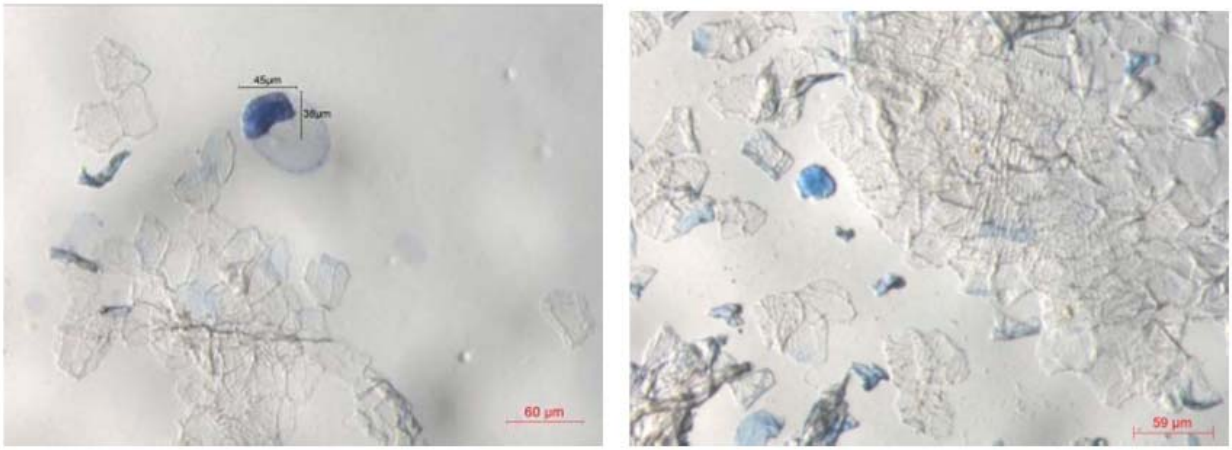



**Figure 27. Bio-Particles From Direct Skin (Forearm) Samples**. Left panel – female forearm, right panel – make forearm.






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 28. "Fluid-Like" Material**. "Fluid-like" material, which seems to be an intermediate stage between intact cell membranes and full cell breakdown, was identified as an area of interest for possible DNA recovery.

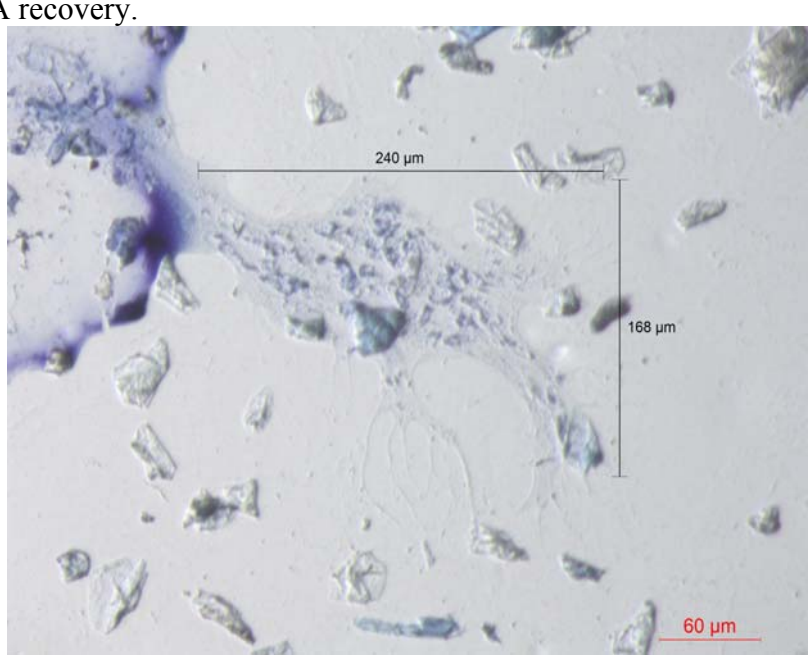
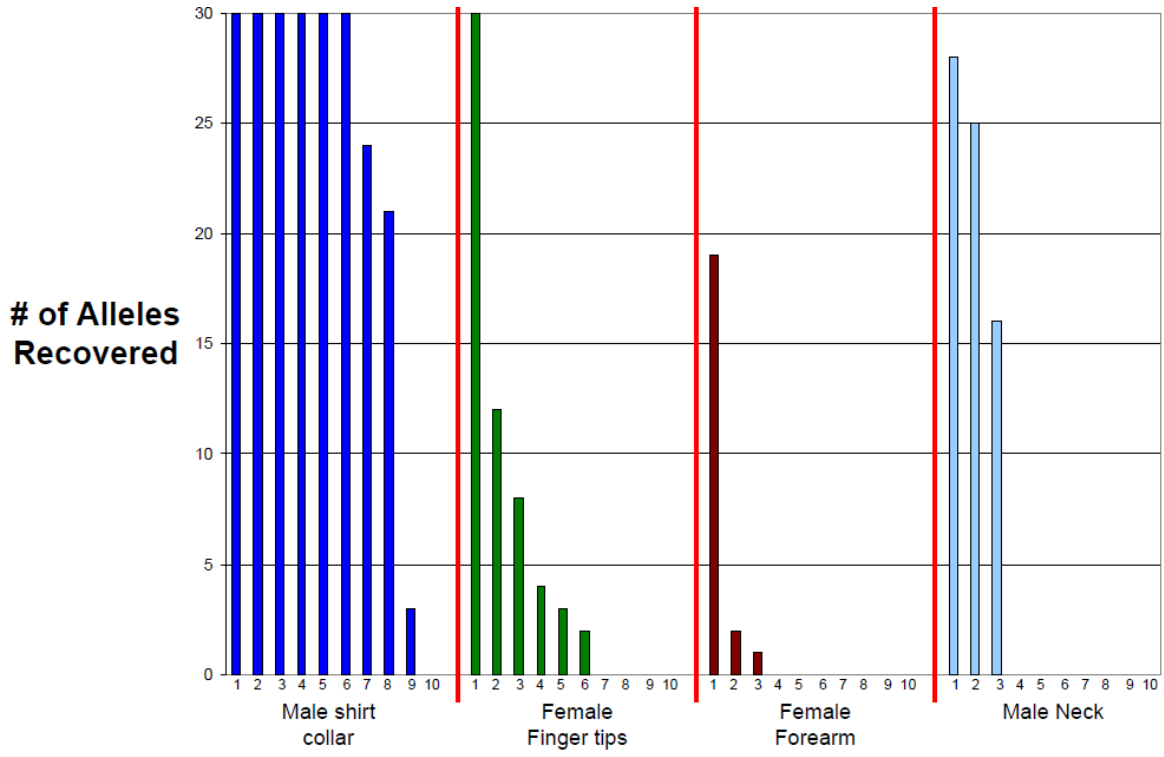


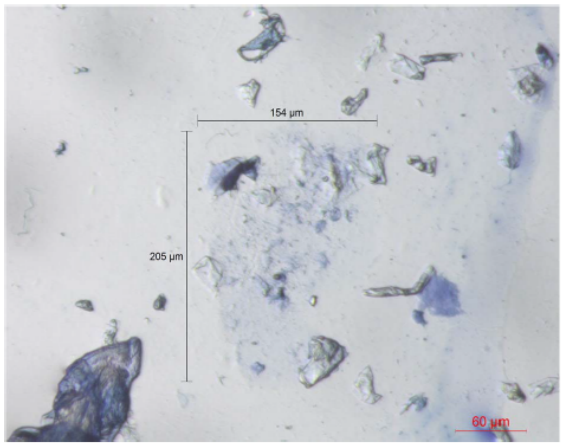

**Figure 29. STR Profile Recovery from "Fluid-Like" Material**. "Fluid-like" material was collected from a male shirt collar, female fingertip, female forearm and male neck. Allele recovery for each "fluid-like" sample is shown. N=10 for each item.

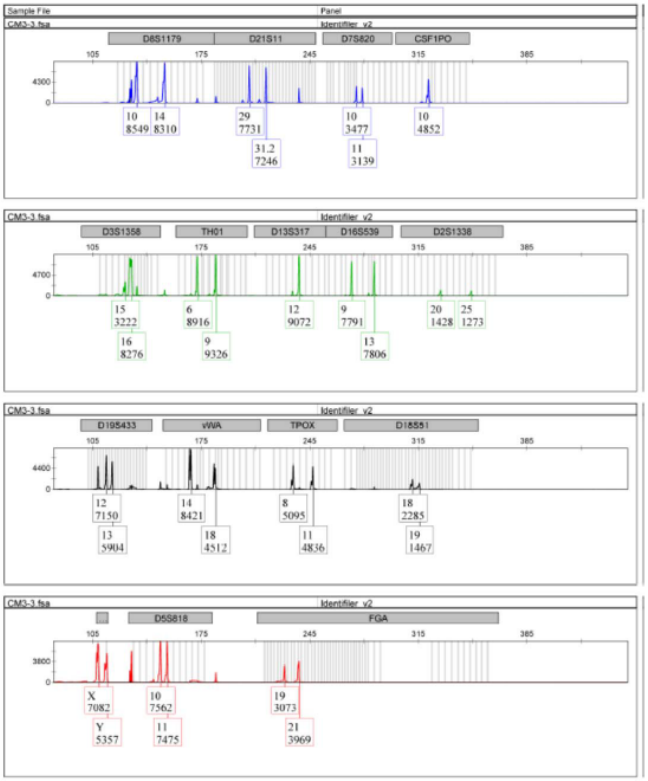





This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 30. STR Profile Recovery from "Fluid-Like" Material**. "Fluid-like" material was collected from a male shirt collar, female fingertip, female forearm and male neck. Allele recovery for each "fluid-like" sample is shown. N=10 for each item.






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 31. Donor Source Attribution of Single And Clumped Bio-Particles Collected from Simulated Physical Assault Mixtures.** 20 single (A) and 20 clumped (B) bio-particles were collected from simulated physical mixture samples (shirt sleeve grab, wrist grab and choking – male donor performed the grabbing in each mixture scenario). The number of bio-particles in which a profile attributed to the female donor and the male donor or no profile is indicated. Inconclusive classifications indicate that too few alleles were present to determine which donor the profile originated from.

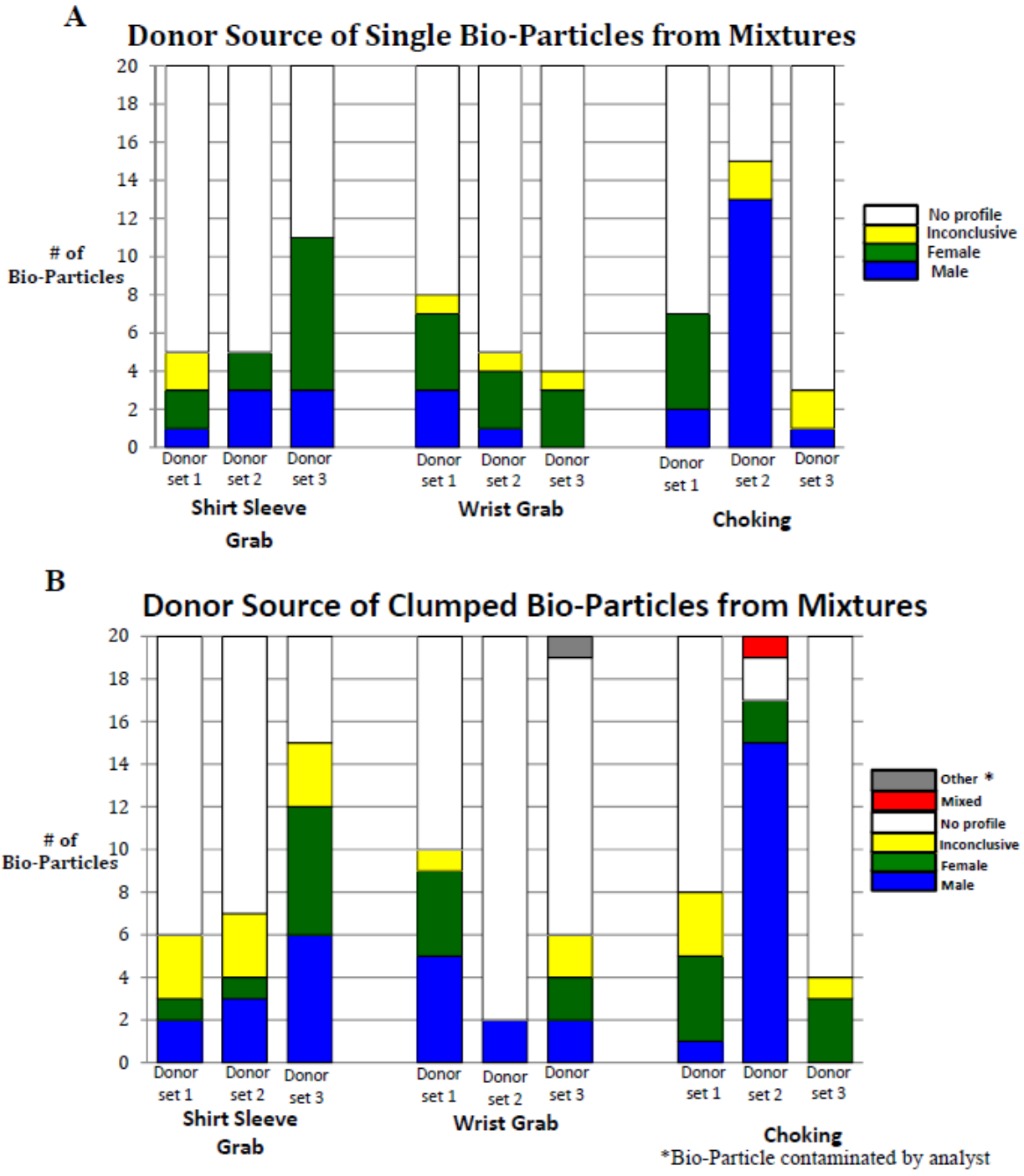


**Figure 32. Donor Source Attribution of Single And Clumped Bio-Particles Collected from Simulated Physical Assault Mixture (Shirt sleeve grab).** 20 single and 20 clumped bio-particles were collected from three simulated physical assaults in which a male donor forcefully grabbed a female ('victim's') shirt sleeve (A – donor set 1, B – donor set 2, C – donor set 3). The number of alleles recovered from each sample is shown: female donor – green, male donor – blue, inconclusive – yellow.

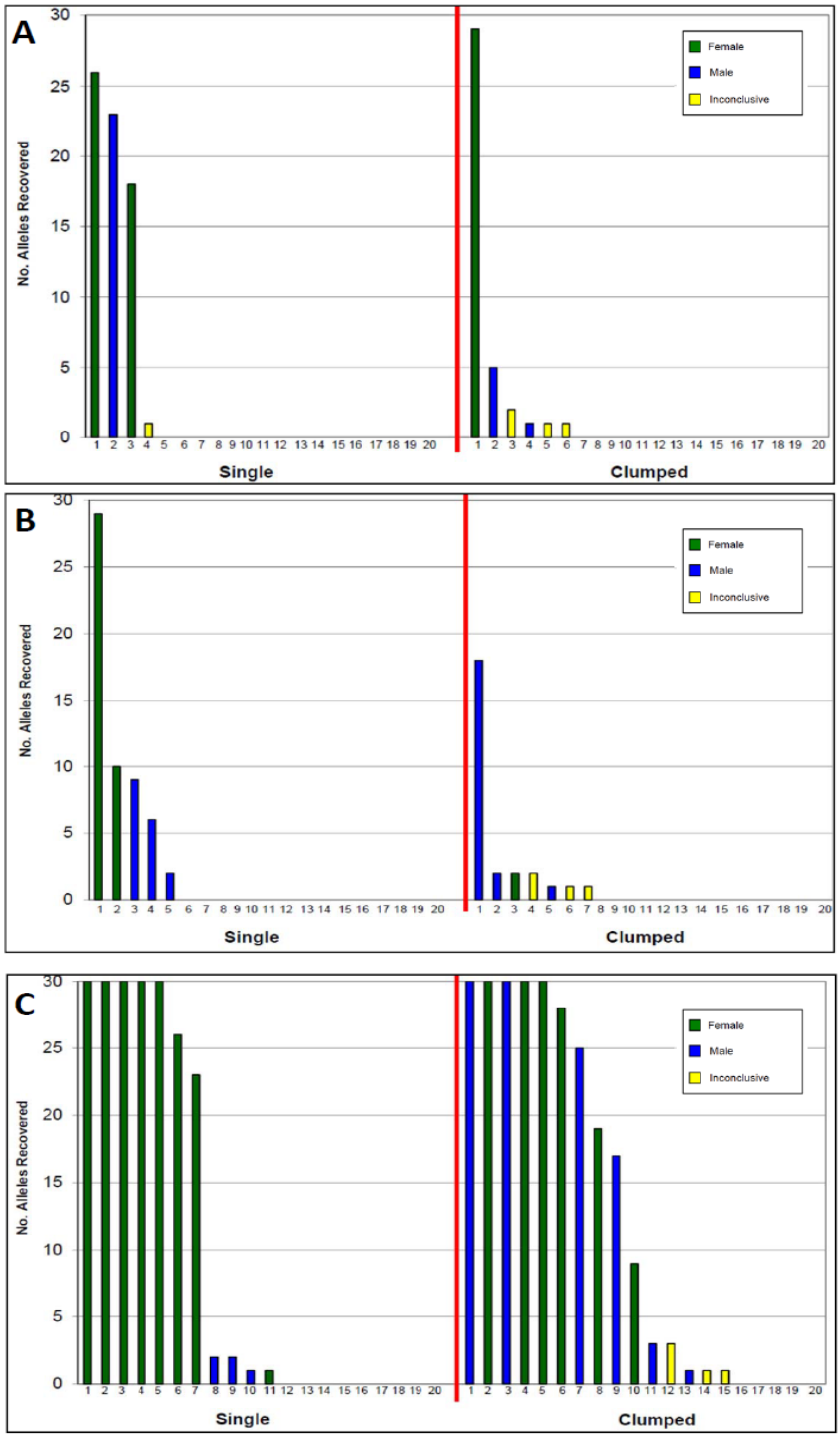
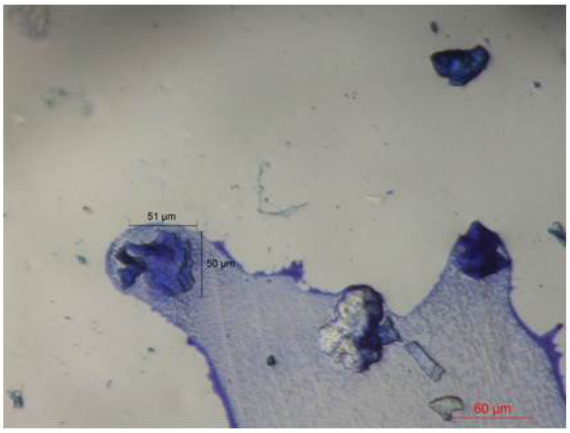
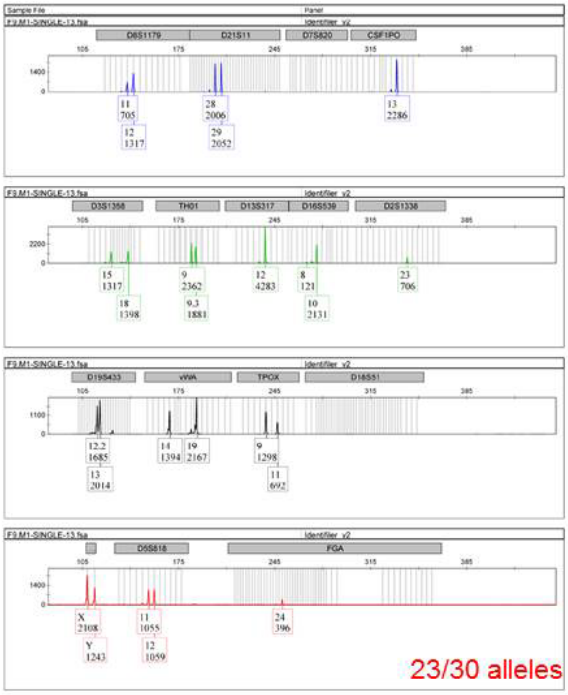


**Figure 33. STR Profile Recovery of the Male "Assailant" in a Simulated Physical Contact Mixture (Skin-Clothing); Shirt Sleeve Grab**. A single bio-particle (left panel) was collected from a simulated physical contact mixture (male donor forcefully grabbing a female's shirt sleeve). The obtained STR profile is shown (5µl$^{+}$ one-step lysis/amplification, Identifiler Plus, 34 cycles).






This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

**Figure 34. Donor Source Attribution of Single And Clumped Bio-Particles Collected from Simulated Physical Assault Mixture (Wrist grab).** 20 single and 20 clumped bio-particles were collected from three simulated physical assaults in which a male donor forcefully grabbed a female's ('victim') shirt sleeve (A – donor set 1, B – donor set 2, C – donor set 3). The number of alleles recovered from each sample is shown: female donor – green, male donor – blue, inconclusive – yellow.

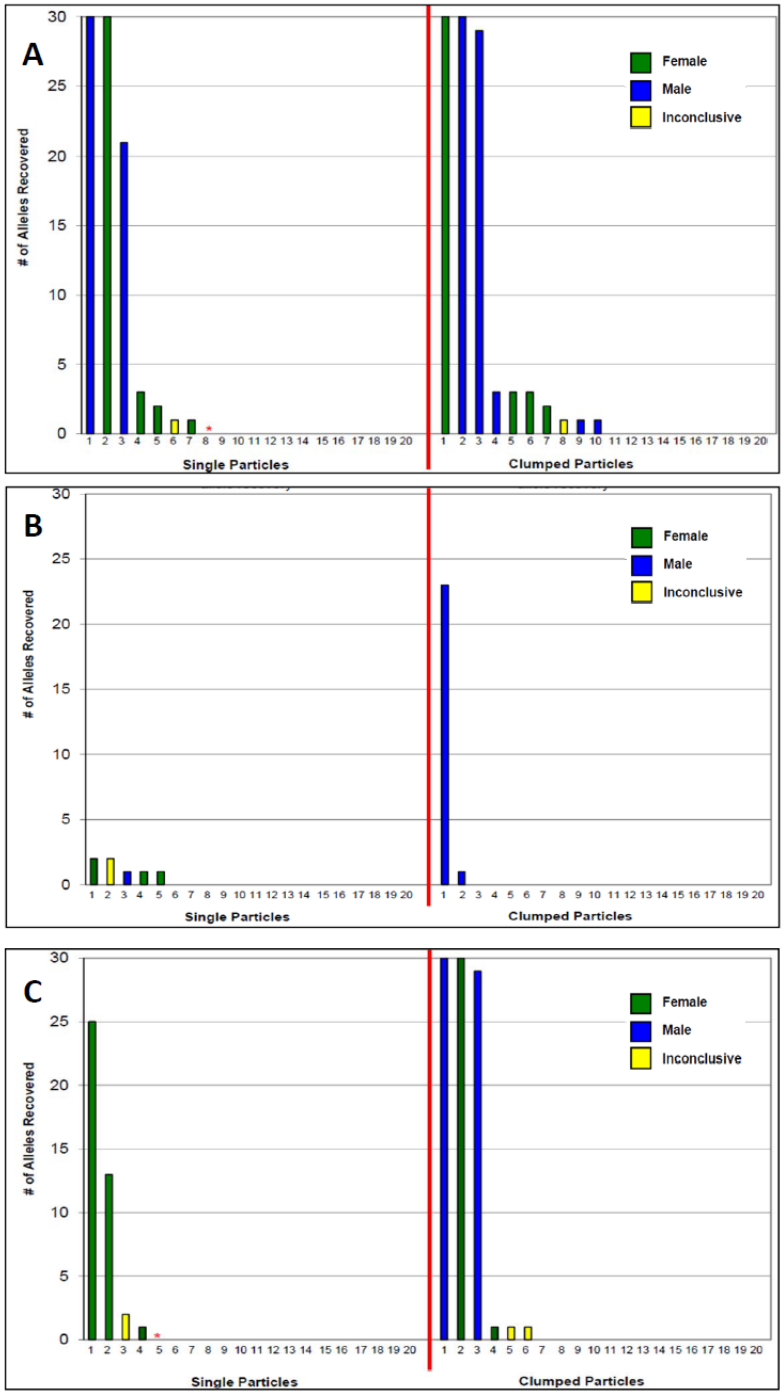


**Figure 35. Donor Source Attribution of Single And Clumped Bio-Particles Collected from Simulated Physical Assault Mixture (Neck grab/choking).** 20 single and 20 clumped bio-particles were collected from three simulated physical assaults in which a male donor grabbed a female's ('victim') neck (A – donor set 1, B – donor set 2, C – donor set 3). The number of alleles recovered from each sample is shown: female donor – green, male donor – blue, inconclusive – yellow, mixture - red.

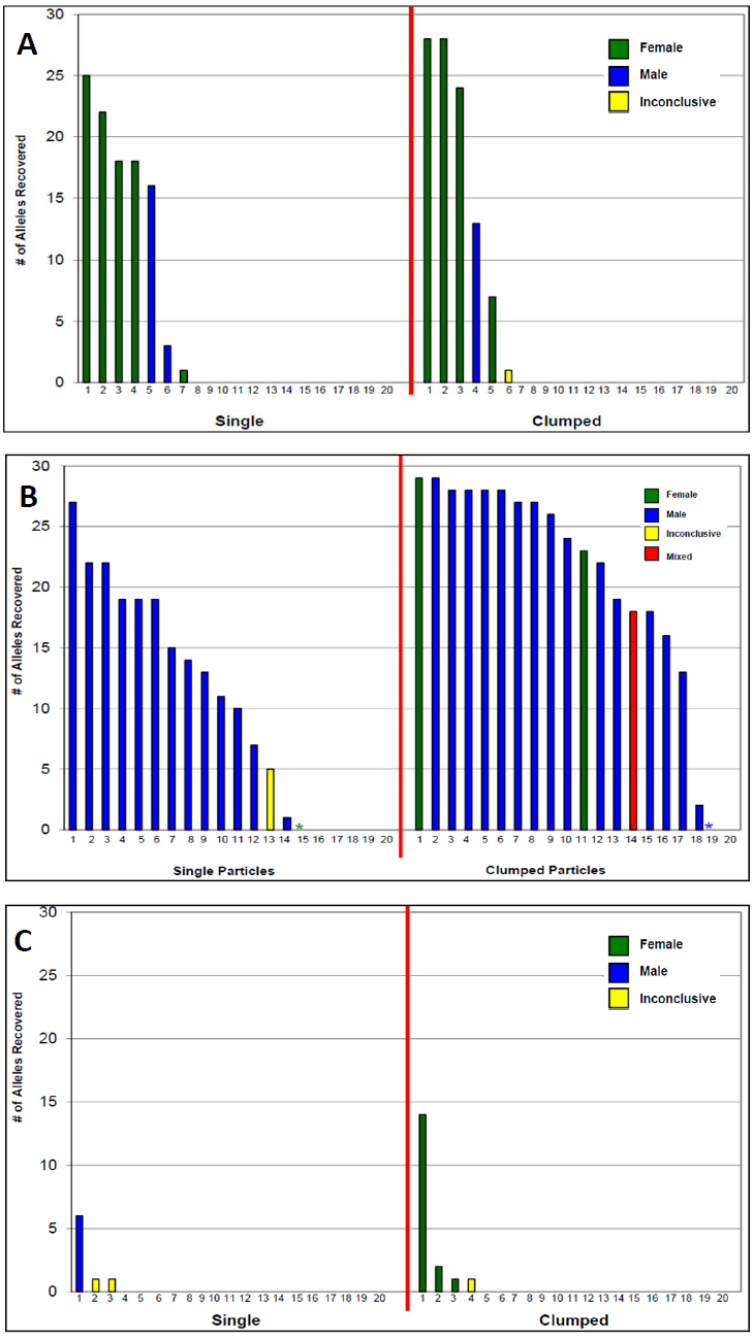


**Figure 36. STR Profile Recovery of the Male "Assailant" in a Simulated Physical Contact Mixture (Skin-Skin; Neck Grab)**. A single bio-particle (left panel) was collected from a simulated physical contact mixture (male donor forcefully grabbing a female's shirt sleeve). The obtained STR profile is shown ($5\mu l^{+}$ one-step lysis/amplification, Identifiler Plus, 34 cycles). ADO = allele drop out.

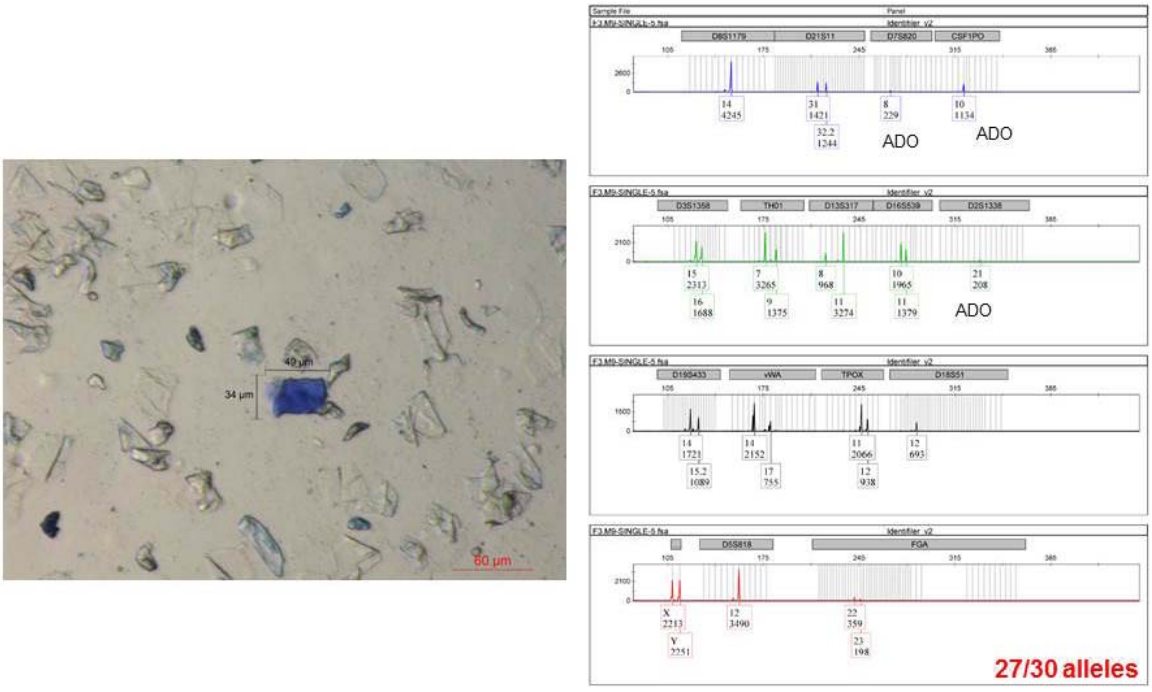


**Figure 37. Donor Source Attribution of Single And Clumped Bio-Particles Collected from Simulated Sexual Assault Mixture (Bedding).** 20 single and 20 clumped bio-particles were collected from three simulated physical assaults in which a male donor made physical contact with a female's ('victim') bedding/sheets (A – donor set 1, B – donor set 2, C – donor set 3). The number of alleles recovered from each sample is shown: female donor – green, male donor – blue, inconclusive – yellow, mixture - red.

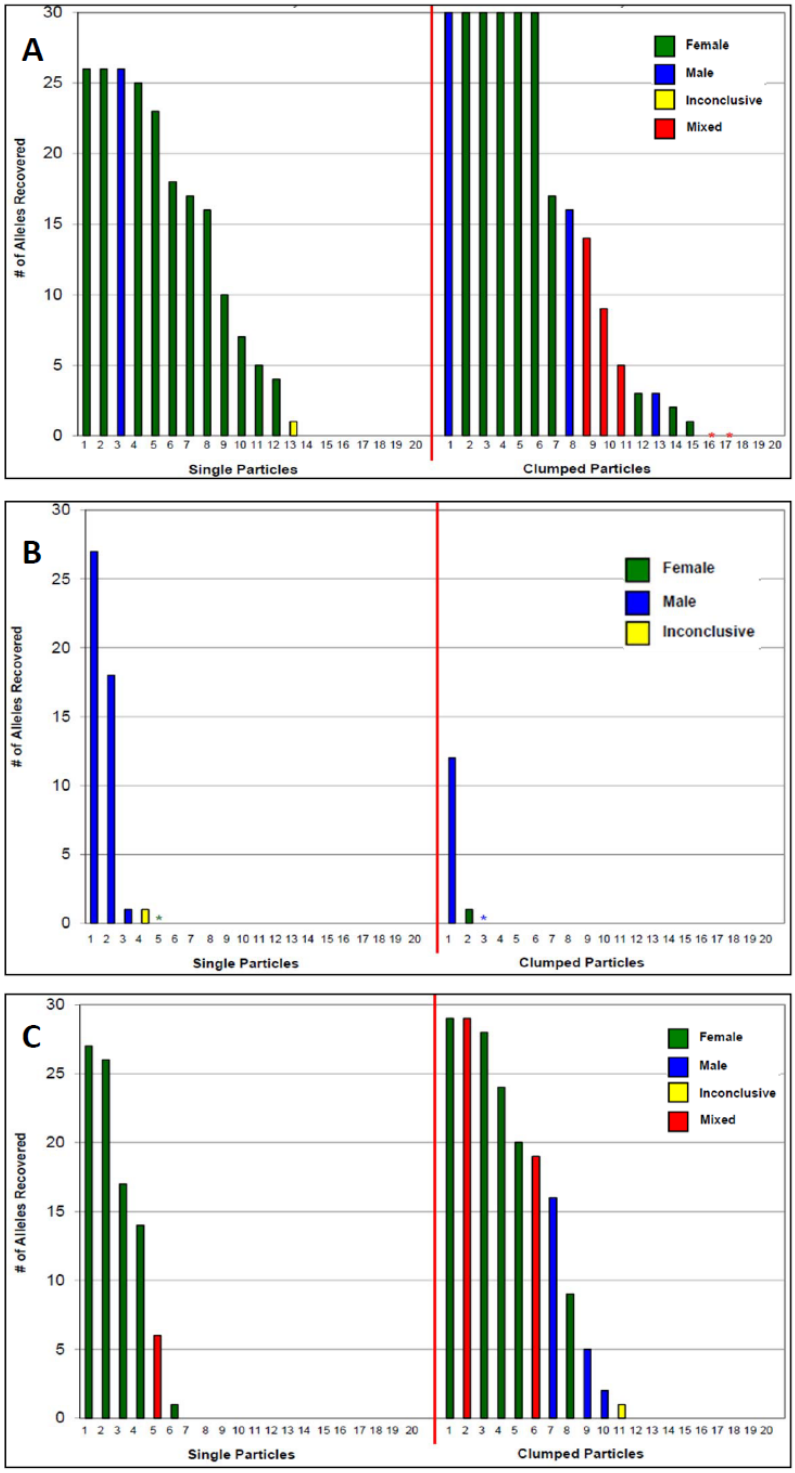


**Figure 38. Singleplex CE assays for skin biomarkers**. RT-PCR reactions were performed using 25 ng of total RNA isolated using a manual organic extraction, with the exception of LCE1C where 100 pg of total RNA was used. Singleplex amplification products are shown (LCE1C – 56/58 bp (A); LCE1D – 142 bp (B); LCE2D – 193 bp (C); CCL27 – 254 bp (D); IL1F7 – 92 bp (E)). Representative electropherograms are shown. Controls without RT (RT-, right panel) were run in parallel to the RT reaction (RT+, left panel).

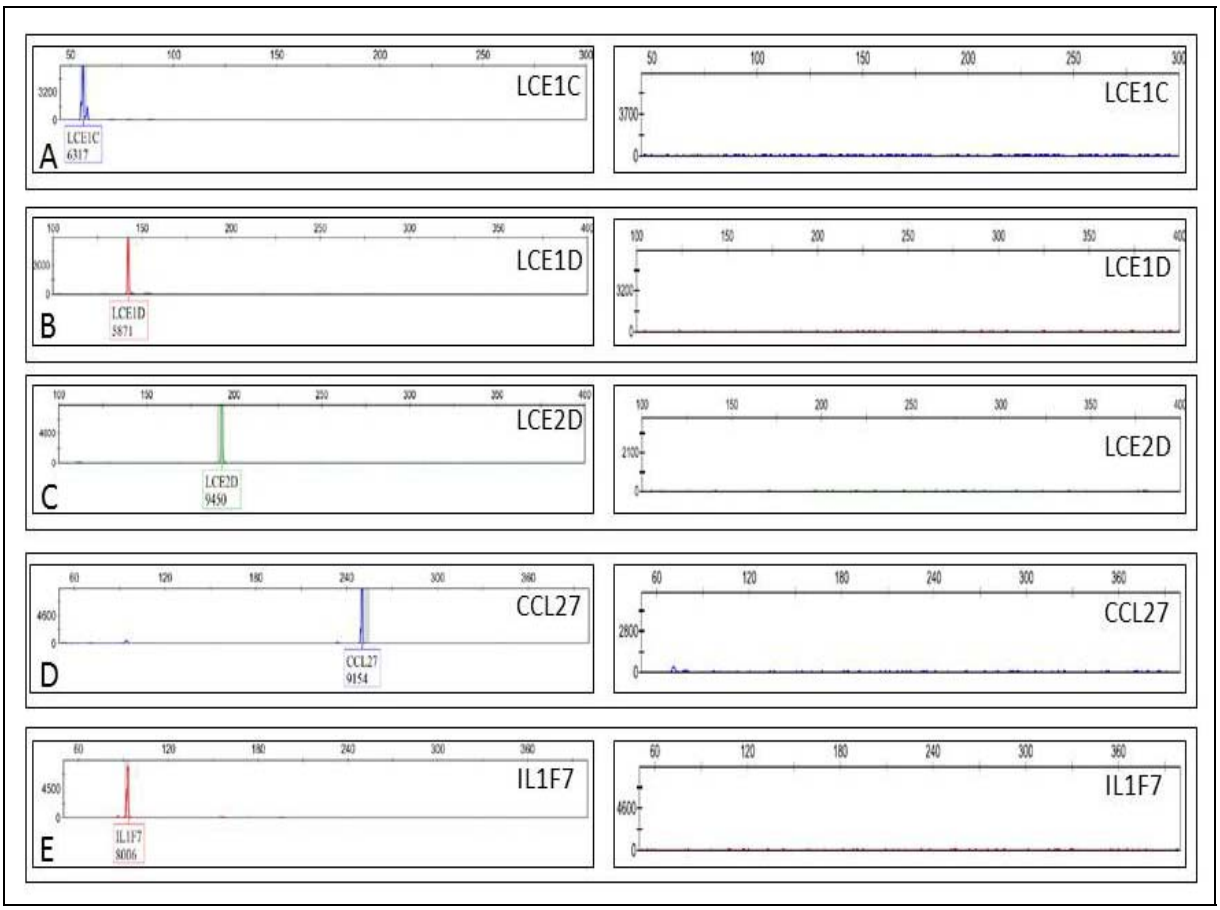


**Figure 39. Skin biomarker detection in human skin and touched objects.** RT-PCR reactions were performed using total RNA extracted from surface swabs of human skin and various touched objects. Each sample was evaluated for the presence of the five skin biomarkers. Representative electropherograms for detected biomarkers are shown for each sample. The 'x 2' designation indicates that positive results for two different computer mouse samples were obtained but only one representative electropherogram is shown.

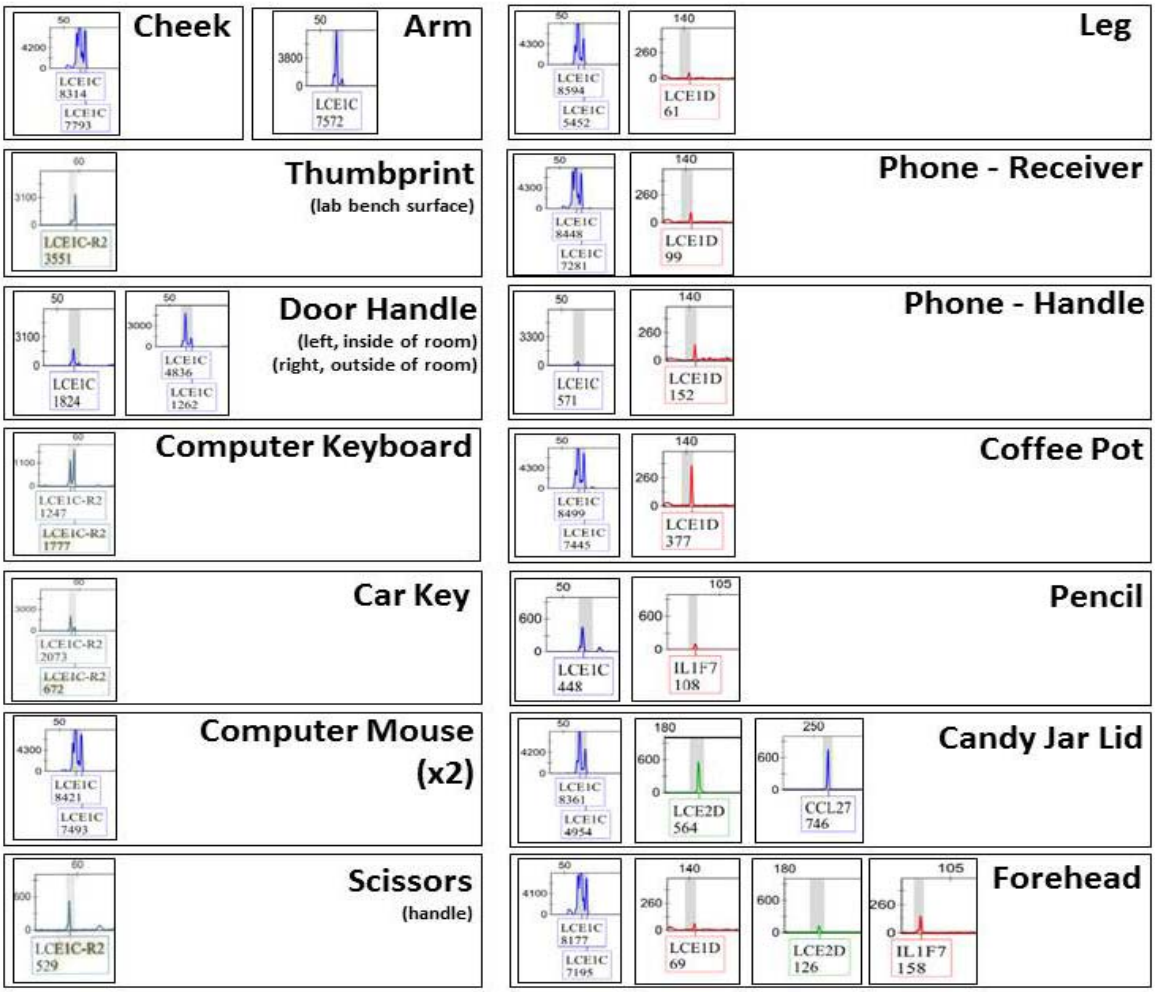


**Figure 40.Tetraplex and pentaplex assays for the identification of skin.** RT-PCR products for the tetraplex (A) and pentaplex (B) assays using 25 ng of total RNA from human skin are shown.

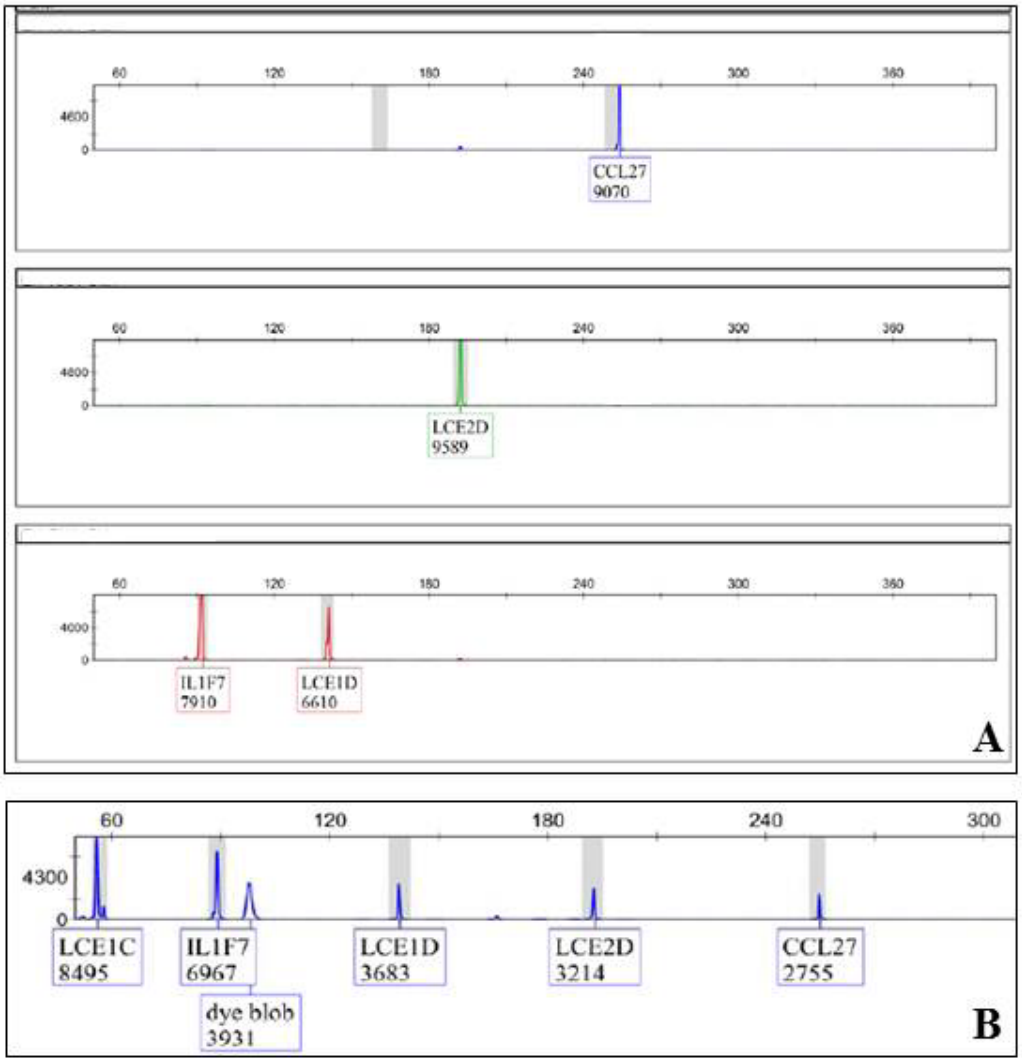


**Figure 41. Skin biomarker detection in isolated bio-particles from worn clothing items using RNA pre-amplification strategies.** Bio-particles from a shirt back and lab coat were collected using micro-manipulation. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent mRNA body fluid identification multiplex (CE-based detection). Representative electropherograms from the shirt back (10 bio-particles) and the lab coat (25 bio-particles) are shown.

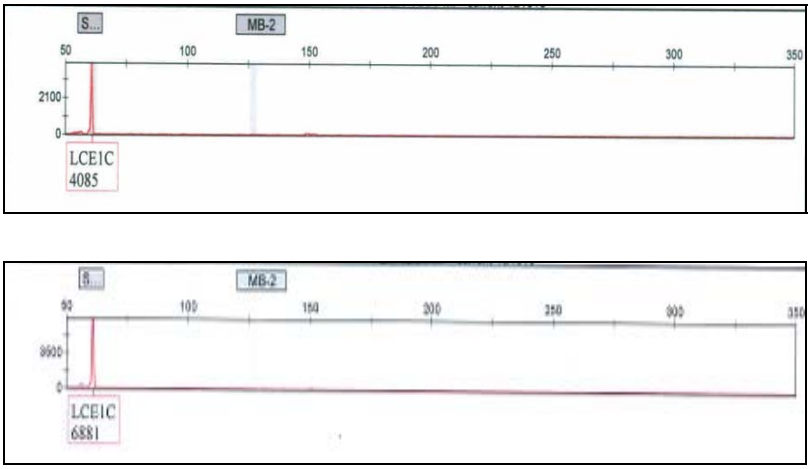


**Figure 42. Skin biomarker detection in isolated bio-particles "clumps" from a shirt collar using RNA pre-amplification strategies.** Bio-particle "clumps (1, 5, 10, 20 and 0) from a shirt collar were collected using micro-manipulation. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent mRNA body fluid identification multiplex (CE-based detection). Electropherograms from 1, 5, 10, 20 and 0 bio-particle clump samples are shown. X-axis – size (in base pairs); Y-axis – relative fluorescence units (RFUs)

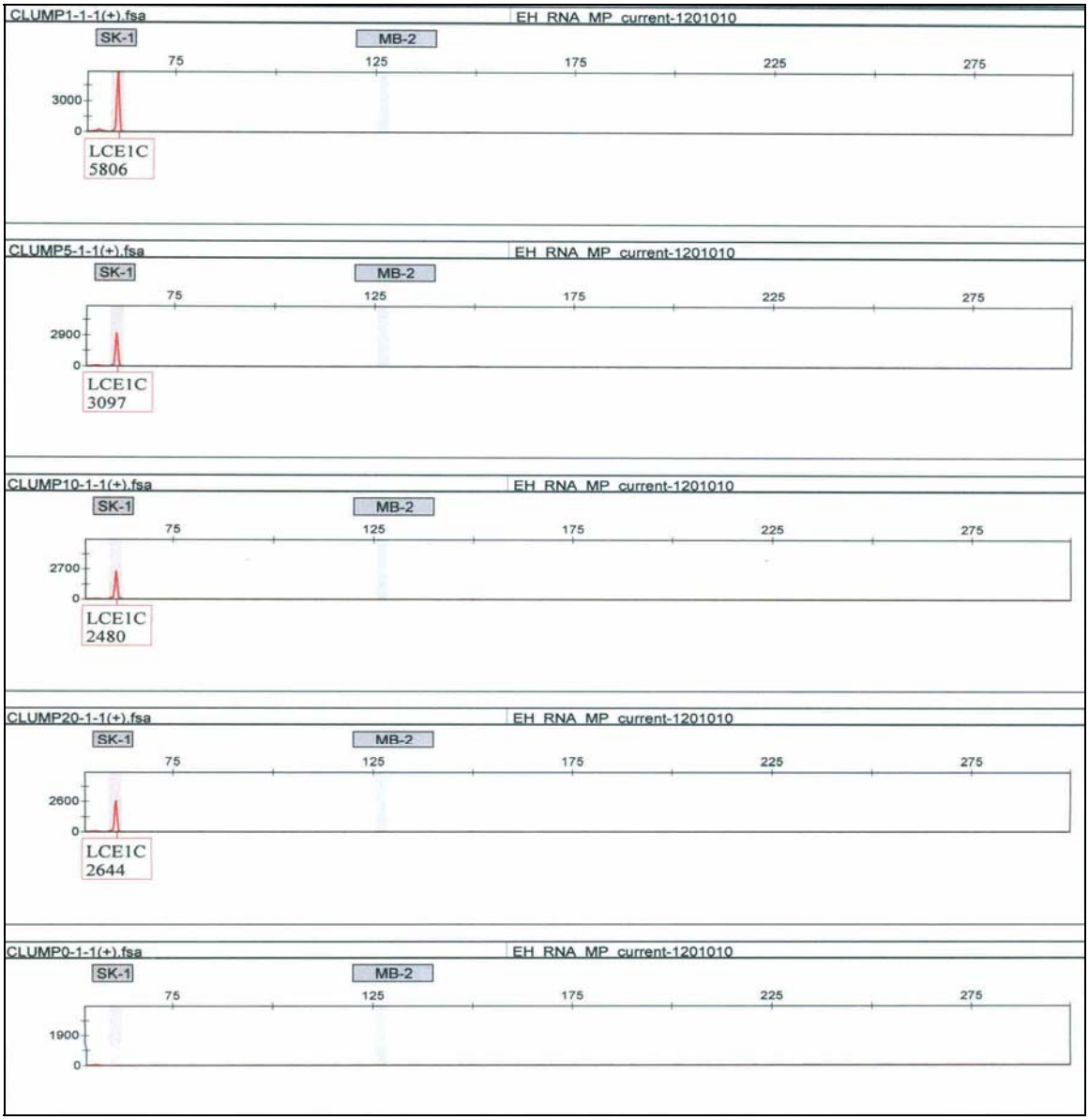


**Figure 43. HRM Analysis of Touch Bio-Particles from a Shirt Collar.** Bio-particle (singles (s) (10, 25 and 50) and "clumps" (c): (1, 5, 10, 20) from a shirt collar were collected using micro-manipulation. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent singleplex LCE1C HRM assay. The melt plots for all samples are shown. X-axis – Tm (°C); Y-axis – dF/dT). The table indicates the Tm value obtained for all samples. Grey cells represent no detection. PC = positive control. RTB = reverse transcription blank.

| No. | Name | LCE1C |
|---|---|---|
| 1 | Shirt Collar-10s | |
| 2 | Shirt Collar-25s | 83.5 |
| 3 | Shirt Collar-50s | 83.5 |
| 4 | Shirt Collar-1c | 83.5 |
| 5 | Shirt Collar-5c | 83.5 |
| 6 | Shirt Collar-10c | 83.5 |
| 7 | Shirt Collar-20c | 83.5 |
| 8 | Shirt Collar-blank | |
| 9 | SK2(+)-PreAmp-PC | 83.0 |
| 10 | RTB(+) | |
| 11 | PreAmp Blank | |
| 12 | SK2(+)-HRM-PC | 82.6 |
| 13 | BLANK | |

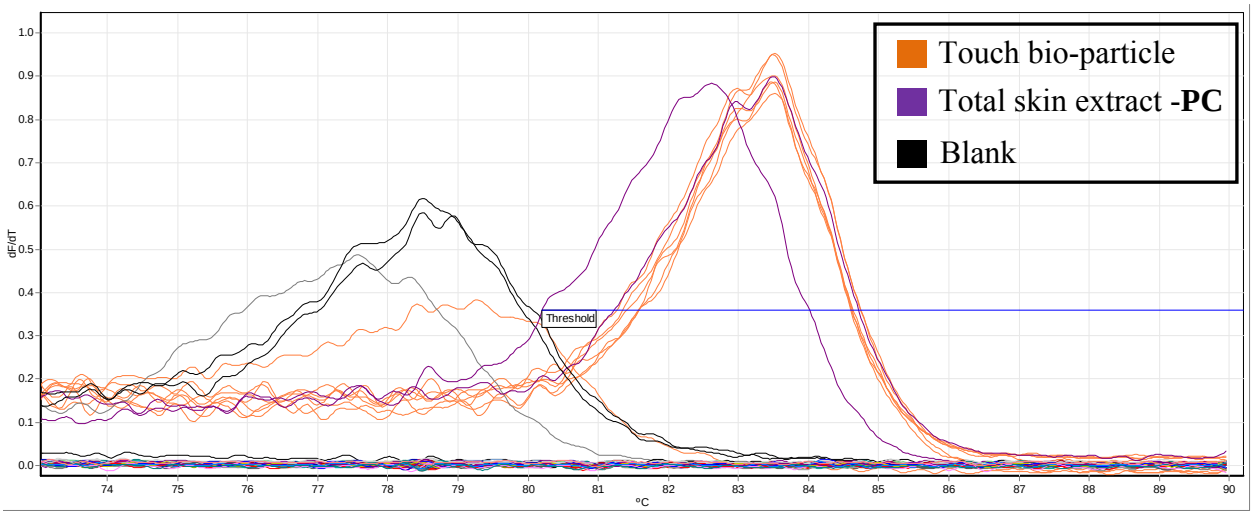
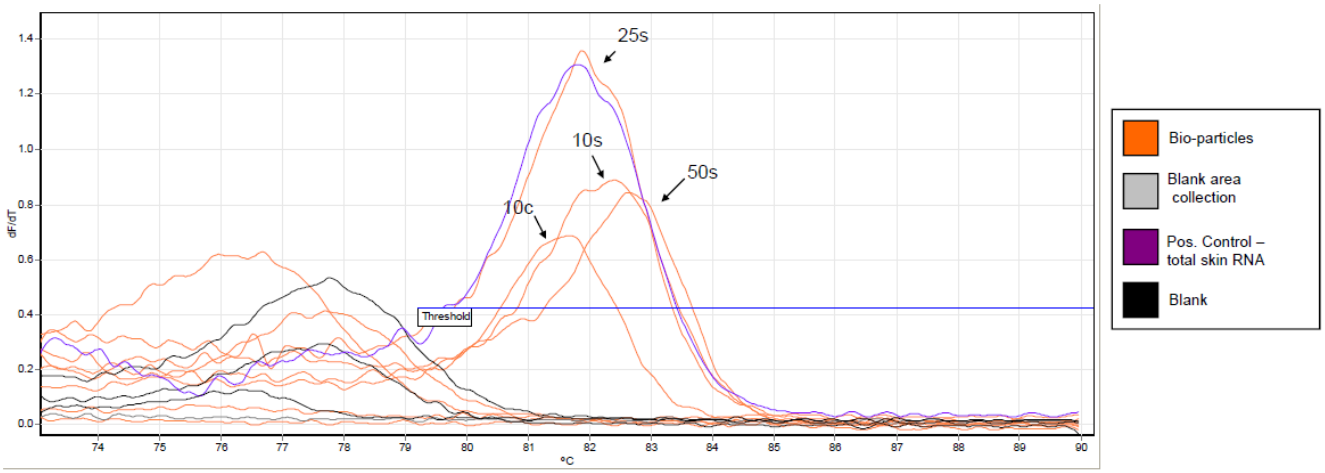


**Figure 44. HRM Analysis of Touch Bio-Particles from a Chair Armrest.** Bio-particle (singles (10, 25 and 50) and "clumps: (1, 5, 10, 20) from a chair armrest were collected using micro-manipulation. RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex LCE1C pre-amplification. Pre-amplifications products were then used in a subsequent singleplex LCE1C HRM assay. The melt plots for all samples are shown. X-axis – Tm (°C); Y-axis – dF/dT). The table indicates the Tm value obtained for all samples. Grey cells represent no detection.




| No. | Name | Peak 1 | Peak 2 |
|---|---|---|---|
| 9 | chair-10s | 81.95 | 82.4 |
| 10 | chair-25s | 79.95 | 81.88 |
| 11 | chair-50s | 82.63 | |
| 12 | chair-1c | | |
| 13 | chair-5c | | |
| 14 | chair-10c | 81.68 | |
| 15 | chair-20c | | |
| 16 | chair-blank | | |
| 17 | SK1(+)-PA-PC | 82.55 | |
| 18 | RTB(+) | | |
| 19 | pre-amp-bl | | |
| 20 | SK1(+)-HRM-PC | 79.73 | 81.8 |
| 21 | BLANK | | |

**Figure 45. 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 – 10 cells, blue; skin – 1 "clump", peach; vaginal – 10 cells, green) are shown to demonstrate the successful use of the triplex assay for tissue/fluid source of origin determination for isolated cells and bio-particles. The melt plots for all samples are shown overlaid to indicate relative positions of each biomarker in the triplex reaction. The observed Tm value for each biomarker is provided. X-axis – Tm ($^{o}$C); Y-axis – dF/dT).

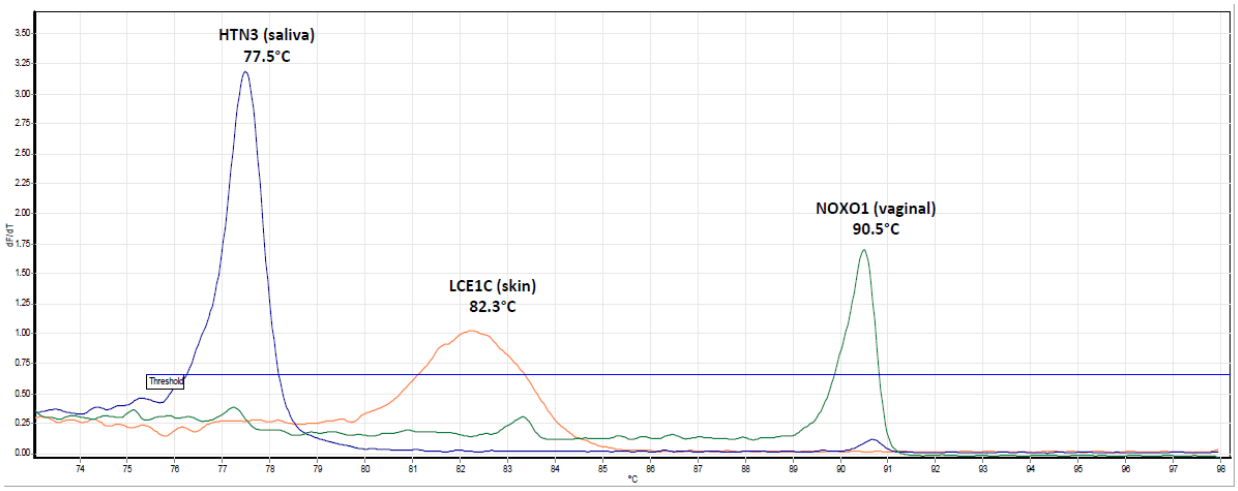
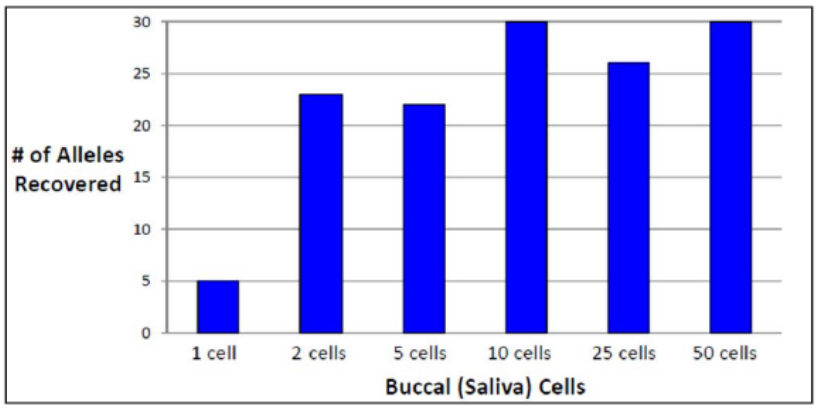
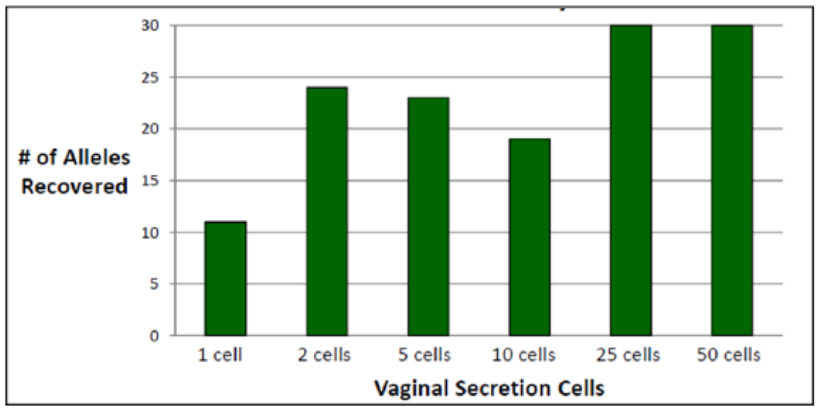


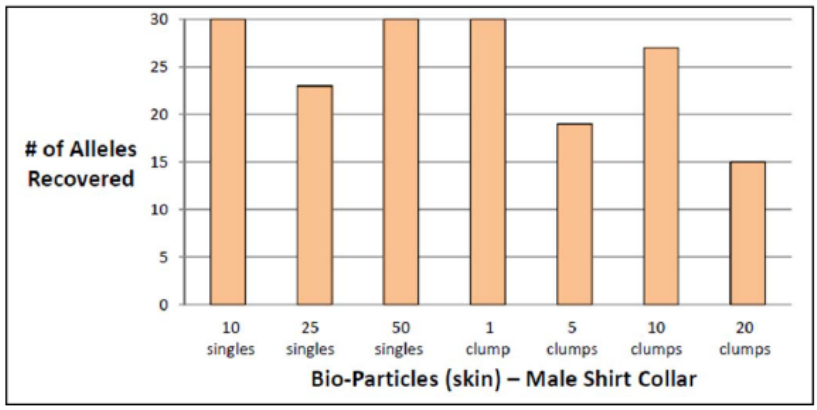
**Figure 46. DNA/RNA Co-Isolation from Isolated Buccal, Vaginal and Skin Cells.** Buccal and vaginal cells (1, 2, 5, 10, 25 and 50 cells) and skin bio-particles (10, 25 and 50 single bio-particles and 1, 5, 10 and 20 clumps) were collected using micro-manipulation and analyzed using DNA/RNA co-isolation micro-volume assays. STR profiles were obtained from DNA lysates (Identifiler Plus, 34 cycles) and the number of alleles observed are provided (profile accuracy was confirmed by comparison to reference samples). RNA was isolated from the recovered bio-particles using a reduced volume direct RT reaction, followed by singleplex pre-amplification (saliva – HTN3, vaginal – NOXO1; skin – LCE1C). Pre-amplifications products were then used in a subsequent singleplex HRM assays. The table indicates the Tm value obtained for all samples. Grey cells represent no detection.




| No. | Name | HTN3 |
|---|---|---|
| 1 | SA10K-1cell | |
| 2 | SA10K-2cells | |
| 3 | SA10K-5cells | 77.1 |
| 4 | SA10K-10cells | |
| 5 | SA10K-25cells | 77.1 |
| 6 | SA10K-50cells | 77.4 |
| 7 | SA10K-0cells | |
| 8 | SA2K(+)-RT-PC | 77.2 |
| 9 | RTB(+) | |
| 10 | pre-amp-bl | |
| 11 | SA6K(+)-HRM-PC | |
| 12 | BLANK | |




| No. | Name | NOX01 |
|---|---|---|
| 1 | 1 cell | |
| 2 | 2 cells | 90.6 |
| 3 | 5 cells | |
| 4 | 10 cells | 90.6 |
| 5 | 25 cells | 90.5 |
| 6 | 50 cells | 90.5 |
| 7 | 0 cells | |
| 8 | FM14(+)-PA-PC | 90.0 |
| 9 | RTB(+) | |
| 10 | pre-amp-bl | |
| 11 | FM14(+)-HRM-PC | 89.8 |
| 12 | BLANK | |




| No. | Bio-particles | LCE1C |
|---|---|---|
| 1 | 10 singles | |
| 2 | 25 singles | 83.0 |
| 3 | 50 singles | 83.2 |
| 4 | 1 clump | |
| 5 | 5 clumps | 83.2 |
| 6 | 10 clumps | |
| 7 | 20 clumps | 83.2 |
| 8 | 0 cells | |
| 9 | SK2(+)-PA-PC | 83.2 |
| 10 | RTB(+) | |
| 11 | SK3(+)-HRM-PC | 82.3 |
| 12 | BLANK | |

## IV. CONCLUSIONS

### A. Discussion of findings

The true nature of touch DNA evidence has remained elusive, generally perceived to be the result of DNA obtained from shed skin cells yet never confirmed with scientific certitude. This is largely due to the perception that it is not possible to ascertain the tissue source of origin of the biological material in touch DNA evidence. The current study has provided the forensic community with feasible methods to identify the tissue source of origin of touch DNA samples and obtain STR profiles of the donor of the biological material.

A major objective of the current work was to perform a comprehensive characterization of the biological cellular material present on touched objects which, in many cases, will presumably be skin. We have collected and analyzed over 3,000 images of the biological (and non-biological material) present in various clothing items, household items and touched objects: shirt sleeve, shirt collar, shirt back, shirt front, pants (upper thigh), pants (leg), sock (ankle), sock (bottom), underwear (front), underwear (back), pillow case (both sides of pillow), sheets, towel, hat and couch, cell phones, computer mouse, door handles, car steering wheels, water bottles and fingerprints. Through the collection of these images, we have developed the first touch DNA micro-particle atlas. These images permitted an evaluation of the morphological features of bio-particles in touch DNA evidence and allowed us to assess the type (e.g. single vs "clumps") and quantity of cellular material present. This atlas could be a useful reference material for operational crime laboratories in support of their analysis of touch DNA evidence.

In order to circumvent the challenges with standard recovery and analysis methods for touch DNA evidence, we have developed a lower-cost, 'smart analysis' method that results in enhanced genetic analysis of touch DNA evidence (e.g. worn clothing items, touched/handled objects, skin/skin mixtures). A one-step 5µl micro-volume lysis/STR amplification reaction was developed that permits the recovery of full or probative STR profiles of the donor of single (or few) bio-particles recovered from touch DNA evidence (e.g. worn clothing items and other household items, touched/handled objects and surfaces, skin/skin mixtures). The use of individual or few (i.e. "clumps") of bio-particles results in the ability to obtain single source profiles. Importantly, even with the collection of clumped bio-particles, admixed DNA profiles were infrequently observed. Additionally, we did not see significant affects from "cell-free" DNA. We demonstrated the successful use of our developed "smart" analysis methods for touch DNA evidence with the ability to recover probative single source profiles from single and "clumped" bio-particles from various touched objects and clothing items. With the feasibility of this method demonstrated, we then used this approach for the detection of male donor DNA (single source) in simulated physical contact/assault mixture samples (e.g. perpetrator grabbing a victim's wrist, neck or clothing, or contact with victim's bedding as in sexual assaults). Probative profiles were obtained from 33% and 40% of the single and clumped bio-particles tested and the male donor was detected in every mixture sample tested.

We have identified novel highly specific mRNA biomarkers for the identification of skin. Gene candidates were identified using both literature searches and whole transcriptome deep sequencing (RNA-seq). Utilizing this dual approach, we identified over 100 gene candidates. Five mRNA markers were identified that demonstrated a high degree of specificity for skin. Using these markers, we were able to successfully detect and identify skin using as little as 5-25 pg of input total RNA from skin, and significantly, in swabs of human skin and various

touched objects. One of the markers, LCE1C, is particularly highly sensitive and was detected in the majority of skin samples tested including touched objects. With highly sensitive and specific biomarkers for the identification of skin, we then developed enhanced RNA profiling strategies suitable for use with few isolated bio-particles and cells. We developed micro-volume cell lysis/reverse transcription reactions, combined with subsequent RNA analysis using capillary electrophoresis (CE) or high resolution melt analysis (HRM) body fluid identification assays, to provide the ability to identify the body fluid origin of individual or few isolated bio-particles. We developed a simple pre-amplification step prior to RNA product detection to improve sensitivity. The pre-amplification method included the use of a triplex pre-amplification step to permit the simultaneous amplification of epithelial cell biomarkers for the identification of skin, vaginal secretions and saliva. This would permit an identification of the tissue source of origin of most isolated epithelial cells. We have also developed an initial DNA/RNA co-isolation strategy to permit tissue source identification and STR profiling from small numbers of bio-particles and cells from touch DNA evidence.

**B. Implications for policy and practice**

Analysis of 'touch DNA' evidence presents a complex challenge for forensic laboratories. A majority of the studies involving attempts to improve the analysis of 'touch DNA' have largely circumvented an identification of the tissue source of origin of the recovered biological material (i.e. skin vs. buccal epithelial cells). Often an identification of the tissue source of biological material present can be crucial to the investigation and prosecution of a criminal investigation. This work sought to help the forensic community in this regard by characterizing the biological material in touch or contact DNA evidence and, unlike all other forensic touch DNA studies, providing strategies for a subsequent molecular identification of the presence of skin, thereby yielding significantly more probative information from trace evidentiary items. Additionally, the strategies for efficient removal of individual biological micro-particles reduce the occurrence of admixed profiles resulting from potential secondary and tertiary transfers. The methodologies developed in this work may also have implications for the analysis of trace biological material in sexual assault cases such as those involving digital penetration where positive identification of trace amounts of skin cells from the perpetrator could be crucial to establish that sexual contact occurred.

**C. Implications for further research**

This work sought to aid the forensic community in the analysis of touch DNA evidence by characterizing the biological material in touch or contact DNA evidence, providing enhanced collection and STR profiling strategies for the analysis of biological material in touch DNA evidence and providing strategies for a subsequent molecular identification of the presence of skin, thereby yielding significantly more probative information from trace evidentiary items.

The "micro-particle atlas" that contains thousands of images of bio-particles recovered from various clothing items, household items and touched objects. These images permitted an evaluation of the morphological features of bio-particles in touch DNA evidence and allowed us to assess the type (e.g. single vs "clumps") and quantity of cellular material present. We are currently attempting to make the atlas available on the Web so that it will be accessible to the

forensic community. Additionally, while the atlas contains thousands of images from touch DNA evidence, it can be expanded to include additional objects of interest.

We have developed micro-volume cell lysis/reverse transcription reactions, combined with subsequent RNA analysis using capillary electrophoresis (CE) or high resolution melt analysis (HRM) body fluid identification assays, to provide the ability to identify the body fluid origin of individual or few isolated bio-particles. We have also developed an initial DNA/RNA co-isolation strategy to permit tissue source identification and STR profiling from the same sample including bio-particles from touch DNA evidence. Further optimization and validation of the co-isolation approach is required. Additionally, alternative cell type identification strategies, such as the use of RNA hybridization probes, could be developed that would permit tissue source determination prior to collection.

The enhanced one-step micro-volume (5µl) lysis/STR amplification reactions that was developed in this work permits the recovery of full or probative STR profiles of the donor of single or few bio-particles. We demonstrate the ability to obtain single source STR profiles from single- and multi-source touch DNA evidence (e.g. worn clothing items and other household items, touched/handled objects and surfaces, skin/skin mixtures). We also demonstrate the ability to apply the developed collection and micro-volume profiling strategies to detect the male donor in simulated physical assault mixture samples. This approach could be further evaluated in additional mixture scenarios such as digital penetration in which only a few bio-particles from the assailant would be present amongst an overwhelming amount of biological material from the victim. Additionally, since this approach developed in the current study permits analysis at the single bio-particle level, it could be used to gain a better understanding of the nature and extent of secondary transfer. A blind-swabbing approach to the analysis of secondary transfer may fail to identify trace amounts of material from the secondary donor. The approach developed here permits the analysis of single or few bio-particles and therefore the results are not confounded by the presence of an overwhelming amount of biological material from the primary donor.

While the focus of the current work was in the characterization and analysis of touch DNA evidence, the strategies developed here can be applied to other sources of biological material and offer an opportunity to obtain genetic information at the single cell level.

## V. REFERENCES


[1] B. Budowle, A.J. Eisenberg, D.A. van, Validity of low copy number typing and applications to forensic science, Croat.Med.J. 50 (2009) 207-217.

[2] N. Gilbert, Science in court: DNA's identity crisis, Nature. 464 (2010) 347-348.

[3] J. Gilder, R. Koppl, I. Kornfield, D. Krane, L. Mueller, W. Thompson, Comments on the review of low copy number testing, Int J Legal Med. 123 (2009) 535-536.

[4] A. Lowe, C. Murray, J. Whitaker, G. Tully, P. Gill, The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces, Forensic Sci Int. 129 (2002) 25-34.

[5] J.A. Bright, S.F. Petricevic, Recovery of trace DNA and its application to DNA profiling of shoe insoles, Forensic Sci.Int. 145 (2004) 7-12.

[6] M. Goray, E. Eken, R.J. Mitchell, R.A. van Oorschot, Secondary DNA transfer of biological substances under varying test conditions, Forensic Sci Int Genet. 4 (2010) 62-67.

[7] D. Hall, M. Fairley, A single approach to the recovery of DNA and firearm discharge residue evidence, Sci Justice. 44 (2004) 15-19.

[8] B.C. Pang, B.K. Cheung, Double swab technique for collecting touched evidence, Leg.Med (Tokyo). 9 (2007) 181-184.

[9] S.F. Petricevic, J.A. Bright, S.L. Cockerton, DNA profiling of trace DNA recovered from bedding, Forensic Sci.Int. 159 (2006) 21-26.

[10] R.A. Wickenheiser, Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact, J.Forensic Sci. 47 (2002) 442-450.

[11] M.K. Balogh, J. Burger, K. Bender, P.M. Schneider, K.W. Alt, STR genotyping and mtDNA sequencing of latent fingerprint on paper, Forensic Sci Int. 137 (2003) 188-95.

[12] A. Barbaro, P. Cormaci, A. Teatino, M.A. La, A. Barbaro, Anonymous letters? DNA and fingerprints technologies combined to solve a case, Forensic Sci Int. 146 Suppl (2004) S133-S134.

[13] A. Castello, M. Alvarez, F. Verdu, DNA from a Computer Keyboard, Forensic Science Communications. 6 (2004).

[14] K.M. Horsman-Hall, Y. Orihuela, S.L. Karczynski, A.L. Davis, J.D. Ban, S.A. Greenspoon, Development of STR profiles from firearms and fired cartridge cases, Forensic Sci Int.Genet. 3 (2009) 242-250.

[15] R.A. van Oorschot, M.K. Jones, DNA fingerprints from fingerprints, Nature. 387 (1997) 767.

[16] N. Port, V. Bowyer, E. Graham, M. Batuwangala, G. Rutty, How Long Does It Take a Static Speaking Individual to Contaminate the Immediate Environment?, Forensic Science, Medicine, and Pathology. 2 (2005) 157-163.

[17] C. Chao, M. Wan, L. Morawska, G. Johnson, Z. Ristovski, M. Hargreaves, K. Mengersen, S. Corbett, Y. Li, X. Xie, D. Katoshevski, Characterization of expiration air jets and droplet size distributions immediately at the mouth opening, Aerosol Science. 40 (2009) 122-133.

[18] F. Alessandrini, M. Cecati, M. Pesaresi, C. Turchi, F. Carle, A. Tagliabracci, Fingerprints as evidence for a genetic profile: morphological study on fingerprints and analysis of exogenous and individual factors affecting DNA typing, J.Forensic Sci. 48 (2003) 586-592.
[19] T. Kita, H. Yamaguchi, M. Yokoyama, T. Tanaka, N. Tanaka, Morphological study of fragmented DNA on touched objects, Forensic Sci Int.Genet. 3 (2008) 32-36.
[20] R.a.G. R v.Reed, [Nos: 2007/04708/B3, 2007/04710/B3, 2007/04800/D4], High Court Justice Court of Appeal (Criminal Division), Royal Courts of Jusic, Strand, London, WC2A, 2LL.2009).
[21] People v.Megnath, [Ind. No. 917/07], Supreme Court, State of New York, Criminal Term, TAPC, Queens County.2010).
[22] C. Haas, B. Klesser, C. Maake, W. Bar, A. Kratzer, mRNA profiling for body fluid identification by reverse transcription endpoint PCR and realtime PCR, Forensic Sci Int Genet. 3 (2009) 80-88.
[23] J. Juusola, J. Ballantyne, Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification, Forensic Sci Int. 135 (2003) 85-96.
[24] J. Juusola, J. Ballantyne, Multiplex mRNA profiling for the identification of body fluids, Forensic Sci Int. 152 (2005) 1-12.
[25] J. Juusola, J. Ballantyne, mRNA profiling for body fluid identification by multiplex quantitative RT-PCR, J Forensic Sci. 52 (2007) 1252-1262.
[26] E.K. Hanson, J. Ballantyne, Highly specific mRNA biomarkers for the identification of vaginal secretions in sexual assault investigations, Sci Justice. 53 (2013) 14-22.
[27] L.M. Milstone, Epidermal desquamation, J Dermatol.Sci. 36 (2004) 131-140.
[28] M. Darmon, M. Blumenberg, Molecular biology of the skin - the Keratinocyte, Academic Press, San Diego, 1993.
[29] E.K. Hanson, J. Ballantyne, "Getting blood from a stone": ultrasensitive forensic DNA profiling of microscopic bio-particles recovered from "touch DNA" evidence, Methods Mol.Biol. 1039 (2013) 3-17.
[30] K. Kelley-Primozic, Sperm Recovery - New Sperm micromanipulation and isolation technique may increase sexual-assault convictions, www.EvidenceMagazine.com. November-December (2008).
[31] E. Hanson, C. Haas, R. Jucker, J. Ballantyne, Specific and sensitive mRNA biomarkers for the identification of skin in 'touch DNA' evidence, Forensic Sci Int Genet. 6 (2012) 548-558.
[32] B. Jackson, C.M. Tilli, M.J. Hardman, A.A. Avilion, M.C. MacLeod, G.S. Ashcroft, C. Byrne, Late cornified envelope family in differentiating epithelia--response to calcium and ultraviolet irradiation, J.Invest Dermatol. 124 (2005) 1062-1070.
[33] D. Marshall, M.J. Hardman, K.M. Nield, C. Byrne, Differentially expressed late constituents of the epidermal cornified envelope, Proc.Natl.Acad.Sci U.S.A. 98 (2001) 13031-13036.
[34] S. Fujimoto, H. Uratsuji, H. Saeki, S. Kagami, Y. Tsunemi, M. Komine, K. Tamaki, CCR4 and CCR10 are expressed on epidermal keratinocytes and are involved in cutaneous immune reaction, Cytokine. 44 (2008) 172-178.
[35] J. Morales, B. Homey, A.P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N.G. Copeland, N.A. Jenkins, L.M. McEvoy, A. Zlotnik, CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells, Proc.Natl.Acad.Sci U.S.A. 96 (1999) 14470-14475.

[36] S.J. Busfield, C.A. Comrack, G. Yu, T.W. Chickering, J.S. Smutko, H. Zhou, K.R. Leiby, L.M. Holmgren, D.P. Gearing, Y. Pan, Identification and gene organization of three novel members of the IL-1 family on human chromosome 2, Genomics. 66 (2000) 213-216.
[37] M.F. Nold, C.A. Nold-Petry, J.A. Zepp, B.E. Palmer, P. Bufler, C.A. Dinarello, IL-37 is a fundamental inhibitor of innate immunity, Nat.Immunol. 11 (2010) 1014-1022.
[38] S.L. Taylor, B.R. Renshaw, K.E. Garka, D.E. Smith, J.E. Sims, Genomic organization of the interleukin-1 locus, Genomics. 79 (2002) 726-733.
[39] E.K. Hanson, J. Ballantyne, Rapid and inexpensive body fluid identification by RNA profiling-based multiplex High Resolution Melt (HRM) analysis, F1000Res. 2 (2013) 281.

## VI. DISSEMINATION OF RESEARCH FINDINGS

### A. Publications

1. Hanson, E.K., Haas, C., Jucker, R. and Ballantyne, J. (2011). Identification of Skin in Touch/Contact Forensic Samples by Messenger RNA Profiling. Forensic Sci Int Genet. Genetics Supplement Series, 3, e305-e306.
2. Hanson E, Haas C, Jucker R and Ballantyne J. Specific and Sensitive mRNA Biomarkers for the Identification of Skin in 'Touch DNA' Evidence. Forensic Sci Int Genet doi:10.1016/j.fsigen.2012.01.004. (2012) 6(5): p. 548-558.
3. Hanson, E.K., and Ballantyne, J. "Getting Blood from a Stone": Ultrasensitive Forensic DNA Profiling of Microscopic Bio-Particles Recovered from "Touch DNA" Evidence. Nucleic Acid Detection: Methods and Protocols, Methods in Molecular Biology. Vol 1039, Springer Science. 2013.

*Manuscripts in preparation (will be submitted for publication by project end date)*

1. *Subject:* Detection of Male Donor DNA in Simulated Physical Contact/Assault Mixture Samples Using Enhanced One-Step Micro-Volume DNA Profiling of Isolated Bio-Particles. Expected submission – June 2014.
2. *Subject:* Ultrasensitive Forensic DNA Profiling of Microscopic Bio-Particles Recovered from "Touch DNA" Evidence. Expected submission – July/August 2014. JoVe – Journal of Visualized Experiments.
3. *Subject:* Identification of Skin as the Tissue Source of Origin of Bio-particles Isolated from Touch DNA Evidence. Expected submission – Fall 2014.

### B. Presentations

1. Identification of Skin in Touch/Contact Forensic Samples by Messenger RNA Profiling. Hanson E., Haas, C., Jucker, R., Ballantyne, J. 24th World Congress of the International Society of Forensic Genetics, Vienna, Austria.
2. Ballantyne, J. 2011. Forensic Biology Research at the National Center for Forensic Science. European DNA Analysis Profiling (EDNAP) Meeting, Athens, Greece
3. Development of Optimized Recovery and DNA Typing Methodologies for the Analysis of "Touch and Contact" DNA Samples. Hanson, E., Kelley-Primozic K., Vigil, B., Bisbing, R. and Ballantyne, J. AAFS Annual Meeting, Atlanta, GA.
4. Identification of Skin in Touch/Contact Forensic Samples by Messenger RNA Profiling. Ballantyne J and Hanson EK. Association of Forensic DNA Administrators and Analysts (AFDAA), San Antonio, TX.
5. Ballantyne, J. 2012. Advanced Topics in Forensic DNA Analysis. Forensic Science Training for Capital Defense Attorneys: Advanced Issues. Bureau of Justice Assistance and the National Clearing House for Science, Technology and the Law, Las Vegas NV.

6. Farash, K., Morgan, B., DeVore, A., Hanson, E.K., Ballantyne, J. Enhanced Genetic Analysis of Bio-Particles Isolated from Single- and Multi-Source Touch DNA evidence Using Micro-Volume DNA/RNA Profiling Strategies. 24th International Symposium on Human Identification. Atlanta, GA.

*Scheduled/Upcoming*

1. Farash, K., Hanson, E and Ballantyne, J. Detection of Male Donor DNA in Simulated Physical Contact/Assault Mixture Samples Using Enhanced One-Step Micro-Volume DNA Profiling of Isolated Bio-Particles. International Symposium on Human Identification (ISHI), Sept 29 – Oct 2, 2014, Phoenix, AZ (submitted for an oral presentation).
2. Farash, K., Hanson, E and Ballantyne, J. Detection of Male Donor DNA in Simulated Physical Contact/Assault Mixture Samples Using Enhanced One-Step Micro-Volume DNA Profiling of Isolated Bio-Particles. 20th World Meeting of the International Association of Forensic Sciences (IAFS), Oct 12-18, 2014, Seoul, Korea (submitted for an oral presentation).
3. EDNAP Fall Meeting. Presentation title – to be determined. Zurich, Switzerland. November, 2014.
4. 67th Annual American Academy of Forensic Science Meeting. Presentation title – to be determined. Orlando, FL 2015.