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KVALITATIVNA I KVANTITATIVNA ANALIZA HUMANIH BIOLOŠKIH TRAGOVA MINIMALNIH GRANIČNIH KOLIČINA U FORENZIČKIM ANALIZAMA DNK

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QUALITATIVE AND QUANTITATIVE ASSESMENT OF BIOLOGICAL TRACES IN FORENSIC DNA ANALYSIS

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1. Ostojic, L., Klempner, S.A., Patel, R.A., Mitchell, A.A., Axler-DiPerte, G.L., Wurmbach, E., (2014): Qualitative and quantitative assessments of single fingerprints in forensic DNA analysis, *Electrophoresis*, 35, 3165-3172. doi: 10.1002/elps.201400177.

2. Ostojic, L. and Wurmbach, E., (2017): Analysis of fingerprint samples, testing various conditions, for forensic DNA identification, *Science and Justice*, 57, 35-40. doi: 10.1016/j.scijus.2016.08.009.

KVALITATIVNA I KVANTITATIVNA ANALIZA HUMANIH BIOLOŠKIH TRAGOVA MINIMALNIH GRANIČIH KOLIČINA U FORENZIČKIM ANALIZAMA DNK

SAŽETAK

Ćelije kože ostavljene na površini bilo kog predmeta nakon kontakta sa njim mogu biti izvor DNK materijala. Ova vrsta biološkog materijala, u odnosu na količinu DNK koju je moguće izolovati iz nje, obično je mnogo manje izdašna u odnosu na tragove koji se rutinski analiziraju (krv, semena tečnost, pljuvačka). Iako visoko osetljive tehnike analize omogućavaju dobijanje rezultata iz minimalnih količina DNK, još uvek postoje znatne poteškoće u radu sa ovakvim uzorcima, i to iz više razloga. Različit kvalitet i kvantitet izolovane DNK, te posebno izraženi stohastički efekti samo su neki od njih. Zbog toga je proces analize DNK materijala izolovan iz ovakvih uzoraka posebno kompleksan. Biološki uzorci ove vrste se vrlo često nalaze u izuzetno malim količinama (doslovno tragovima), što se postojećim rutinskim metodama analize DNK ne mogu pouzdano interpretirati.

U cilju poboljšavanja rezultata analize DNK materijala dobijenog izolovanjem biološkog materijala zaostalog na površini dodirnutih predmeta, analizirano je više od 700 otisaka prstiju. Svaki korak procesa je testiran posebno u cilju dobijanja STR profila koji su bar 70% kompletni, te stoga mogu biti uneti u baze podataka. Kompletnost profila je određivana na osnovu broja dobijenih alela iz 15 testiranih lokusa (najviše moguće 30 alela). Testirani su sledeći koraci u proceduri dobijanja DNK profila: prikupljanje uzoraka, izolovanje DNK, umnožavanje STR lokusa kao i detekcija proizvoda umnožavanja kapilarnom elektroforezom. Ćelije su prikupljene korišćenjem mikrosfera, lepljivih traka, lepila ili pamučnih briseva natopljenih različitim rastvorima. DNK materijal je izolovan korišćenjem interno razvijenih metoda i/ili komercijalno dostupnih kompleta hemikalija za njenu izolaciju. Izolovana DNK je umnožavana *Identifiler*® kompletom, korišćenjem različitog broja PCR ciklusa.

Studija je rezultirala utvrđivanjem najoptimalnije metode za utvrđivanje STR profila pogodnih za unošenje u baze podataka, i to u gotovo 70% analiziranih uzoraka. Odabrani metod testiranja uzoraka i analize DNK materijala podrazumevao je prikupljanje biološkog materijala iz otisaka prstiju korišćenjem pamučnih briseva natopljenih rastvorom deterdženta, izolovanje DNK korišćenjem komercijalno dostupnih kompleta za izolaciju, čiji se mehanizam zasniva na enzimskoj aktivaciji na visokim temperaturama, umnožavanje DNK koriščenjem većeg broja PCR ciklusa, i analiza kapilarnom elektroforezom sa parametrima dužeg injekcionog vremena i višeg električnog napona.

U okviru ove studije ispitivan je i uticaj vremena proteklog od ostavljanja otisaka prstiju do dobijanja DNK profila. Pokazano je da je kompletnost DNK profila obrnuto srazmerna vremenu proteklom od ostavljanja uzorka, ali tek nakon 3-10 dana od momenta ostavljanja otisaka prsta. Pokazano je, takođe, da je u 17% ispitivanih uzoraka dobijen kompletan DNK profil i nakon 40 dana od ostavljanja otiska.

Poznato je da vrsta materijala na kome je ostavljen otisak prsta može da menja kvalitet dobijenog DNK profila, i to direktnim uticajem na neki od koraka analize biološkog materijala. Zbog toga su u ovom eksperimentu kao površine na koje su ostavljani otisci prstiju korišćeni različiti materijali, i to oni koji se najčešće koriste u kućnim i kancelarijskim uslovima, kao što su staklo, plastika, hartija i metal (kovani novac). Najveći broj DNK profila sa najmanje 70% utvrđenih alela je dobijen analizom biološkog materijala iz otisaka prstiju sa stakla (62%), plastične mase (25%), te hartije (12.5%). DNK profili pogodni za unos u baze podataka nisu dobijeni analizom biološkog materijala iz otisaka prstiju ostavljenih na metalnom novcu.

Kontaktni tragovi na predmetima veoma često se karakterišu mešavinom DNK materijala osoba koje ne mogu biti međusobno razlučene. Analize mešavina bioloških uzoraka poreklom od istih tipova ćelija je uvek kompleksna zbog većeg broja alela koje nose različiti donori biološkog materijala nađenih u mešavini, te disbalansa njihovog intenziteta. Na taj način, izuzetno je složeno odrediti DNK profile pojedinačnih donora sa sigurnošću. U ovoj studiji analizirane su mešavine DNK materijala, ostavljene na 36 staklenih boca koje su uzastopno držane u rukama tri osobe. Umesto rutinske metode prikupljanja biološkog materijala uzimanjem jednog brisa sa čitave površine boce, usitnjene su ciljne površine, i vršeno je uzimanje briseva

sa 6 različitih regiona svake boce ponaosob. Najveći broj utvrđenih profila prikupljenih sa boca na ovaj način je pokazao prisustvo biološkog materijala dva ili samo jednog donora ("čist profil"). Ustanovljeno je da ovakav pristup uzorkovanja smanjuje mogućnost dobijanje kompleksnih mešavina koje sadrže DNK profile koje je vrlo često nemoguće pouzdano interpretirati. Analiza više od 200 ovakvih uzoraka pokazala je nedvosmisleno da je moguće dobiti bar 2 različita DNK profila sa istog predmeta.

Način uzimanja biološkog materijala, "po sekcijama" površina, može da se primeni i u slučajevima drugih vrsta ćelija, kao što su epitelne ćelije sa drugih lokacija ljudskog organizma, te semene ćelije koje mogu biti prisutne kod slučajeva sa više lica uključenih u seksualni delikt. Fizičko razdvajanje ćelija sa površina pre izolovanja DNK materijala pojednostavljuje analizu *de facto* mešavina biološkog materijala. U ovoj studiji su testirane različite tehnike manipulacije ćelijama, i to mikroskopom *Axiozoom* .V16 uz robotsku (aureka® sistem) i manuelnu manipulaciju ćelija, te mikroskopsko lasersko katapultiranje ćelija *P.A.L.M.*® mikroskopskim sistemom. Primena svake od ovih metoda se pokazala uspešnom za utvrđivanje DNK profila podobnih za unos u baze podataka, i to iz samo 10 ćelija koje poseduju jedro. *Axiozoom* .V16 mikroskop uz primenu *aureka*® sistema je pokazao dobre rezultate pri izolaciji ćelija sa različitih predmeta, bioloških razmaza, lepljivih traka i slično. Isti mikroskopski sistem uz primenjenu ručnu manipulaciju ćelijama je takođe veoma pouzdan, ali mnogo zavisi od veštine operatera. Metod prikupljanja i razdvajanja biološkog materijala korišćenjem *P.A.L.M.*® mikroskopskog sistema je veoma jednostavan, efikasan i brz, ali zahteva posebnu pripremu biološkog razmaza ćelija, što ograničava upotrebu u forenzici.

Analizom DNK izolovane iz biološkog materijala iz otisaka prstiju kao primera kontaktnih tragova, ova studija pruža značajan doprinos boljem razumevanju problema forenzičkih uzoraka, posebno onih sa minimalnim graničnim količnama DNK materijala, te predlaže efikasnije načine tretiranja ove vrste bioloških tragova. Metod skupljanja biološkog materijala "po sekvencama" sa različitih predmeta posebno obećava kada je u pitanju interpretacija DNK profila dobijenih iz mešavine bioloških uzoraka. Ovaj metod je veoma koristan u utvrđivanju upotrebljivih DNK profila, podobnih za unošenje u baze podataka, posebno u slučajevima uzoraka tipa

kompleksnih mešavina za koje se ranije smatralo da se ne mogu uspešno analizirati postojećim metodama analize DNK.

QUALITATIVE AND QUANTITATIVE ASSESMENT OF BIOLOGICAL TRACES IN FORENSIC DNA ANALYSIS

SUMMARY

Skin flakes left on an object after it has been touched or handled could be a source of DNA. These skin flakes tend to be deposited in considerably smaller amounts than from routinely tested cells of blood, semen or saliva. Although, highly sensitive DNA analysis procedures are able to provide results from trace amounts of DNA there are still some fundamental difficulties inherent to these samples, including variability in quality and quantity of extracted DNA and exaggerated stochastic effects, making it hard to reliably interpret DNA profiles of these samples. These types of samples could also carry skin flakes in trace, which currently applied methodology of testing frequently cannot interpret.

In order to improve the results from touched DNA samples, over 700 fingerprints were tested. Each step of the workflow for genotyping was assessed with the goal to generate STR profiles that were at least 70% complete and therefore database eligible. The profiles were calculated from the number of obtained alleles with a maximum of 30 for the 15 amplified STR loci. The steps evaluated in the workflow included sample collection, DNA extraction, STR amplification and detection utilizing capillary electrophoresis. Cells were collected using microglobes, tapes, glues, or cotton swabs moistened with different solutions. DNA extraction was assessed with methods designed in the laboratory and commercially available extraction kits. Extracted DNA was amplified with Identifiler® kits using various number of PCR cycles.

These comparisons led to the best method that generated a database eligible STR profiles from almost 70% of tested fingerprints. This method suggested collection of fingerprints by swabbing with cotton swab moistened in detergent solution, then extracting DNA using a commercially available extraction kit that uses enzyme activated at a high temperature, followed by amplification at higher PCR cycle number and analysis at longer injection time and higher voltage during capillary electrophoresis.

Using this efficient method to process fingerprints, the impact of time was investigated. Fingerprints that were deposited for different time intervals, days and weeks, were tested. It was demonstrated that the completeness of DNA profiles declined over time, and it was also shown that the decrease in DNA profile completeness was not significant for 3 days, but at day 10, as well as for longer time periods, this decrease became significant. Nevertheless, it was also found that 17% of the tested fingerprints generated complete DNA profiles even 40 days after the fingerprint deposition.

The type of evidentiary substrates could also have an effect on the quality of DNA profiles by influencing the DNA analysis process. Therefore, fingerprints were deposited on most commonly used substrate types in household and offices, which included glass, plastic, paper and metal (a Quarter dollar). The greatest number of samples with at least 70% complete profiles were generated on glass (62%), followed by plastic (25%) and then paper (12.5%). No profile was obtained from metal.

Touched evidence frequently contains DNA mixtures whose DNA profiles could not be determined. Biological mixtures of the same cell types are challenging for interpretation because profiles of mixed DNA samples can contain multiple alleles at multiple locations. Also, due to allele sharing and imbalance of allele heights, it is often difficult to assign DNA profiles to their individual contributors'. In this study, DNA mixtures were assessed thoroughly by testing 36 bottles, touched by three individuals, consecutively. Instead of sampling the bottle by swabbing it entirely (which is routinely done in forensic laboratories), six sections of the bottle were swabbed and tested separately. The majority of samples from the bottle were either two-person mixtures or single source samples. By taking samples in sections, this approach reduced the complexity of three-person mixture. Examining over 200 samples indicated that this sectioned sampling approach was able to generate two different DNA profiles from the same bottle.

The sectioned sampling approach could also be applied to mixtures of cells of similar type, such as epithelial or sperm cells. Generating multiple samples of a complex mixture could simplify its interpretation. Various methods for collecting cells were tested such as: Axiozoom .V16 microscope handled with robotic manipulator aureka®, or by manual manipulations and P.A.L.M.®. All methods were able to

generate database eligible STR profiles from as little as 10 nucleated cells. Axiozoom .V16 microscope with aureka® manipulations can be very resourceful by isolating cells of various items, smears, tapes, and it generates reproducible results. Axiozoom .V16 microscope with manual manipulations could also have versatile applications, but rely on the dexterity of the operator. P.A.L.M. ® collection of the cells was simple and time efficient, however it could be applied only on a cell smear which limits its application in forensic work.

This study provided more insights on touched samples with thorough fingerprint analysis, and suggested ways to more efficiently process them. The sectioned sampling approach was proven to be a promising method in improving mixed sample interpretation, and was found to have potentials in generating database eligible DNA profiles from evidences that were previously considered unsuitable for forensic identification.

Keywords: fingerprints, touched DNA, short tandem repeats (STRs), biological mixtures, micromanipulations

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CONTENT

1. Abbreviations

ABI	Applied Biosystems
AG	Arabic gum/glycerol spheres
ANOVA	Analysis of variance
bp	base pair
BSA	Bovine Serum Albumin
°C	Degree Celsius
Cm	centimeter
CODIS	Combined DNA Index System
Cu	copper
DAPI	4', 6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphates
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ESS	European Standardization Systems
EtOH	ethanol
FBI	Federal Bureau of Investigation
g	relative centrifugal force
h	hour
HCl	hydrochloric acid
HIDI	highly deionized formamide
HighSens	High Sensitivity DNA extraction protocol
H_2O	water
ID28	AmpF*l*STR® Identifiler® PCR Amplification Kit for 28 cycles
ID31	AmpF*l*STR® Identifiler® PCR Amplification Kit for 31 cycles
ID+	AmpF*l*STR® Identifiler® Plus PCR Amplification Kit for 32 cycles
IRB	Institutional Review Board
LCM	Laser Capture Microdissection
LCN	Low Copy Number
LIZ	GeneScan® 500 Liz® Size Standard

M	molar concentration
$MgCl_2$	magnesium chloride
min	minute
ml	milliliter
mm	millimeter
NaN_3	sodium azide
NFR	Nuclear Fast Red
Ni	nickel
NTC	Non Template Control
NYC	New York City
NYPD	New York Police Department
OCME	Office of the Chief Medical Examiner
P.A.L.M.	Positioning and Ablation with Laser Microbeam
PCR	Polymerase Chain Reaction
PEN	polyethylene-naphthalate
pg	picogram
POP4	performance optimized polymer 4
ProK	Proteinase K
RC	rubber cement spheres
RFU	relative fluorescence unit
rpm	revolutions per minute
s	second
SDS	sodium dodecyl sulphate
SIRCHIE	Fingerprint lifting tape from “SIRCHIE”
SNP	Single Nucleotide Polymorphism
SPSS	Software Package for the Social Sciences
ssDNA	singe stranded Deoxyribonucleic acid
STR	short tandem repeat
SWGDAM	Scientific Working Group on DNA Analysis Methods
SZX	Stereo Zoom X times
U.S.	The United States of America
UV	ultraviolet

Zygem	PrepGEM® Tissue extraction mixture
µl	microliter
9947A	AmpF*l*STR® 9947A control DNA
3D	three-dimensional
%	percent
9/11	September 11

2. Introduction

2.1 Forensic DNA testing

In forensic science DNA profiles are generated by testing highly polymorphic markers on DNA called STRs (Butler, 2007; Dumache et al., 2016; Hameed, 2014). Thus, DNA profiles are also known as STR profiles. STRs are microsatellite markers that consist of repeat nucleotide sequences. Each repeat sequence is built of two to six nucleotides in length, tandemly repeated a specific number of times for an individual (Gill et al., 1985; Hameed, 2014; Jeffreys et al., 1985a, b). The number of repeats in STR markers can be highly variable among individuals, which make STRs effective for human identification purposes (Butler, 2004; Ellegren, 2004; Gill et al., 1985; Jeffreys et al., 1985a, b). STRs are not associated with phenotypic description of a person (Katsanis and Wagner, 2013) and are tested using commercially prepared kits (Butler, 2007; Edwards and Gibbs, 1994). Kit is a standardized chemical and enzymatic mixture that enables simultaneous amplification of multiple STR loci using fluorescently labeled primers. STRs are distinguished from one another with fluorescence detection following electrophoretic separation (Butler, 2005; Edwards and Gibbs, 1994; Mayrand, 1992). These markers were found throughout non coding regions of the human genome, located either on a distinct chromosome, or if they are located on the same chromosome these should be widely separated (Butler, 2006).

In criminal investigation, forensic DNA testing has a significant importance by its ability to generate unique STR profiles from biological material obtained from crime scenes, and through comparison to DNA profiles of known individuals or profiles stored in the database, it could provide identification or exclusion (Roewer, 2013). Other applications of STR profiling were to identify victims of the 9/11 terrorist attack (Biesecker et al., 2005), of natural disasters such as Hurricane Katrina in the United States (Dolan et al., 2009), and of recent wars, such as in the former Yugoslavia in 1990’s (Huffine et al., 2001) or victims of mass killing during the dictatorship in Argentina (1976-1983) (Corach et al., 1997). STRs are also used as tools for a wide range of other applications, for instance, parentage testing, analysis of genetic structure of populations and the assessment of phylogenetic relationships (Montinaro et al., 2012; Yao et al., 2017).

From 2000 to early 2017, forensic biology laboratories were routinely testing sixteen STR loci and thirteen of those were a uniform set of core loci required for inclusion in the Federal Bureau of Investigation's (FBI) CODIS database (Budowle et al., 1999; Butler, 2006), which included: CSF1PO, D3S1358, D5S818, D7S820, D8S1179, D13S317, D16S539, D18S51, D21S11, FGA, TH01, TPOX, and vWA (Butler, 2006). This standardization of STR loci enabled the U.S. forensic testing laboratories to share criminal DNA profiles nationally. It was also possible to internationally exchange this information, due to some overlap in core loci among CODIS and European databases (Gill et al., 2006a; Gill et al., 2006b; Martin et al., 2001; Welch et al., 2012). However, there was an overlap in only 8 loci between these databases, which include: D3S1358, D8S1179, D16S539, D18S51, D21S11, FGA, TH01, and vWA. Therefore, to enable international cooperation, data sharing and to reduce adventitious matches, locus overlap between U.S. and European databases was expanded. This was obtained by adding the following seven ESS (European Standardization System) (Welch et al., 2012) core loci: D1S1656, D2S441, D2S1338, D10S1248, D12S391, D19S433 and D22S1045 to the U.S. database requirement and thus expanding CODIS core loci from 13 to 20 (Hares, 2012; Oostdik et al., 2014). Additional reason for expanding CODIS core loci in the United States was to increase the discrimination power between related individuals and thus aid missing person's investigations (Li et al., 2015; Zaken et al., 2013). In 2017 this expanded system was expected to be in use for upload and search of DNA profiles.

The first multiplexing was obtained by testing only four- STR loci in 1994 (Kimpton et al., 1994; Lygo et al., 1994). The following year it was replaced with a multiplex that tested six-loci (Gill et al., 2015). The 6-plex was made by adding two newly discovered STRs to the profiling kit of the 4-plex, thereby decreasing the match probability between two unrelated individuals from 1 in 10,000 to 1 in 50 million (Gill, 2002). This 6-plex enabled the creation of the National DNA database (Gill et al., 2015). As the database was becoming larger, it was necessary to ensure that the match probability was sufficiently small enough to minimize the chance of two unrelated individuals matching accidentally. In 1999 a new multiplex reaction was applied. It tested ten- loci and further increased the power of discrimination (Gill, 2002; Gill et al., 2015; Jobling and Gill, 2004). The six-loci of the older system were retained to enable continuity of

existing DNA database and to find matches with already entered DNA profiles. Later, new systems appeared testing twelve- and sixteen-loci (Butler, 2006; Gill et al., 2015). 16-plex kits were provided by two companies, Promega Corporation and Applied Biosystems, who released the PowerPlex® 16 in 2000 and the Identifiler® kit in 2001, respectively (Moretti et al., 2001a; Moretti et al., 2001b). In addition to the 13 CODIS core loci and Amelogenin, Powerplex® 16 tested two pentanucleotide loci referred to as Penta D and Penta E (Krenke et al., 2002), while Identifiler® kit tested an additional two tetra-nucleotide loci D2S1338 and D19S433 (Collins et al., 2004). Until recently, the New York City Office of the Chief Medical Examiner laboratory (NYC OCME) was using Identifiler® kit. Although D2S1338 and D19S433 were not considered core loci for database inclusion, they were beneficial for increasing the power of discrimination; they also overlapped with European STR system, and helped with mixture interpretation. 16-plex kits further decreased probability of a match between two unrelated people to less than 10^{-13} (Gill, 2002).

Since 2012, 24-plex kits were commercially available, such as PowerPlex® Fusion 5C provided by Promega Corporation (Oostdik et al., 2014; Verzeletti et al., 2013), and GlobalFiler™ by Life Technologies (Flores et al., 2014; Martin et al., 2014). Also, Investigator® supplied by Qiagen was available in the U.S. since 2015. These kits tested 24-loci using 5-dye technology, which was compatible with the Applied Biosystems® 3130 and 3500 Series Genetic Analyzer capillary electrophoresis instruments, and did not require upgrades to existing collection and analysis software. These kits have made an easy transition from 16-plex to 24-plexes for many forensic biology laboratories. In 2015, Promega provided a 27-plex Powerplex® 6C Fusion system that tested 27 loci in 6 dyes. It contains 20 autosomal loci (expanded CODIS core loci) as well as Amelogenin and DYS391 for gender determination. The Penta D, Penta E and SE33 loci were also included to increase discrimination and allow searching of databases that include profiles with these loci. Finally, two rapidly mutating Y-STR loci, DYS570 and DYS576, were also included to improve mixture interpretation (Ensenberger et al., 2016). However, this 6-dye system required upgrades in electrophoresis instruments, collection and analysis software.

Further addition of STR loci to multiplexes challenges technological limits which are inherent to any technique. Such is the case of PCR amplification especially when more

STR loci are being tested. This may disrupt the efficiency of any single reaction during the amplification and during capillary electrophoresis. In the electrophoresis stage, difficulties may arise in the separation of the dyes, which can result in spectral overlap of very similar size ranges.

Currently, the CODIS database stores over 700,000 unknown forensic profiles on the national level. Offenders and arrestees are almost an additional 15 million profiles. Thereby, it is of paramount importance to generate an STR profile from the crime scene, and through comparisons to already obtained DNA profiles (either from known profiles within the case context or in database) it may lead to identification of a person who left a biological sample at the crime scene.

2.2 Crime scene biological samples

Most commonly tested biological material includes blood, semen, or saliva. Blood could be soaked in fabrics or deposited on items used to commit a crime such as a knife to stab a victim or a baseball bat in an assault case. Semen of an assailant could be found on a victim's intimate clothing or swabs taken from body cavities of sexually assaulted persons, and saliva is often collected as a dried secretion from a victim's body, a bottle or of cigarette butts left by an assailant who smoked at the crime scene. STR profiles could also be obtained from items without biological staining (Schneider et al., 2011). These STR profiles are generated from objects that have been touched or worn by an individual (Balogh et al., 2003b; Bright and Petricevic, 2004; Wiegand and Kleiber, 1997). Handling weapons or other objects associated with a crime, touching surfaces, or wearing clothing, may represent sufficient contact to transfer a small number of DNA bearing cells to enable STR analysis (Alessandrini et al., 2003; Caragine et al., 2009). Apart from blood, semen or saliva stains, which are typically in abundance of DNA and tend to successfully generate STR profiles (Verheij et al., 2012), touched samples produce variable results, including poor profiles or no results (Ostojic et al., 2014; Templeton and Linacre, 2014; van Oorschot et al., 2010).

2.3 DNA testing of touched samples

Touched samples usually contain less than 100 pg of DNA (Kloosterman and Kersbergen, 2003), and often fail to generate conclusive profiles after 28 cycles of

amplification with routine testing methodology. Therefore, additional measures were implemented in order to improve sensitivity and increase the chance to obtain usable STR profiles. Most commonly is increasing the number of PCR cycles, such as to 30 (Roeder et al., 2009), 31 (Caragine et al., 2009) or even 34 in some laboratories (Gill et al., 2015; Kloosterman and Kersbergen, 2003; Phipps and Petricevic, 2007; Roeder et al., 2009). Further increase in cycle number did not provide an advantage but increased the number of artifacts (Gill et al., 2000). The approach of increasing sensitivity of testing by adding PCR cycles could lead to more scorable peaks, but it also resulted in artifacts and stochastic effects, such as increased stutters and peak height imbalance of heterozygote alleles, which can further complicate the interpretation of profiles (Budowle et al., 2009a; Caragine et al., 2009; Gill, 2001; Gill et al., 2000).

Another strategy to improve the signal is reducing the volume of PCR amplification (Gaines et al., 2002), which concentrates the DNA and reagents. PCR inhibitors may be present in the sample, therefore, this approach may not be able to consistently increase the efficiency of the method (Mulero et al., 2008; Opel et al., 2010; Shrader, 2012; Watson and Blackwell, 2000; Wilson, 1997). Additional attempts to obtain a signal can be done by performing nested PCR (Snabes et al., 1994), however it requires opening the tube to add new reagents for the second round of PCR, which may increase the possibility of contamination.

The sample can also be purified after amplification to lower the salt level used for PCR amplification, which would otherwise compete with DNA fragments for the injection in the capillary (Forster et al., 2008). The downside of cleaning the sample could be loss of DNA and exposure of the sample to a possible laboratory based contamination. Detection of the PCR products can be improved by increasing the amount of PCR products added to the capillary electrophoresis by raising the voltage of the electrokinetic injection, or lengthening time of the injections, or both (Benschop et al., 2015; Caragine et al., 2009; Fonnelop et al., 2015; Westen et al., 2009).

Regardless of the approach taken to test touched DNA samples, they all aim to increase sensitivity of testing, which could ultimately result in appearance of sporadic alleles often from background DNA in a sample, known as drop-ins, and increased risk of detecting collection and laboratory based contamination (Budowle et al., 2009a).

Consequently, touched samples, also known in forensics as trace DNA samples (van Oorschot et al., 2010), are considered as very challenging forensic samples.

2.4 Difficulties in forensic DNA profiling

In addition to touched DNA samples, difficulties arise for forensic DNA testing when dealing with degraded DNA. Degradation can result from environmental insults especially when DNA is influenced by harsh factors such as exposure to UV light, humidity, and high temperature that can speed the process of hydrolysis and oxidation of DNA (Bus and Allen, 2014). Also, the time lag between a criminal act and the collection of biological samples for laboratory analysis may have an effect on DNA recovery and ability to generate an STR profile (Raymond et al., 2009). Often environmental influences, such as radiation can produce single and double strand DNA breaks, intra- and inter-strand crosslinks and formation of dimers that affect amplification of STR loci on DNA (Alaeddini et al., 2010). Furthermore, ionizing radiation can produce oxygen-derived species such as superoxide radical and hydrogen peroxide or even reactive hydroxyl radicals in cells by interacting with cellular water causing oxidative damage to nitrogen bases or crosslinkages or even removal of bases which ultimately may block PCR amplification (Hoss et al., 1996; Teoule and Cadet, 1978). High temperatures (Bruskov et al., 2002) as well as storing forensic samples in aqueous environments (Lindahl and Andersson, 1972) may cause accumulation of damage in DNA and ultimate degradation. Longer STR fragments on DNA are more likely to be degraded then shorter fragments, which can be easily detected in the electropherogram as peaks with declined heights or being absent, while smaller loci are preferentially amplified. To overcome problems of degraded samples mini-STRs could be a solution where primers are positioned as close as possible to the repeat motif (Butler et al., 2003; Ip et al., 2014; Opel et al., 2006; Phipps and Petricevic, 2007). Another possibility is single nucleotide polymorphism (SNP) typing (Cho et al., 2014; Mehta et al., 2017; Sanchez et al., 2006; Sobrino et al., 2005; Sobrino and Carracedo, 2005), which produces results even when STR typing fails (Onori et al., 2006). However, restraints of using SNP over STRs are due to the lack of a SNP database.

Further challenges in forensic biology are PCR inhibitors from soil, leather, denim, etc. (Mulero et al., 2008; Opel et al., 2010; Shrader, 2012; Watson and Blackwell, 2000;

Wilson, 1997) which may affect PCR results producing false negative results from a biological stain. Inhibitors generally express their effect through direct interaction with DNA or interference with thermostabile DNA polymerases, by interacting directly to block enzyme activity or react with $MgCl_{2,}$ a cofactor of DNA polymerase, thus inhibiting PCR (Alaeddini et al., 2010; Schrader et al., 2012). For example, hematin may be encountered in dried blood stains and acts as a metal chelating molecule (Akane et al., 1994); humic acid, a component in soils (Young et al., 1993), often found in samples that have been buried, particularly skeletal remains, inhibits the PCR reaction through sequence specific binding to DNA, limiting the amount of available template; tannic acid, an agent found in leather (Wilson, 1997), appears to be a Taq inhibitor by affecting availability of the DNA template; and Indigo which is a dye used in certain types of fabrics, that may be encountered in a DNA sample extracted from stains on denim or other dyed fabrics (Larkin and Harbison, 1999). Indigo can affect the PCR results possibly by quenching the fluorescence of a dye reporter, such as SYBR Green I in qPCR, thus affecting efficiency of the reaction (Opel et al., 2010). Calcium is another PCR inhibitor, which is a major inorganic component of bones (Bickley et al., 1996). Calcium is a Taq inhibitor, which competes with magnesium and reduces the reaction efficiency and total amount of PCR product (Opel et al., 2010; Schrader et al., 2012).

Inhibitors could be removed by DNA purification upon extraction (Akane et al., 1994; Shutler et al., 1999), however this may lead to some DNA loss, which can be critical when the extracted DNA is from biological traces. Inhibition can also be caused by components of cell lysis processes, such as by proteinases or denaturants, carried over to amplification. The strategy to prevent inhibition in this case is to customize the buffer system by adding components such as BSA (Abu Al-Soud and Radstrom, 2000; Kreader, 1996) an alternative substrate for proteinase, thus preventing inhibition of PCR amplification (Wilson, 1997). Commercially available forensic STR amplification kits specifically designed to be more resistant to inhibitors (Ip et al., 2014) is another way to relieve inhibition. Improvements in STR profiling of inhibited samples was obtained with the Identifiler® Plus Kit (Wang et al., 2012). This kit includes a buffer mix with optimized thermocycling parameters to overcome the inhibitors encountered in forensic samples (Ip et al., 2014; Romanini, 2011).

2.5 Touched samples originating from skin

Low levels of DNA from touched items or pieces of clothing can mostly be attributed to its origin, which is skin. The skin is the largest organ of the human body, accounting for approximately 15% of total body weight. It makes the outer covering. (Walters, 2002; Watt, 2014). From the perspective of forensic biology, the skin is important for shedding cells from the outer- most layer, which tends to transfer on an object by the person who touches it or onto clothing that is worn. To replenish lost cells, skin goes through constant proliferation and renewal. Keratinocytes, which are skin's main cells, form an adhesive network organized into multiple layers (Figure 1). At the basal layer they actively divide and progress to the upper epidermal layer where they exit the cell cycle, grow larger and establish robust intercellular connections. As they move outward to replace shed cells, they undergo a dramatic transformation (Simpson et al., 2011), become flattened, rich in keratohyalin granules, and have condensed nuclei that lose shape (Gandarillas et al., 1999). Finally, in the most outer layer of the skin, lysosomal enzymes are released to degrade major organelles and nuclei, leading to complete squamous cells that are tightly crosslinked together to complete the cutaneous barrier (Simpson et al., 2011).

These are the cells that largely shed from the skin, approximately 400,000 a day (reviewed in (Wickenheiser, 2002)). Despite these morphological changes, some cells still have stripped nuclei and contain DNA that could be suitable for STR profiling (Kita et al., 2008).

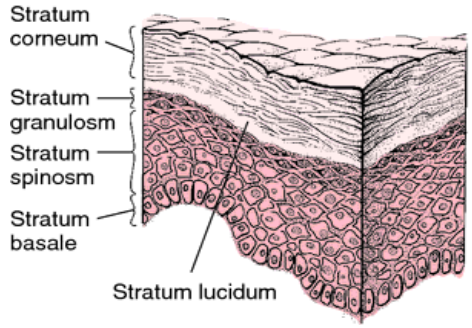


Figure 1. Layers of epidermis

The skin's epidermis is a stratified squamous epithelia that is composed of a few cell layers: 1) basal layer or stratum basale, composed of mitotically active cells, which gives rise to differentiated cell layers of: 2) stratum spinosum; 3) stratum granulosum, composed of flattened granular cells; 4) stratum lucidum, composed of several layers of clear, transparent cells in which the nuclei are indistinct or absent; and 5) stratum corneum, composed of flattened, cornified, and non-nucleated cells.
Source: Dorland's Medical Dictionary 2000.

Aside from shed cells, additional potential DNA sources on touched objects are cell free DNA coming from sweat and oil glands (Quinones and Daniel, 2012; Vandewoestyne et al., 2013). Each square centimeter of skin contains approximately 100 sweat glands, and 10 oil glands (Wickenheiser, 2002). Secretions produced within these glands make their way to the skin surface through ducts and pores, and being exposed to large numbers of DNA bearing cells, the secretions may also carry some cell free DNA to the skin's surface (Wickenheiser, 2002). In addition, the hands may act as vectors of transmission of nucleated cells by rubbing the face, nose, mouth, biting fingernails, and other unconscious acts, and transferring them to the contacted object (Wickenheiser, 2002). These various origins of DNA on touched objects could explain diverse DNA profiling results (Ostojic et al., 2014; Templeton and Linacre, 2014; van Oorschot et al., 2010).

2.6 Quality control and interpretation measures taken with touched samples

Touched samples are generally expected to contain low levels of DNA, thereby, sensitivity is increased during testing by some of the previously listed measures. The goal is to obtain results and generate more peaks, but could also lead to stochastic

effects and increased risk of contamination from personnel within the laboratory, consumables, and between samples (Gill et al., 2000; Petricevic et al., 2010). In order to minimize possible laboratory-based contamination, rigorous quality control measures must be implemented, such as physically separating preamp- and postamp- procedures, UV irradiating consumables, running negative controls with every test, wearing appropriate protective attire, etc. (Gill, 2001; Gill et al., 2000). If negative controls tested positive, it would indicate contamination in chemicals and/or consumables used. Therefore, associated touched samples would not be used in interpretation.

Moreover, to account for stochastic variations, such as exaggerated peak height imbalance at heterozygote locations, elevated stutters, and allele drop-in or allele drop-outs, which are typically observed at larger loci, samples should be amplified a few times (Petricevic et al., 2010). Only alleles which are observed more than once among at least two replicate analyses should be retained and listed in the consensus profile (Caragine et al., 2009; Cowen et al., 2011; Grisedale and van Daal, 2012; Prinz et al., 2006; Taberler, 1996). Additional alleles that do not repeat are called spurious alleles. They most likely do not originate from DNA fragments inherent to the sample collected from the crime scene, but instead from contaminating DNA.

2.7 NYC OCME strategy to test touch samples

The NYC OCME laboratory validated and implemented the High Sensitivity testing or Low Copy Number (LCN) methodology to test samples with low amounts of DNA (below 100pg). This procedure encompasses enhancements of every step from the sample collection to the analysis of DNA (Budimlija and Caragine, 2012; Caragine et al., 2009; Prinz et al., 2006; Schiffner et al., 2005). It involves using a specialized swab (Caragine et al., 2009) moistened in 0.01% SDS to collect cells off of an item. SDS is also used in the DNA extraction process to help in opening of the cell membrane and thus release the DNA, however, it can inhibit PCR (Rossen et al., 1992; Schrader et al., 2012), therefore it must be removed from the sample. Microcon® column, pre-coated with carrier DNA or RNA to minimize DNA loss, is used to purify a sample and also to concentrate DNA upon extraction (Prinz et al., 2006; Schiffner et al., 2005). The LCN procedure with increased sensitivity of testing, also includes raising the cycle number in the PCR amplification to 31, reducing the reaction volume, and doubling the annealing

time (Caragine et al., 2009). Each sample is also amplified in triple individual analysis to help the interpretation and reliability of assignment of STR results to contributing profiles (Caragine et al., 2009; Prinz et al., 2006). Amplified PCR products were injected in capillary electrophoresis with altered injection times and voltage (Caragine et al., 2009). This included injecting samples for a longer period of time under higher voltage (Caragine et al., 2009). Furthermore, interpretation guidelines were developed through intensive validation studies, which account for stochastic variation inherent to low template DNA samples and helped reliable interpretation of STR results (Caragine et al., 2009).

The LCN methodology has been implemented into routine workflow at the NYC OCME laboratory since 2006 to process thousands of samples with no visible biological staining for 'touch' DNA. This allowed the generation of DNA profiles from a wide range of exhibits not previously examined, such as jewelry, vehicles, paper, glass, window, firearms, etc. (Balogh et al., 2003b; Bright and Petricevic, 2004; Horsman-Hall et al., 2009; Petricevic et al., 2010; Polley et al., 2006; Raymond et al., 2009; Rutty, 2002; Sewell et al., 2008; Van Hoofstat et al., 1999; Webb et al., 2001; Wickenheiser, 2002). Thereby, there was an increase in submission of touched items and firearm related cases for examination to the NYC OCME laboratory, with an increase from 10,000 in 2013 to over 12,000 cases in 2016 (Figure 2).

Touched evidence could be received for examination from various cases, such as homicides, assaults, and burglaries. Sometimes these items could be the only evidence available for examination. Also, in sexual assaults, touched item(s) may lead to identification of a perpetrator when intimate swabs or pieces of clothing fail to generate results, or if the perpetrator is an azospermic donor. Therefore, obtaining STR profiles from touched evidence is of high importance for criminal investigations.

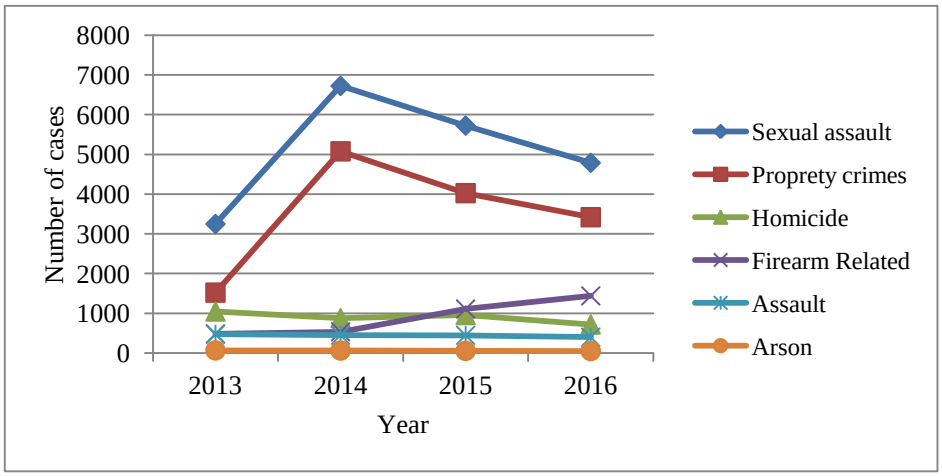


Figure 2. Number of cases by type received by the NYC OCME laboratory for examination for period 2013-2016. There was an increase in the number of firearm related cases due to a gun initiative by the NYPD and NYC District attorney's office. Other types of cases were in stagnation or in decrease.

2.8 Touched biological mixtures

Touched items are routinely examined by entirely swabbing an item and submitting the swab(s) for DNA analysis. These analyses could lead to a DNA mixture if an item was touched by multiple individuals. At the NYC OCME laboratory it was documented that the majority of tested "touched" samples were DNA mixtures. Sometimes, DNA mixtures could be easily resolved if one person deposited more cells and cell free DNA then other people that touched the same object, therefore the DNA profile of that major DNA donor could be determined. It is also possible that all individuals leave equal and/ or small amounts of DNA making it difficult to identify any DNA profile.

The forensic biology accrediting bodies and leading scientific group, SWGDAM, recommend that positive associations, such as a suspect to a major DNA donor or a suspect to a mixture, should be accompanied with statistical calculations (Ladd et al., 2001). Single source or major DNA profiles can be statistically evaluated by using the random match probability. This describes the rarity of the profile in general population (Bille et al., 2013). However, the association of a suspect to a nondeductible mixture requires very complex statistical calculations usually in the form of likelihood ratio

(Bille et al., 2014; Budowle et al., 2009b; Cooper et al., 2015; Cowell, 2016; Gill et al., 2006a; Gill and Haned, 2013; Ladd et al., 2001; Mitchell et al., 2012; Prieto et al., 2014; Puch-Solis et al., 2010). Additional difficulties when working with mixtures are interpretations of STR data, which are rather subjective and under guidelines developed within each laboratory, and thereby may not lead to standardized deductions among laboratories (Budowle et al., 2009b; Dror and Hampikian, 2011).

Ideally, if complete STR profiles could be obtained from one cell, mixtures would be resolved by testing a single cell, which would ultimately lead to identification of DNA profiles of all cell donors to a mixture sample. In this case, physical isolation of target cells from a mixture is required. There are many techniques available that enable physical isolation of a cell, such as manually scraping or plucking a cell by using sterile hypodermic needles (Brauns and Goos, 2005; Stouder, 2001). These physical isolation techniques are widely used in biology to isolate individual cells or parts of a tissue for further analysis (Klein et al., 1999; Walch et al., 2000). They are simple and inexpensive and can be performed using common equipment in the laboratory. This involves use of a microscope to observe a biological material and various dissection tools, such as needles, tweezers, or surgical blades (Hunt and Finkelstein, 2004) to select and isolate cells of interest (Hunt and Finkelstein, 2004; Kehr, 2003).

Collecting individual skin cell flakes could be a promising method to identify cell donors on an object touched by multiple individuals. However, it may not be sufficient to standardize DNA profiling procedure due to apoptotic processes individual cells/ flakes go through (Gandarillas et al., 1999) and thereby may have insufficient amounts of DNA for analysis. Single fingerprints may gain more interest for forensic identification as they are already widely used in biometrics (Jain AK and A, 2015; Trauring, 1963). Also, cellular and extracellular DNA contributions (Quinones and Daniel, 2012) to fingerprint samples may enrich a sample with DNA rather than relying on a presence of sporadically not degraded DNA from a single skin cell (Kita et al., 2008). Since the DNA was detected within fingerprints (Haines et al., 2013), there is a promise to obtain usable STR profiles from a single fingerprint sample. The ability to retrieve the maximum amount of DNA from a fingerprint and generate a database eligible DNA profile is of high importance in resolving a case.

First analysis of sloughed epithelial cell from hands was done by Oorschot et al. in 1997 (van Oorschot and Jones, 1997). It was recognized that cells were instantaneously transferred to a substrate upon handling (van Oorschot and Jones, 1997). Transfer of cells depends on the type of substrate, where porous substrates collected sloughed cells more readily than non-porous substrates (Wickenheiser, 2002). Also, the possibility for secondary transfer was indicated (van Oorschot and Jones, 1997) which may further complicate the interpretation of STR results. Secondary transfer happens due to previous touches, such as other people or objects that may carry some cell material. These cells are then transferred to the following item, which is an object for forensic examination, together with cells of the direct donor (van Oorschot et al., 2010; Zoppis et al., 2014).

There are many obstacles when working with fingerprints, including the uncertainty in the amount of DNA found in the sample. Fingerprints could be deposited on an item days prior to their examination and collection, which can also impact the quality of DNA. Therefore, fingerprints are considered the most challenging forensic samples and need further analysis in order to understand their potentials and limitations for forensic biology.

3. Thesis problem and goal of research

There is a need to more effectively test evidence from different types of crimes. Often, evidence is touched by multiple people, not just a single perpetrator, or carries body fluids from different donors, such as in a sexual assault. Mixtures of fingerprints from various donors are often difficult to be interpreted, due to low amounts of DNA left by individuals. Also mixtures of cells from various bodily fluids, such as sperm cells from an assailant and epithelial cells from a victim could be incompletely separated. Especially, it could be hard to identify a sperm cell donor when epithelial cells are greatly exceeding the sperm cells in the sample.

The objective of this thesis is to perform a study and learn how to more effectively process DNA from touched or trace biological materials and biological mixtures. There is a need to 1) maximize DNA recovery from biological traces, because of so little DNA; and 2) to separate cells from various cell donors, because of mixtures that are often generated. This cell separation could help to simplify DNA analysis of mixtures samples where the major DNA donor can be easily deduced or ideally could lead to single source DNA samples. This may provide more information from biological samples and lead to identification of more DNA donors.

Therefore, a comprehensive study was done on the most challenging forensic samples, touched samples/ fingerprints. A comparative study between various available methods to lyse samples and amplify DNA was done to understand which method is able to more effectively process DNA from biological traces. Finally, various cell separation techniques were tested and compared for their sensitivity and reproducibility. The results of this comparative evaluation will provide insight on how to more effectively process DNA from touched, trace biological materials and identify the majority of or even all cell donors to a mixture.

4. Instruments and Materials

4.1 Instruments

Aureka® (Aura Optik GmbH, Jena, Germany)

Axiozoom .V16 (Carl Zeiss AG, Göttingen, Germany)

Dumont N5 dissecting tweezers (Ted Pella, Stockholm, Sweden)

GenAmp® 9700 thermal cycler (Life Technologies Applied Biosystems, Foster City, CA, USA)

GeneMapper® ID v.3.2 software (Life Technologies Applied Biosystems, Foster City, CA, USA)

Hypodermic needle (Terumo Medical Corporation, Elkton, MD, USA)

Microsurgical blade (FEATHER Safety Razor Co., Ltd. Osaka, Japan)

Needle micro-tool (McCrone Group, Westmont, Il, USA)

NuAire biosafety cabinet (NuAire, Plymouth, MN, USA)

Olympus SZX-16® stereomicroscope (Olympus of the Americas, Central Valley, PA, USA)

P.A.L.M.® (Carl Zeiss MicroImaging GmbH, Bernried, Germany)

Rotorgene™ Q (Qiagen, Valencia, CA, USA)

Thermobrite® StatSpin® (Iris, Westwood, MA, USA)

Thermomixer® (Eppendorf International, NY, NY, USA)

Tungsten needle (McCrone Group, Westmont, Il, USA)

X-Acto knife (Elmer's Products, Inc., Westerville, OH, USA)

ABI Prism® 3130*xl* Genetic Analyzer (Life Technologies Applied Biosystems, Foster City, CA, USA)

4.2 Materials

Arabic gum (Winsor & Newton, London, UK)

DIFF-lift tape (Lynn Peavey Forensics, Lenexa, KS, USA)

Cotton swab (Dynarex, Orangeburg, NY, USA)

Cytodex™3 microcarrier (GE Healthcare, Upsala, Sweden)

"Elmer's stick" (Elmer's Products, Inc., Westerville, OH, USA)

Fingerprint lifting tape (SIRCHIE, Youngsville, NC, USA)

Latent print tape (Remco, Lancaster, CA, USA)

Microcon® 100 filter (Millipore, Billerca, MA, USA)

PEN membrane microscope slide (Carl Zeiss Microimaging GmbH, Bernreid, Germany)

"Pritt" glue (Henkel AG&Co, Düsseldorf, Germany)

Rubber cement (Elmer's Products, Inc., Westerville, OH, USA)

Scotch tape (Scotch, St. Paul, MN, USA)

Solo Hill Engineering microcarriers (Solo Hill Engineering, Ann Arbor, MI, USA):
polystyrene beads coated with collagen, ProNectin® F, and CT

"tesa" tape (Tesa, Hamburg, Germany)

Tubes 0.1 ml (Qiagen, Valencia, CA, USA)

Tubes (0.2 ml, 1.5 ml) (Life Technologies Applied BioSystems, Foster City, CA, USA)

4.3 Reagents

AmpF*l*STR® Identifiler® Plus PCR Amplification Kit (Life Technologies Applied Biosystems, Foster City, CA, USA)

AmpF*l*STR® Identifiler® PCR Amplification Kit (Life Technologies Applied Biosystems, Foster City, CA, USA)

Allelic ladder (Life Technologies Applied Biosystems, Foster City, CA, USA)

AmpF*l*STR® 9947A control DNA (Life Technologies Applied Biosystems, Foster City, CA, USA)

Chelex®-100 (Bio-Rad, Hercules, CA, USA)

DAPI (VectaShiel®, Burlingame, CA, USA)

DTT (Sigma-Aldrich, St. Louis, MO, USA)

EB1 and EB2 (Integrated DNA Technologies, Coralville, IA, USA)

Fish sperm DNA (Crescent Chemicals, Islandia, NY, USA)

GeneScan® 500 Liz® Size Standard (Life Technologies Applied Biosystems, Foster City, CA, USA)

Glycerol (Thermo Fisher Scientific, Waltham, MA, USA)

HCl (Sigma-Aldrich, St. Louis, MO, USA)

HIDI formamide (Life Technologies Applied BioSystems, Foster City, CA, USA)

NFR (Sigma-Aldrich, St. Louis, MO, USA)

PrepGEM® Tissue extraction mixture (ZyGEM, Corporation Ltd, New Zealand)

SDS (USB®, Cleveland, OH, USA)

SYBR green I (Life Technologies Molecular Probes, Grand Island, NY, USA)

Trehalose (Thermo Fisher Scientific, Waltham, MA, USA)

Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA)

4.4. Enzymes

Ampli*Taq* Gold® DNA polymerase (Life Technologies Applied BioSystems, Foster City, CA, USA)

Proprietary EA1 enzyme (ZyGEM, Corporation Ltd, New Zealand)

Proteinase K (Life Technologies Applied BioSystems, Foster City, CA, USA)

5. Methods

5.1 Sample collection

This research project was approved by the New York City Department of Health and Mental Hygiene Institutional Review Board that oversees research involving human subjects for the OCME (IRB# 12-058) and approves policies for ethical standards and methods for human testing. Sample collection and experimentation were commenced only after the IRB review process was completed and written approval for this study was received.

Volunteers who participated in this study were assigned a numerical code to anonymize the sample. Epithelial (buccal) cells, fingerprints and/or sperm were collected. Along with these testing samples, additional buccal samples were obtained from volunteers in order to generate reference DNA profiles, which were used for comparison purposes. The fraction of the profiles obtained from samples tested in this study that matched the reference profile was calculated.

Buccal cells:

Sterile cotton swabs were used to collect cells from the inside of the cheek of volunteers. Cells were collected by rubbing a cotton tip of the swab against the inner cheek and gum areas of the mouth. While still wet, the swab was used to smear the cells on the surface of the PEN membrane slide by rubbing the cotton tip of the swab against the membrane. A membrane of the PEN slide is clear and transparent, and does not interfere with the DNA testing process. If a swab dried prior to smearing, it was wetted with few drops of distilled water to enable an easier transfer of cells from the swab to the membrane. Immediately afterwards the cells were put on a water steam to spread them on the surface of the slide, followed by heat-fixation at 56^{o}C for 2 min using a Thermobrite® StatSpin® and then stained with NFR for 10 min. NFR stain is prepared with 0.07 M aluminum sulfate and 0.05% NFR. NFR is a member of the anthraquinone group of dyes. It is applied with aluminum sulphate to generate a complex that has a high affinity for the phosphate groups of DNA. This interaction is probably non-covalent (Wang et al., 2011). When the stain is applied on cells, it penetrates into the nucleus and stains the entire nucleus red. Washes with H_2O and EtOH were applied to

remove excess dye that was not specifically bound. Then the slide was placed back on a thermobrite at 56^{o}C for 2 min to dry. Slides were stored at room temperature until use.

Sperm:

Volunteers provided seminal fluids in 50 ml plastic, conical tubes that were kept at 4^{o}C until use. By using a cotton swab, a portion of seminal fluid was taken to spread cells onto a PEN membrane slide. Immediately afterwards the cells were heat fixed on a membrane at 56^{o}C for 2 min. Then, cells were stained with NFR for 10 min. After washes with H_2O and EtOH the slide was placed back on a thermobrite at 56^{o}C for 2 min to dry. Slides were stored at room temperature until use.

Fingerprints:

In order to mimic real scenarios, all volunteers were asked to refrain from washing their hands for at least two hours prior to sampling. Fingerprint samples were taken by pressing right and left thumbs for a few seconds separately on various substrates (glass, plastic, paper, metal and tape). Glass microscope slides were used as well as 50 mm x 80 mm pieces cut out of white office paper and plastic sheet protector made of polypropylene Avery®, respectively. A U.S. Quarter (25 cents) was used as metal substrate, which is 24.26 mm in diameter, and made of 91.67% Cu and 8.33% Ni. Fingerprints were always deposited on the same side of the Quarter. The adhesive side of a piece cut out of "SIRCHIE" tape affixed on a metal frame slide was used to deposit a fingerprint. For a mixture study, an empty beer bottle (amber glass, 354.8 ml fluid volume) was used, where three persons held the bottle consecutively with their dominant hand, which may allow the palm as well as the fingers to touch the bottle. Each person held the bottle for 60 s. The order of persons touching the bottle was alternated. The trunk of the beer bottle (height of bottle: 23 cm, height of trunk: 12 cm, diameter of trunk: 6 cm), of which the label was removed, was initially etched into six equivalent sections; each size measured 6 cm x 6.3 cm. The beer bottle was stored at room temperature until the next day when it was then swabbed.

Prior to collecting fingerprints, substrates were decontaminated by immersing the entire object in 10% bleach, followed by water, and 70% ethanol, except for the paper and tape that were UV irradiated for 30 min in the NuAire biosafety cabinet.

After fingerprints were deposited they were kept at room temperature in the laboratory avoiding direct sun exposure until being swabbed. Some fingerprints on glass

microscope slides were immediately examined under an Olympus SZX-16® stereomicroscope to observe the individuals' propensity to shed skin cells. A score of 1 to 5 was assigned to each print. A lowest score of 1 referred to no cells or skin flakes, while the highest score of 5 referred to fully populated print immersion. Grading was performed by the same trained analyst in order to avoid subjective variability by numerous analysts. Visual inspection was followed by swabbing the fingerprints of the slides. Other fingerprints on glass microscope slides were left in closed or open, decontaminated, boxes for the following time periods prior to swabbing: 1, 3, 10, 20, and 40 days. Fingerprints on plastic, paper, and metal were stored in closed, decontaminated boxes for 3 days. Fingerprints on tape and beer bottles were kept on the bench and were swabbed after one day following touching.

Fingerprints were swabbed in their entirety under the Olympus SZX-16® stereomicroscope using a small portion of a sterile, UV irradiated cotton swab, held with reverse clamp tweezers (Dumont N5 dissecting tweezers) and moistened with 4 µl of water or 5% Triton X-100. Triton X-100 is a non-ionic detergent that disturbs noncovalent interactions in protein-lipids or lipid-lipids and solubilizes hydrophobic molecules. Due to the presence of extracellular DNA in the fingerprint sample, such as in sweat and oil (Quinones and Daniel, 2012; Stanciu et al., 2016; Zoppis et al., 2014), using a detergent may improve DNA extraction. Detergents were shown to improve DNA yield from touched samples (Thomasma and Foran, 2013) and Triton X-100 should not influence the PCR (Radstrom et al., 2004).

Fingerprints on tape were swabbed under the Axiozoom .V16 using 10 µl of prepGEM® tissue extraction mixture diluted 1:2 in H_2O. The buffer of this extraction mixture contains a detergent that does not impact the PCR process; however, components of the extraction buffer were not disclosed by the manufacturer.

A clean substrate was swabbed alongside each batch of samples as a negative control.

5.2 Picking and transferring single cells and fingerprints

Various tools were evaluated for their ability to pick individual cells and transfer fingerprints from a piece of evidence, including microcarriers, tapes, and needles.

The microcarriers tested were commercially available microglobes and glue spheres. The principle underlying microcarriers is physical adhesion. When evaluating

microcarriers for their suitability in picking and transferring cells from evidence into a test tube, it was important to observe that the adhesive force of the microcarrier surface was greater than the adhesive force of the surface on which the cells rested. Upon picking, cells were deposited in the extraction buffer of a 0.2 ml reaction tube. The entire process of lifting and transferring a cell into the test tube was observed under the stereomicroscope (Olympus SZX-16® or Axiozoom .V16).

Commercially available microglobes used were from Solo Hill Engineering: polystyrene beads coated with collagen, ProNectin® F, and CT, a modified proprietary coating. Also, Cytodex™3 was used, a dextran microglobe coated with collagen.

Glue spheres were manually made by smearing glue on a glass microscope slide and rolling a portion of glue into a spherical shape using a needle micro-tool. The entire process was monitored under the Axiozoom .V16 stereomicroscope where the size of a glue sphere was measured and adjusted for single cell picking. Glues evaluated included: "Pritt" glue, "Elmer's stick", and glue made in-house from Arabic gum in glycerol 1:5 to 1:9, and rubber cement.

Tapes may be used to transfer a fingerprint from a physical object. Various adhesive tapes were evaluated for the ability to lift cells from evidence and their compatibility with micromanipulations. In micromanipulations the evaluation focused on the ability to cut a tape around a target cell and the ease of transfer to a test tube for DNA analysis. Tapes tested were: latent print tape, DIFF-lift tape, fingerprint lifting tape, "tesa" tape, and Scotch tape.

5.3 Micromanipulation of single cells and fingerprints

Micromanipulation refers to techniques that use microscopy and physical manipulation techniques often in conjunction with cell staining to separate the cells of interest. Three instruments were tested for their efficiency to physically isolate a single cell from other cells for DNA profiling success. The instruments evaluated were: (i) P.A.L.M.®, (ii) the robotically assisted micromanipulator aureka® integrated to the Axiozoom .V16 stereomicroscope and (iii) manual manipulations performed under the Axiozoom .V16 stereomicroscope.

P.A.L.M. ® is an inverted, computer-controlled microscope that uses an UV laser to cut and transfer areas or cells of interest from a slide into a reaction vessel (reviewed in

(Espina et al., 2006). It combines light microscopic instrumentation with laser beam technology and allows targeting specific cells or tissue regions that need to be separated from others. Isolation is done under direct visualization (Vandewoestyne and Deforce, 2010).

Cells smeared on a PEN membrane-coated slide were located and marked for capture using the instrument software. Selection of spermatozoa was performed under 400 fold magnification and epithelial cells under 200 fold magnification.

The cells of interest were cut out of the membrane with a laser beam that was directed through the objective lens onto a microscope slide. The cut material was subsequently catapulted from a slide by a defocused UV laser pulse of the laser beam that generates a photonic force to propel the material off the slide. The dissected material is sent upward (up to several millimeters) to a tube cap of a 0.2 ml microfuge tube mounted onto the collector. The tube cap was flat and filled with 20 µl of extraction buffer.

Aureka® robotically-assisted micromanipulator is integrated to the Axiozoom .V16 stereomicroscope (magnification up to 200 fold) custom fitted with interchangeable toolboxes on a robotic arm adapted to carry a pair of microtweezers, or a tungsten needle or microsurgical blade. The attached microtweezers could be opened and closed. Micromanipulator and functions of the tools are intuitively controlled with a position control device (3D joystick) with micron precision. The aureka® motorized unit can be moved in 4 directions via the joystick: left-right, forward-backward, up-down, and diagonally. The motorized micromanipulator can memorize positions for tweezers, tungsten needle or microsurgical blade until turned off, and then a resetting is needed (Schneider et al., 2012). This instrument was used to pick up single cells, swab small areas (on slides or other objects), to cut tape or membrane, and for transfer to reaction tubes.

Manual microdissection using the Axiozoom .V16 stereomicroscope was done by manually scraping or lifting cellular material off the microscope slide or other objects using tools such as a sterile hypodermic needle, tungsten needle, or manual microtweezers coupled with adhesive microspheres. In addition, cells were manually cut out of sticky tape or membrane slides using a microsurgical blade or the X-Acto knife. When working with fingerprints, due to dual origin of DNA within fingerprint samples from: 1) fragmented nucleus of keratinocytes that went through apoptosis (Gandarillas

et al., 1999) and 2) extracellular DNA from sweat and oil (Quinones and Daniel, 2012), fingerprints were analyzed by swabbing rather than picking individual cells. Fingerprints on glass or other substrates, such as plastic, metal, and paper, were swabbed under the Olympus SZX-16® stereomicroscope (magnification up to 40 fold) using a small portion of a cotton swab, and for tiny areas using a sphere made of rubber cement. Fingerprints on lifting tape (SIRCHIE) were swabbed under the Axiozoom .V16 stereomicroscope.

5.4 DNA extraction and quantification

When comparing extraction procedures, DNA was isolated from fingerprints swabbed off glass slides using one of the three protocols: (i) One-tube, (ii) High-sensitivity, and (iii) Zygem extraction. These protocols could be applied on epithelial (buccal) cells, but not on spermatozoids. Disulfide-linked integral membrane proteins are abundant in the nuclear membrane of spermatozoids; hence additional treatment is needed in order to release the DNA from sperm. Therefore, the protocol routinely applied by the NYC OCME laboratory for sperm cells was used. The crucial components of this extraction buffer were Chelex beads and DTT reducing agent.

Negative controls were added to each extraction protocol to test for possible contamination. If these controls were positive all data associated with this extraction batch would be discarded.

(i) <u>One-tube extraction protocol</u> was developed in-house. Samples (swabs containing a single fingerprint) were incubated in 20 µl of digestion buffer, which contained: 3% trehalose (a α-linked disaccharide), 1.44 mg/ml Proteinase K, and 1mM DTT. Incubation was done on a Eppendorf Thermomixer® at 56^{o}C and 600 rpm for 45 min, followed by 10 min at 95^{o}C with no shaking, and 5 min on ice. Trehalose in the extraction was used to provide support and stability to DNA. Each OH- group of a α-linked disaccharide forms a large number of hydrogen bonds with the backbone of DNA thus providing structural protection (Smith and Morin, 2005; Zhu et al., 2007). This could be important for samples with low levels of DNA as usually expected from fingerprint samples or other biological traces, which are prone to DNA degradation.

ProK used was isolated from the fungus *Engyodontium album*. It is an endolytic serine protease that digests proteins and inactivates nucleases, thereby providing protection to

released DNA. ProK remains active over a wide pH range (optimal activity is between 6.5 and 9.5), so any fluctuation in the extraction buffer during cell lysis will not disrupt its activity. It also remains active under denaturating conditions, such as in the presence of SDS, or metal chelating agents, such as Chelex resins. Elevation of the reaction temperature from 37^{o}C to 60^{o}C increases ProK's activity (Chellappan et al., 2011). ProK's activity is enhanced by making its substrate cleavage site more accessible. Temperatures above 65^{o}C inactivate the enzyme. 95^{o}C temperature of inactivation was chosen to insure complete inactivation of the enzyme upon lysis so none of the active enzyme was transferred to the amplification.

Skin cells are highly keratinized, where filaments are quite stabilized by numerous cross-linking disulfide bonds, therefore DTT, a reducing agent, may aid in releasing DNA from the cells by loosening keratin and extracting it (reviewed in (Bragulla and Homberger, 2009).

The length of incubation for this extraction was determined based on comparative studies of various incubation times followed by visual inspection of cells under the microscope. Upon 45 minutes of incubation at 56^{o}C no intact cells were observed and therefore full digestion was suspected.

(ii) High-sensitivity extraction is an extraction method developed in-house and is routinely applied in casework at the OCME to extract DNA from "touched" samples. The swabs used to collect fingerprints were incubated in digestion buffer containing 0.05% SDS and 0.8 mg/ml ProK. SDS is an ionic detergent that disrupts noncovalent interactions between protein and lipids, lipids, and proteins, and solubilizes hydrophobic molecules during lysis of the cell. ProK digests enzymes and proteins and facilitates release of DNA from the cell. Following incubation on the Eppendorf Thermomixer® at 1400 rpm for 30 min at 56^{o}C, which is a temperature of optimal activity of ProK, samples were incubated without shaking for 10 min at 99^{o}C, to inactivate ProK. The ProK that was used is the same as in the One tube extraction. Finally, samples were concentrated and purified using a Microcon® 100 filter twice, which was pretreated with 200 µl of fish sperm DNA at a concentration of 5 ng/µl, to capture ssDNA from the extract. In addition, the microcon column enables removal of SDS, which if left in the extract can inhibit PCR (Schrader et al., 2012). Finally, DNA was eluted into 20 µl UV irradiated water.

(iii) Zygem extraction (Zygem) is a commercially available extraction kit and the extraction was performed based on the manufacturer's instructions. Samples were incubated in 20 µl of prepGEM® tissue extraction mixture for 15 min at 75°C, followed by 5 min at 95°C using a GenAmp 9700 thermal cycler. The extraction mixture contains proprietary EA1 enzyme that operates at high temperatures, 75°C, therefore more efficient degradation of proteins can be achieved than with other enzymes, such as with ProK, which operates at lower temperatures. Because of this distinctive temperature characteristic, DNA extraction using Zygem was conducted in a single closed tube, which decreased sample preparation time and minimized the chance of sample contamination. The manufacturer's protocol suggested that the sample not be purified after DNA extraction, since the proteinase and all the reagents in the buffer were intentionally selected to be fully compatible with PCR. The Zygem removes the need for any purification steps by extracting in mild buffers and selectively releasing DNA into solution using conditions that do not solubilise most inhibitors. Raising the temperature to 95°C inactivated the Zygem enzyme.

Chelex DNA extraction from sperm: Sperm cells were incubated in extraction buffer, containing 200 µl of 5% Chelex® 100 (from a well-resuspended Chelex solution), 1 µl of 20 mg/ml ProK, and 7 µl of 1 M DTT. Chelex beads are styrene divinylbenzene copolymer beads with iminociacetate ions that chelate polyvalent metal ions, such as divalent cations, as magnesium, calcium, manganese or zinc, which act as a cofactor necessary for nuclease activity (Nishino and Morikawa, 2002) and therefore reduce nuclease activity *in vitro*.

Sperm cells were initially incubated for 2 h at 56°C, followed by vortexing at high speed for 10-30 s. Then samples were incubated at 100°C for 8 min and vortexed for 10-30 s. Finally, samples were centrifugated for 2 min at 10-15,000 g, leaving DNA in the supernatant and cellular debris in the pellet.

DNA quantification: Following extraction, DNA was quantified using an *Alu*-based real-time PCR for human DNA, based on the method described by Nicklas and Buel (Nicklas and Buel, 2003). *Alu* sequences are short (approximately 280 bp in length), highly repetitive elements that are interspersed throughout the primate genome, which makes them an excellent target or marker to detect primate DNA (Mighell et al., 1997; Schmid, 1996). The quantitative assay used is a modified version of Nicklas and Buel's

method that included the addition of 0.3 µl of 100X SYBR Green I employed as the fluorescent reporter, and 0.525 mg/ml BSA in 25 µl reaction volume. BSA was added to the reaction to overcome the effect of the inhibitors (Schrader et al., 2012) that may be present in the sample originating from a substrate where forensic biological material was collected (Opel et al., 2010). The inhibitors could include indigo, tannic acid, humic acid, etc. These inhibitors could commingle with the DNA sample upon exposure to different environmental conditions and/or co-extract with the DNA sample (Shrader, 2012).

BSA is used in the reaction to bind various inhibitory substances in the sample thereby preventing them from binding and inactivating Taq polymerase. Also, BSA can relieve inhibition in the sample containing endogenous protease activity by providing an alternate substrate and thereby protecting Taq DNA polymerase (Kreader, 1996; Ralser et al., 2006).

Real-time PCR monitors the amount of PCR amplicons as the reaction occurs. SYBR Green I specifically binds double-stranded DNA, and fluoresces only when bound to DNA. The resulting DNA-dye-complex absorbs blue light (λ_{max} = 497 nm) and emits green light (λ_{max} = 520 nm). Detection of the fluorescent signal occurs after each PCR cycle.

The amount of DNA in the sample was established based on a standard curve. In order to construct a standard curve, a set of standards were made through a serial dilution: 1600 pg/µl, 400 pg/µl, 100 pg/µl, 25 pg/µl, 6.25 pg/µl, 1.56 pg/µl, 0.39 pg/µl, and 0 pg/µl. All samples, standards, and master mix were vortexed thoroughly and then centrifuged at 3000 rpm for 3 s. The master mix consisted of a cocktail of: irradiated GIBCO™ ULTRA PURE™ distilled water, 10X PCR Buffer, 25 mM $MgCl_2$, 5 mg/ml BSA, 2.5 mM dNTPs, DMSO, 1/100 dilution of 10,000X SYBR Green I, 20 pmol/µl Primer EB1, 20 pmol/µl Primer EB2, and 5 U/µl Ampli*Taq* Gold.

DMSO in PCR binds to the DNA at the cytosine base and changes its conformation which makes the DNA more labile to heat denaturation. This lowers the melting temperature in GC regions. Thus, DMSO indirectly facilitates the annealing of primers to the template and enhances amplification (Hardjasa et al., 2010).

A 23 µl aliquot of master mix was added into each 0.1 ml tubes followed by 2 µl of standards, NTC, and samples into each respective tube. 0.1 ml rotorgene tubes were

transferred into the Rotorgene™ machine. PCR begins with incubation at 95°C for 10 min, which is also known as a "hot start". This condition reduces non-specific amplification and offers the convenience of reaction set up at room temperature. Hot start is enabled by using the polymerases inhibited at room temperatures through different mechanisms, including antibody interaction, chemical modification such as Ampli*Taq* Gold® DNA Polymerase, and aptamer technology (D'Aquila et al., 1991). At 95°C the polymerase dissociates from its inhibitor to commence polymerization. Hot start PCR is followed by 35 cycles of 94°C for 15 s, 68°C for 60 s, and 72°C for 30 s. The run is completed with final extension at 72°C for 15 s. Following the run which lasts 100 minutes, the results were analyzed using Rotorgene™ 6 software. The amount of DNA in the sample was measured through comparison to the standard curve. Measurements were within 30% of their expected value. The NTC threshold was set to 0.1 pg/µl.

Quantification was performed in triplicate, duplicate or singletons.

5.5 Amplification of STR loci

Samples were amplified using the AmpF*l*STR® Identifiler® PCR Amplification Kit for 28 (ID28) or 31 (ID31) cycles or the AmpF*l*STR® Identifiler® Plus PCR Amplification Kit for 32 (ID+) cycles. The ID+ kit was specifically designed to overcome inhibitors that may be present in the sample (Ip et al., 2014).

The Identifiler® kits are short tandem repeat (STR) multiplex assays that amplify 15 tetranucleotide repeat loci and the Amelogenin gender-determining marker in a single PCR amplification. Besides D2S1338 and D19S433, the remaining thirteen autosomal loci are the required core loci for the Combined DNA Index System (CODIS) (Budowle et al., 1999).

For ID28 and ID31 the PCR reaction contained 2.5 µl Primer Mix (sequences are from ABI and is proprietary information), 5 µl Reaction Mix, and 0.5 µl Ampli*Taq* Gold® DNA Polymerase (5 U/µl), whereas for ID+ the PCR per reaction contained 5 µl of Reaction Mix that includes AmpliTaq Gold® DNA Polymerase, and 2.5 µl Primer Mix. Only two tubes in the Identifiler® Plus amplification (Reaction and Primer Mix tubes) were used and they streamlined reaction setup. 5 µl or 6.6 µl extracted DNA was used

as template. 6.6 µl of extracted DNA was used for samples that were not quantified, because no extract was left for quantification.

The AmpFLSTR® Identifiler® Plus PCR Amplification kit includes the same primers and allelic ladder as the AmpFLSTR® Identifiler® PCR kit.

Components of the reaction mixture are: $MgCl_2$, deoxynucleotide triphosphates (dNTP's), and BSA in buffer with 0.05% sodium azide. For one-tube extracts, the $MgCl_2$ concentration was increased to 0.25 mM.

Using the AmpFLSTR® Identifiler® Kit (ID28 or ID31) PCR occurred through the consecutive stages of:

- preincubation or "hot start" (at 95°C for 11 min)
- 28 or 31 cycle:
    - Denaturation (94°C for 60 s),
    - Annealing (59°C for 2 min),
    - Extension (72°C for 1 min),
- 60 min incubation at 60°C
- Storage (indefinitely at 4°C).

Using the AmpFLSTR® Identifiler® Plus Kit (32 cycles) PCR occurs through the consecutive stages of:

- preincubation or "hot start" (at 95°C for 11 min),
- 32 cycle:
    - Denaturation (94°C for 20 s),
    - Annealing/ Extension (59°C for 3 min)
- Final extension (60°C for 10 min),
- Storage (indefinitely at 4°C).

Preincubation or "hot start" PCR at 95°C for 11 min prevents nonspecific primer binding, activates DNA polymerase and therefore prevents generation of nonspecific PCR products. After the "hot start", denaturation occurs at 94°C during which hydrogen bonds are broken and the DNA strands separate. At the annealing step, the temperature decreases to 59°C to enable primers to bind to the template. Next is extension at 72°C, which is an optimal temperature for Taq polymerase activity. Primers are extended by

adding dNTP's to nascent strand based on complementarity to bases in the template DNA. AmpliTaq Gold® is a modified form of the *Thermus aquaticus* DNA polymerase. It often adds an extra Adenine to the 3' ends of double-stranded PCR products (Clark, 1988) producing "N-band" after the last cycle. An extension at 60°C for 60 min ensures complete adenylation of all double-stranded PCR products to avoid split peaks. Finally, at the end of PCR run, samples are left at 4°C.

A negative control was used for each amplification method, and if positive, the entire batch was disregarded. Along with the samples, 9947A control DNA was amplified to test for accuracy of the thermal cycler activity. Control DNA contains 0.10 ng/µL human female 9947A DNA in 0.05% sodium azide and buffer.

Samples were amplified either once or in triplicate depending on the testing method used.

5.6 Capillary electrophoresis, STR analysis

Separation of amplicons by size was obtained through capillary electrophoresis using an ABI Prism® 3130*xl* Genetic Analyzer. 5 µl of each PCR product and 0.375 µl of GeneScan™ 500 Liz® Size Standard were prepared with HIDI formamide for a total volume of 50 µl for separation using capillary electrophoresis. HIDI formamide is highly deionized formamide used to resuspend samples and denature DNA before electrokinetic injection on capillary electrophoresis systems, thereby, the amount of ions in the sample that could compete with DNA for injection into the capillaries is diluted. Each sample was mixed with size standard before electrophoresis and each sample was run in its respective capillary. GeneScan™ 500 LIZ® Size Standard is used for obtaining precise sizing results for PCR products. It contains 16 single-stranded labeled fragments of: 35, 50, 75, 100, 139, 150, 160, 200, 250, 300, 340, 350, 400, 450, 490 and 500 bp nucleotides. Because the sizes (in bp) of the size standard peaks are known, sizes of sample peaks are determined through relative comparison of migration speeds during electrophoresis (Moretti et al., 2001a).

For samples amplified in triplicate, 5 µl of each of the 3 replicates were combined and mixed to form a pooled sample. 5 µl of this pooled sample was also injected. Along with samples, 0.5 µl of allelic ladder and 1 µl of a 1/10 dilution of the 9947A control DNA were prepared with 0.375 µl of LIZ and HIDI formamide in a total volume of 16

µl. Allelic ladders were used to calibrate PCR product sizes to STR repeat number for genotyping purposes. Allelic ladders contain all common STR alleles that have been previously characterized.

Upon preparation, all the samples were spun down in a centrifuge for 1 min at 1000 rpm and then placed in a GeneAmp® PCR System 9700 for denature/chill, which denaturated the samples at 95°C for 5 min and chilled at 4°C for 5 min. The samples were re-spun in a centrifuge for 1 min at 1000 rpm and placed into the 3130*xl* Genetic Analyzer containing separation matrix POP4 polymer. PCR products were separated on a 3130*xl* Genetic Analyzer at 3 kV for 20 s. The settings for the Genetic Analyzer were: oven temperature: 60°C, pre-run voltage: 15 kV, pre-run time: 180 s, injection voltage: 3 kV, injection time: 20 s, run voltage: 15 kV, run time: 1500 s. Sometimes oversaturated samples with too much DNA could be present. They appeared with lots of spurious peaks, such as numerous pull-ups due to the software tool (matrix) unable to compensate for a spectral overlap between fluorescent dyes resulting in a peak of another color under main peaks. Also, noise signals could be elevated and above detection threshold. To remove these numerous artifacts, oversaturated samples were reinjected at 1 kV for 22 s. Conversely samples with the peaks below threshold for allelic identification could be present due to very low amounts of DNA. These were reinjected at 6 kV for 30 s to pull these peaks up and thus obtain more information from the sample.

Data analysis was performed using GeneMapper® ID v.3.2 software. The peak amplitude threshold in GeneMapper was set to 75 RFUs. Peak ratio cut off value for tetra-nucleotide markers was set to 0.1. This filter removes labels from peaks that are less than 10% of the height of the highest peak at each locus. Stutter filters provided by the manufacturer were as follows: 6% (TPOX and THO1), 9% (D7S820 and CSF1PO), 10% (D13S317 and D5S818), 11% (D3S1358, vWA, and FGA), 12% (D8S1179), 13% (D21S11 and D16S539), 15% (D2S1338), 16% (D18S51), and 17% (D19S433). For single source samples, stutter peaks were removed if they were less than 20% of the height of the main peak. All profiles were edited for DNA artifacts, which included pull-ups, dye-blobs, spikes and elevated baseline.

5.7 Data interpretation

Samples that displayed the presence of more than 2 allele peaks at more than 2 loci were categorized as a mixture; otherwise samples were interpreted as a single source.

First step in a mixture analysis is to establish a minimum number of contributors to a mixture. This can be determined based on loci that exhibit the greatest number of alleles (Haned et al., 2011a; Haned et al., 2011b; Paoletti et al., 2005). Locus-by-locus allele counting can provide an estimate of the minimum number of contributors to a mixture, however due to allele sharing between individuals it may not indicate the actual number of contributor, particularly those with three or more DNA donors (Buckleton et al., 2007; Paoletti et al., 2005). A sample with three or more labeled alleles at two or more loci can be considered to contain a minimum of two contributors. A sample may also be deemed a two-person mixture even if there is a maximum of two alleles present in all loci. This could be attributed to heterozygote loci that are more imbalanced than the laboratory's empirically determined limit. This limit by the NYC OCME laboratory is 68% height of smaller peak to larger peaks at the heterozygote locus. A sample with five or more labeled alleles at two or more loci can be considered to contain a minimum of three contributors (Clayton et al., 1998). A mixture can range from components being present in equal amounts to one component being greatly in excess. Therefore, the following step in mixture analysis is determining the ratio or proportion of donors in the mixture followed by providing the genotype combination of DNA donors. The ratio is established based on the height of peaks at each locus. If the peaks are similar heights, then equal amounts of DNA from the donors is present, and the donors' DNA profiles cannot be determined. As the ratio of a mixture increases, interpretation of the major DNA donor becomes less complicated (Clayton et al., 1998). For 2-people mixtures, if the ratio was equal or higher than 1:3, the major donor was inferred and the mixture was categorized as a "deducible". For 3-people mixtures, the major was deduced only if obvious. A major DNA profile can be fully deduced or allele intensities could be such that the major contributor can be deduced in some but not at other loci, providing a partial DNA profile.

For samples that were amplified only once, all labeled alleles were considered when assigning a donor's profile. For samples amplified in triplicates, only alleles that were

present in at least 2 of 3 replicates were used to determine whether the sample is a mixture or a single source, and were used to assign a DNA profile of the sample's donor.

In order to assign alleles to single source samples, the interpretation criteria were employed:

- For samples amplified once, a heterozygous locus was identified by 2 peaks, while a homozygous locus has only one allele.
- For samples amplified in triplicates, a heterozygous locus was assigned if the 2 tallest peaks were in at least 2 amplifications and the peak heights were at least 50% of one another. If one repeating allele was less than 50% of the height of the major peak in 2 out of 3 amplifications, the possibility of homozygotes was considered, and a "Z" was assigned next to the major allele to indicate the possible presence of a second allele. Assigning homozygotes is more challenging. When amplifying small amounts of DNA template (usually bellow 20 pg) stochastic effects were inherent, such as exaggerated peak imbalances, allele drop-outs and allele drop-ins. Therefore, if one allele is clearly major and all other alleles are less than 30% of the major peak height in all 3 amplifications, the allele is considered a true homozygote.

When working with mixed samples, only a clear major component can be interpreted. In routine workflow, mixtures with apparently equal ratios of contributors as well as those where the ratios of contributors were varying at several loci could only be used for direct comparison. In direct comparisons a known DNA profile was compared to the mixture to establish whether an individual can be included or excluded.

The following interpretation guidelines were used when assigning alleles to the major donor to a mixture sample:

For samples amplified once, a heterozygote locus was identified by the two tallest peaks and heights of these peaks were at least 68% of one another, while homozygote locus has only one major allele at a locus.

For samples amplified in triplicates, alleles were assigned to a major component if they appeared in all three amplifications and they were the major peaks in at least two amplifications. Also, peak balance has to be greater than or equal to 50%. If one peak was clearly the major peak and the minor peaks (even if they

repeated) were less than 30% of the major peak in all amplifications, an allele was assigned as a true homozygote. In cases, where the height of the second allele was between 30% and 50% of the height of the main peak, and it was not clear whether the major contributor was heterozygous of homozygous, a major peak was assigned to the major component along with a "Z". When alleles could not be assigned to the major component, the locus was deemed inconclusive.

Deduced or assigned profiles were subsequently compared to the reference profile to determine if the profile matched the donor. The match was established if DNA test results at each locus were identical. The DNA profiles were expressed as the percentage of determined alleles. Percentage of alleles was calculated by dividing the number of determined alleles with the expected number of alleles for that individual.

5.8 Data analysis

Data analysis was performed using SPSS version 21 (IBM, Armonk, NY) and Microsoft Excel. The calculations included bivariate statistics: means, t-test, ANOVA, correlation, as well as prediction for numerical outcomes: linear regression and logistic regression.

6. Results

6.1 Collection of single cells

Cells from individual contributors can often be discriminated on a microscopic level by cell morphology or location, and with the help of specific staining techniques. Sperm cells can be clearly distinguished from epithelial cells based on differences in their size and shape. Epithelial cells are large and round, whereas spermatozoa are smaller and have a tail structure. Here, effective strategies were determined to transfer single cells measured by PCR using the AmpF*l*STR® Identifilier® kit for STR typing and calculating the percent of STR profiles as an outcome. Microdissection was carried out by preparing a slide or a whole specimen. Epithelial cells and spermatozoa were stained using NFR stain, as it works in the visible light spectrum and doesn't affect STR PCR amplification ((Burton et al., 1998; Sanders et al., 2006)). DAPI staining was considered but was dismissed, since DAPI is excited with UV-light, which can affect the DNA by inducing formation of pyrimidine dimers (Sinha and Hader, 2002; Tewari et al., 2012) and can lead to DNA degradation, thereby affecting the downstream results and completeness of the STR profiles. Cells were stained in order to clearly visualize nucleated cells and accurately keep count of cells that were picked and placed in a test tube, and thus estimate the amount of DNA in the sample. Fingerprint samples were not stained. This is because skin cells appear entirely red (when stained with NFR) or entirely fluoresce (when DAPI is applied), disabling visualization of a nucleus. Consequently, it was difficult to distinguish nucleated from non-nucleated cells and thus estimate the amount of DNA in a sample. Difficulty in clearly visualizing the nucleus within skin cells could be explained by apoptosis and nuclear fragmentation that keratinocytes suffer as they shed from our body (Gandarillas et al., 1999). Although, some skin cells may still have nuclear DNA (Kita et al., 2008), the majority of skin cells/flakes do not contain nuclei. The presence of extracellular DNA in touched samples (Quinones and Daniel, 2012) was the additional reason for not staining the fingerprint samples. Additionally, this procedure includes washing steps to remove excess staining, which could also wash away an extracellular DNA.

In this study, various tools and adhesive materials were evaluated for their ability to lift and transfer a cell to a test tube. Three micromanipulation techniques were used and

compared: 1) the P.A.L.M.® system, 2) manual manipulations under the Axiozoom .V16 microscope using tools such as a sterile hypodermic needle, tungsten needle or microtweezers coupled with adhesive microspheres or other adhesive materials, and 3) using robotic micromanipulator aureka® and observing robotic manipulations under Axiozoom .V16.

The P.A.L.M.® system and manual manipulations tools where available in the laboratory of the NYC OCME, whereas aureka® manipulator, a state of the art technique for single cell manipulation, was procured for these comparisons. By evaluating various tools and instruments, strategies were discussed on how to reliably transfer various cell types for DNA testing.

6.1.1 Microcarriers to collect single cells

Microcarriers are tiny spheres that range in sizes of 90-300 μm in diameter. They were held by tweezers with the correct position controlled using a mirror (Bruck et al., 2011). The microcarrier had to be positioned far enough forward between the tweezer tips so that it protrudes below them, while still being firmly gripped, so that a microcarrier can establish a contact with the cell of interest. The mirror was placed on the microscope stage at an angle of 45°, and was used to monitor the best position of the microcarrier. Microcarriers were assessed for the ability to adhere on a selected cell and hold it with greater force than the cell holds on to the surface, thereby, selected single cells can be collected and transferred into a reaction tube. The entire process was monitored under the microscope. Microcarriers evaluated in this study were commercially available microglobes and spheres made of glue.

Three types of commercially available microglobes from Solo Hill Engineering were tested. These included polystyrene beads coated with collagen, ProNectin® F, and CT. As well as Cytodex™3 a dextran microglobe coated with collagen. In addition, office glues (“Pritt”, “Elmer’s” and rubber cement) and homemade spheres (Arabic gum mixed with glycerol (1:5 to 1:9) were tested.

Glue spheres were manually made by smearing glue on a microscope slide and rolling a portion of the glue into a sphere using a needle micro-tool. This entire process was monitored under an Axiozoom .V16 stereomicroscope where the size of a glue sphere was measured and adjusted for single cell picking. The best size suitable for single cell

picking was around 100 μm in diameter.

Adhesive properties of microglobes:

Microglobes from Solo Hill Engineering were easy to handle. They appeared firm and were easily picked and held with microtweezers. Sperm or epithelial cells were not attaching to microglobes, but skin cells were partially adhering. It was difficult to lift cells consistently and the adhesive force was insufficient to deposit selected cellular material into a reaction tube. Treating of the microglobes such as heating, drying, or cooling (according to the manufacturer's suggestion) did not increase adhesiveness. Glue pre-treatment, using "Elmer's" or "Pritt" glue also did not increase adhesiveness. After the treatment, microglobes were still not adhesive enough to reliably collect cells. Therefore, microglobes from Solo Hill Engineering were not further considered for single cell picking. The Cytodex™3 microglobes were adhering one cell, however the Cytodex™3 were soft and often burst under the pressure of tweezers, which made them difficult to handle. They were too small to pick more than one cell per globe. In addition, capillary forces tended to accumulate extraction buffer between the tweezer's tips when releasing a sphere in the tube resulting in loss of the microglobe.

When evaluating glue spheres, Arabic gum/glycerol (AG) spheres exhibited adhesive properties to efficiently lift single cells. The sphere came easily off the tungsten needle when transferred into the reaction tube because of dissolving in the extraction buffer. As a result, AG spheres were found as a good tool to pick single cells. Rubber cement (RC) spheres were less adhesive and could not be used to pick up single buccal or sperm cells, but RC spheres were suitable for swabbing tiny areas of a fingerprint to collect skin cells (partial prints). Rubber cement spheres were best manipulated with tweezers (aureka® or Dumont).

"Pritt" glue was very sticky and lifted single cells efficiently. These spheres were carried with tungsten needles. When used to swab cellular material from fingerprints, it smeared and left glue stains on the microscope slide, therefore these spheres could be used for picking individual cells. However, "Pritt" glue spheres could not be easily released into the reaction tube because they do not dissolve in the extraction buffer and they tend to stay on the needle due to their stickiness. "Elmer's" glue, which is soluble in water, was difficult to roll into a sphere, and could not be used to lift cells.

Compatibility of spheres with PCR reaction

The microglobes from Solo Hill Engineering and Cytodex™3, Arabic gum/glycerol, rubber cement, and the glues "Pritt" and "Elmer's" were tested for PCR inhibition using the AmpF*l*STR® Identifiler® Plus amplification kit. Only Cytodex™3 showed PCR inhibition if more than one microglobe was added to the reaction tube.

Outcome from these evaluations

Single epithelial (buccal) and sperm cells were successfully collected with AG spheres held by a tungsten needle. These spheres could also be efficiently used to pick individual skin cells or flakes. However, fingerprints and dried skin flakes were swabbed rather than picked up due to the origin and nature of these cells (Gandarillas et al., 1999; Kita et al., 2008). RC spheres were used to swab partial prints and cotton threads rolled into a sphere for the whole print. RC spheres or cotton threads were held by tweezers.

If fingerprints were present on an object that is too large, they were transferred by tape and swabbed rather than direct microscopic visualization because of its difficulty in sampling.

6.1.2 Needles to collect single cells

Hypodermic and tungsten needles can be used to scrape cells. However, skin cells and flakes do not exhibit electrostatic interactions and are therefore difficult to transfer by a needle. Upon amplification using AmpF*l*STR® Identifiler® Plus, directly or following DNA one-tube extraction, no data or sporadic results were obtained. Epithelial (buccal) cells spread onto microscope slides can be scraped off using the sharp part of the hypodermic needle. Tungsten needles are not as effective as hypodermic needles because of their pointed shape. Their top is not as sharp and wide as hypodermic needles' therefore they are less efficient to scrape cells off the surface. Amplification, following the one-tube extraction, using AmpF*l*STR® Identifiler® Plus led to full STR profiles. However, the use of needles to collect single cells was not further considered, since the scraping procedure would capture too many cells.

6.1.3 Tape to collect prints and skin cells

Tapes can be used to preserve the position of cells collected from an object for further scanning or digitalizing fingerprints. Fingerprints on large objects, such as door knobs or parts of a wall, can be lifted by tape and transferred to analyze by microscope (Olympus SZX-16® or Axiozoom .V16 stereomicroscope). Tapes could also be used to collect cells from evidence to minimize the transfer of PCR inhibitors such as indigo dye from denim or other dyed fabrics (Opel et al., 2010). Fingerprints can be swabbed off of a tape. Nucleated cells, such as buccal or sperm cells can be cut out of tape and patches can be transferred into the 0.2 ml test tube for DNA analysis. Five types of tape were tested: Latent print tape, "DIFF-lift" tape, "SIRCHIE", "tesa", and "Scotch" tape. Thickness, structure, and clearness of the different tapes were inspected, as well as their impact on PCR. Physical characteristics of the tapes were initially evaluated looking at the ease of cutting the tape as well as visibility of the cells lifted by the tape at the end. Latent print tape and "DIFF-lift" tape were either too thick or textured and, therefore, too difficult to be used for manual cutting. Because of this, they were excluded from further studies. "SIRCHIE", "tesa", and "Scotch" tape could be used for manual cutting, however, previous studies evaluating different tapes for collecting touched material from objects showed that "Scotch" tape was significantly outperformed in DNA recovery, and therefore was excluded (Verdon et al., 2014). When evaluating tapes with micromanipulation instruments, it was found that tapes can only be used for manual and robotically-assisted micromanipulations, but not for laser capture microdissection, since the laser cannot cut through the tape.

In addition, tapes cannot be used together with cell staining since the tape absorbs the color of the stain making it difficult to locate cells.

<u>Compatibility of tapes with PCR reaction</u>

The two tapes, "SIRCHIE" and "tesa", were amplified using the AmpF*l*STR® Identifiler® Plus PCR amplification and showed no PCR inhibition. Since "SIRCHIE" tape was produced for forensic applications, it was selected.

6.2 Partial fingerprints

Partial profiles, below 70%, were not considered as database eligible. In order to compare the outcome, the completeness of STR profile was used rather than the number

of full CODIS loci in the profile which, in routine forensic workflow, defines database eligibility. Here, the threshold of 70% was conservatively established. Profiles of at least 70% completeness could have drop-outs at various loci (CODIS and non-CODIS loci) and still be suitable for database searches.

When evaluating various tools for single cell collection, individual skin cells were hardly producing any results. Therefore, sample size was expanded to five skin cell clumps/flakes per sample. The obtained DNA profiles were partial, but very low, barely reaching 20% completeness. Consequently, a small area of the fingerprint was swabbed. One individual fingerprint was divided into eight similar size sections, which were processed separately for DNA extraction and STR amplification. Zygem extraction followed by ID31 amplification was the methodology used. The qualities of profiles showed a broader variation, some of them reached 40% completeness. Based on this data, it was concluded that fingerprints should be collected by swabbing in their entirety in order to obtain meaningful results that could be database eligible.

6.3 Protocol for DNA extraction and STR amplification from fingerprints

In order to find a method that can maximize DNA recovery from fingerprints, various swabbing solutions, extraction and amplification protocols were compared. For each experiment, fingerprints from at least five volunteers were collected for five or more days, aiming for 50 prints per experiment. Table 1 shows an overview of the experiments performed.

Table 1. Overview of experimental procedures on fingerprint samples

Experiment	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Swab	Water	Water	Water	Water	Water	5% Triton X-100	5% Triton X-100	5% Triton X-100	5% Triton X-100	5% Triton X-100
Extraction	One-Tube	One-Tube	One-Tube	One-Tube	One-Tube	Zygem	Zygem	HighSens	HighSens	One-Tube
Quant	Single	Single	Triple	Single	Triple	Single	Single	Duplicate	Duplicate	Single
Amplification	ID+ (Single)	ID+ (Triple)	ID31 (Single)	ID31 (Triple)	ID28 (Single)	ID+ (Triple)	ID31 (Triple)	ID+ (Triple)	ID31 (Triple)	ID31 (Triple)
Total number of prints	222	46	43	41	36	45	43	49	93	92
Number of mixtures	24	2	1	5	2	5	3	1	3	0
Number of samples ≥2 pg/µl	111	30	16	23	19	32	34	43	86	41

6.3.1 DNA extraction and swabbing solutions to collect fingerprints

Different extraction protocols were compared in order to find a reliable method to extract DNA from biological traces with a high recovery rate. Amounts of extracted DNA were quantified and compared throughout the tested protocols. To quantify the samples, an *Alu*-based real-time PCR, adapted from Nicklas and Buel (Nicklas and Buel, 2003) was used, which has a 30% error rate.

Fingerprint samples were used in this study. Fingerprints were deposited on microscope slides and were swabbed in their entirety with a moistened cotton swab, using either water or 5% Triton X-100, under the Olympus stereomicroscope® SZX-16. DNA was isolated using one of three protocols: HighSens, one-tube, or Zygem. The HighSens protocol is routinely used at the NYC OCME to extract DNA from touched samples and in comparison to other routinely used DNA extraction methods it led to the highest DNA recovery (Schiffner et al., 2005). This DNA extraction includes multiple steps, such as purifying a sample and multiple tube exchange. Conversely, using one-tube and Zygem extractions, a sample is extracted in one step, whereby only one tube is used throughout the entire extraction and the sample is not purified.

The DNA concentration of the extracted fingerprints using each of the three extraction methods was compared (Figure 3). Figure 3 represents the fraction of samples extracted using each of the three extraction protocols that obtained at least 2, 3, 4, 5, and 6 pg/µl. Over 90% of samples extracted using the HighSens extraction protocol yielded DNA concentrations greater than 2 pg/µl. Zygem and the one-tube extraction resulted in at least 2 pg/µl of DNA in 70 and 40% of the samples, respectively (Figure 3). The mean values of the DNA concentration were 32.2 pg/µl for HighSens, 11.8 pg/µl Zygem, and the lowest for one-tube of 9.9 pg/µl. Fisher's exact test showed that both, HighSens and Zygem extractions, were significantly better than the one-tube extraction ($p=1x10^{-13}$, $p=6.5x10^{-5}$). Moreover, the HighSens extraction was better than the Zygem extraction ($p=0.001$).

Samples extracted using the one-tube extraction protocol, were quantified in single and triple measurements (of which a median value was used in comparisons). Triplicate measurements were done to reveal consistency of quant results for very low amounts of DNA. It was found that single measurements led to higher values, most likely due to outliers (Figure 3). The difference was significant (Fisher's exact test $p=0.028$),

however, the measurement procedure cannot improve the outcome; rather, using the median value of three measurements eliminated extreme values that can sometimes occur with a single measurement. Therefore, triplicate measurements are considered to be more accurate. However, because each sample was extracted in a total volume of only 20 µl, and each amplification required 5 µl of DNA extract, the triplicate quantification was not performed in order to preserve sample for STR amplification.

The two swabbing solutions, water and 5% Triton X-100, were compared using the one-tube extraction protocol (Figure 3). It was found that swabbing solutions were not associated with higher or lower DNA yield (Fisher's exact test p=0.132).

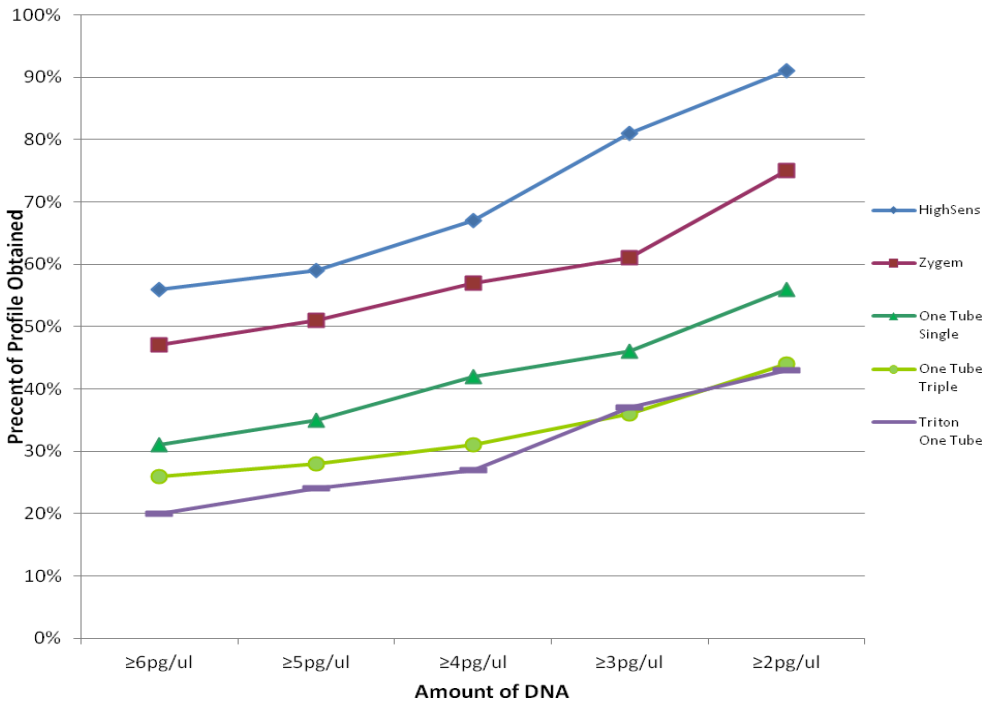


Figure 3. Comparison of DNA extraction protocols:
X-axis: distribution of DNA concentrations in the fingerprint samples, and Y-axis: fraction of fingerprint samples swabbed with water or Triton X-100, extracted using one-tube, HighSens, or Zygem protocols, and quantified by using *Alu*-based real-time PCR (in single or triple measurments for one-tube samples) that contain at least 2, 3, 4, 5, and 6 pg/µl of DNA.

6.3.2 STR amplification and partial profiles

Following DNA extraction, the DNA of the fingerprint samples were amplified using ID28, ID31 and ID+. The amplification protocols were compared in order to find the protocol that would reliably result in database eligible profiles (>70%). The samples extracted using the one-tube extraction protocol were amplified with ID28, ID31 and ID+, while samples extracted using HighSens and Zygem protocols were amplified with ID31 and ID+ (Figure 4). More drop-outs are expected to be observed with ID28 than ID31 amplification, which was investigated only with samples extracted in one-tube extraction protocol. Of the 756 fingerprint samples processed, 710 revealed to be single source and 46 were mixtures (6%). Mixtures were detected by additional signals at multiple loci (Budowle et al., 2009b; Caragine et al., 2009; Cowell et al., 2007). Mixtures were excluded from data analysis leaving 710 single source samples. Most of the STR profiles obtained were partial.

DNA concentration was an important predictor of the completeness of the DNA profile obtained from a sample. For samples of which less than 2 pg/µl of DNA was obtained (n=275), only 21 resulted in profiles that were database eligible. Therefore, samples with higher or equal concentration to 2 pg/µl DNA (n=436) were compared to find out which of the STR amplification protocols would lead to the best outcome.

Figure 4 shows the percentage for each experimental procedure that reached ≥70% complete profiles. Logistic regression was performed using 70% of profile or greater as the desired outcome. Only samples with concentration greater than or equal to 2 pg/µl were included (n=436).

The one-tube extraction followed by ID31 was considered as baseline for the comparison. Using the one-tube extraction procedure followed by ID31 amplification, almost 70% of tested samples (≥2 pg/µl) reached at least 70% completeness. Using ID+ increased the outcome to approximately 90%, while ID28 showed a decrease (Figure 4). Linear regression demonstrated that ID+ amplification was associated with a 12.7% increase ($p=1.36x10^{-6}$) in percent profile obtained over ID31, and ID28 was associated with a 2.5% decrease relative to ID31 (p=0.677). Additionally, we compared the effects of single versus triplicate STR amplification using linear regression for samples extracted by the one-tube protocol (Figure 4). In all comparisons the triplicate amplification showed an increase between 1 to 8% of the profile compared to single

amplification, which were not significant (p-values varied between 0.2 to 0.7).

For Zygem extracted samples, using ID+ had no effect. The ID31 amplification resulted in over 90% of samples that were at least 70% complete (Figure 4). The same trend was observed for samples extracted with HighSens, but lower than for the Zygem extraction (Figure 4).

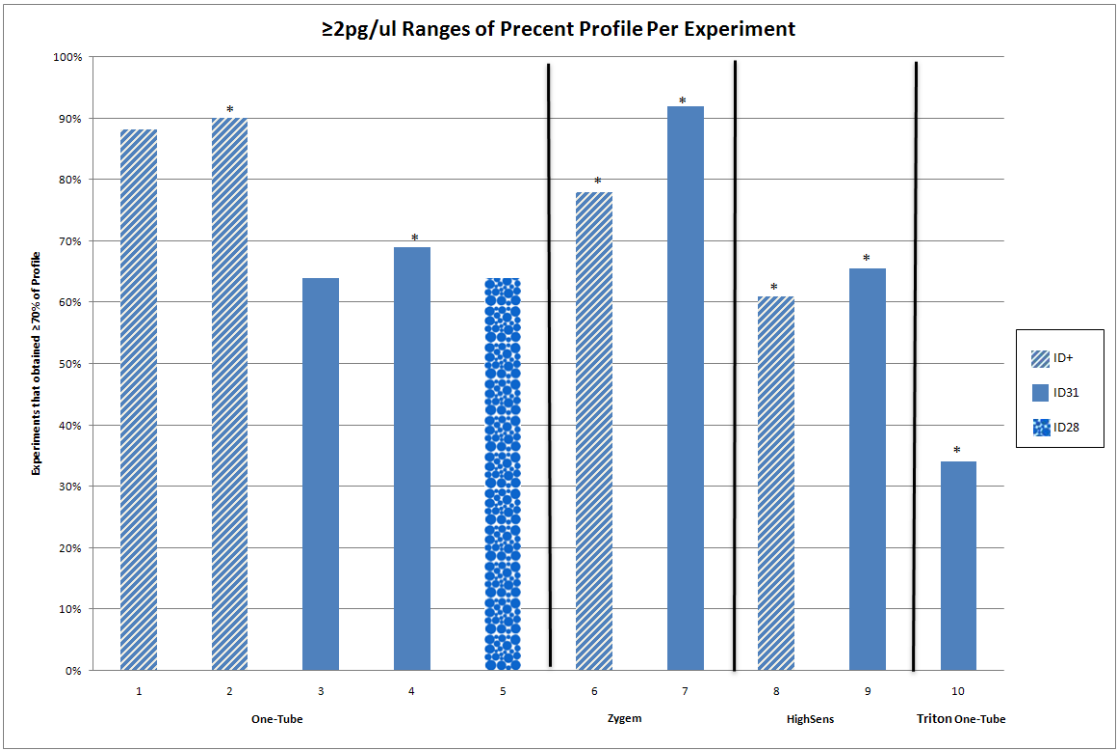


Figure 4. Percent of each experimental procedure (extraction and amplification) with ≥ 2 pg/µl that reached ≥70% profile:
X-axis: experimental procedures [detailed in Table 1. (*, amplification was performed in triplicate)], and Y-axis: percent of non-mixture fingerprint samples with DNA concentrations ≥2 pg/µl for which at least 70% of the donor's profile was obtained.

When Zygem extracted samples were amplified with ID31 and ID+, the outcome was increased in comparison to the one-tube extraction followed by ID31. There was a 67% increase in the probability of obtaining at least 70% of the donor's profile, which was not significant (p=0.285). HighSens extracted samples followed by either ID31 or ID+ amplifications, were associated with a 65% decrease in the probability of obtaining at least 70% of the donor's profile in comparison to the one-tube extraction followed by ID31, which is a significant decrease in the outcome (p=0.005).

These data show that a significantly greater number of better quality profiles were obtained from DNA ≥ 2 pg/µl using the Zygem extraction than the one-tube extraction followed by ID31. However, the HighSens extraction did not lead to improved profile quality. Using ID+ instead of ID31 for amplification following the one-tube extraction also resulted in significantly more quality profiles.

Furthermore, linear regression was performed using the percent of the profile obtained as the outcome of interest. For this test, data from all fingerprints except mixtures were included (n=710). Independent variables included the shedding score for the fingerprint, DNA extraction protocol used, DNA concentration of the extract, and amplification protocol. All variables were associated with the percent of profile obtained in simple linear regression (data not shown) and all remained significant in a multiple linear regression model. In the full model, Zygem and HighSens extractions were associated with an increase of 18.6 and 18.7% in percent of profile obtained, relative to the one-tube process, which were significant ($p=1.9x10^{-6}$ and $2.6x10^{-5}$, respectively). ID+ and ID28 were associated with a 13.2% increase ($p=1.2x10^{-6}$) and 1.3% decrease (p=0.83) in percent profile obtained, respectively, relative to ID31. Each unit in shedding score was associated with a 4.2% increase in the percent profile obtained. This association was significant ($p=4.8x10^{-4}$).

The effect of the extraction for HighSens protocol was very prominent, over 90% of samples yielded more than 2 pg/µl DNA (Figure 3), the outcome was still better for the HighSens procedure than for the one-tube extraction followed by ID31 (Figure 4).

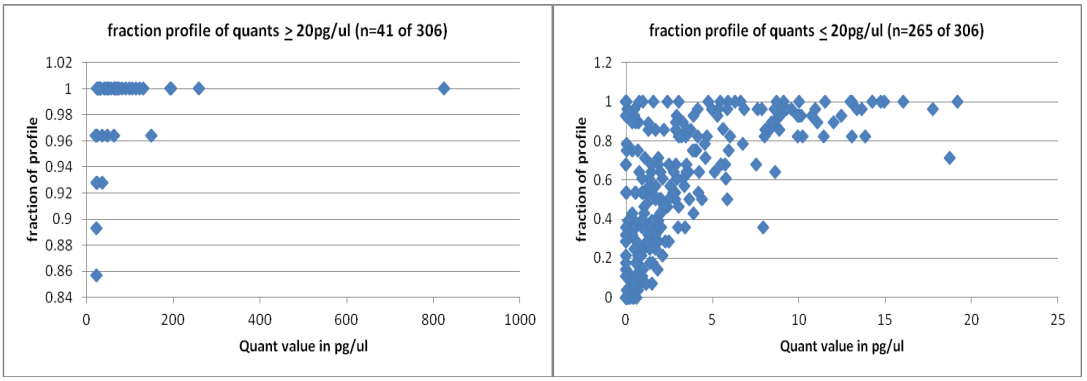
Finally, when including the results of the extraction method, the best protocol is Zygem DNA extraction followed by ID31, while HighSens followed by ID31 and the one-tube extraction followed by ID+ have a similar but lower success rate.

6.3.3. Correlation between quant and DNA profile completeness

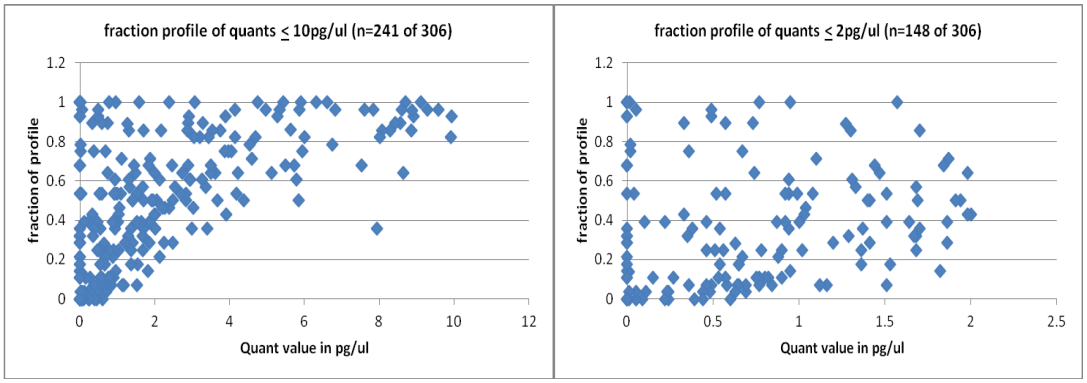
DNA obtained from touched samples is usually in a very low range, less than 20 pg/µl. Touched samples extracted using PrepGem® tissue extraction kit, followed by estimating the amounts of DNA with the *Alu*-based real-time PCR method and ID31 amplification were analyzed to understand if any correlation exists between these low DNA quantities and profile qualities. Here, Pearson's correlation coefficient (R) was calculated, which is in statistics a measure of the linear correlation between two

variables giving a value between +1 and −1 inclusive, where values closer to 1 indicate strong positive correlation, 0 is no correlation, and -1 is strong negative relationship between two variables. 41 of the 306 samples tested obtained > 20 pg/µl, but approximately 50%, or 148 fingerprints, had < 2 pg/µl. Quant and fraction of DNA profiles were plotted together, and a correlation coefficient was calculated (Figure 5a-d). For all samples Pearson's correlation was positive, not strong but significant (r=0.265).

Fingerprints with quant values ≥ 20 pg/µl (n=41 of 306) resulted in more complete profiles, and clear correlation between quant and fraction of profiles was seen (Figure 5a). If quant was ≥ 10 pg/µl, database eligible profiles (higher than 70%) were obtained. The lower the quant was, the weaker the correlation was (Figure 5b and c). For 148 measurements with less than 2 pg/µl of DNA, very weak positive correlation (r=0.135) was found. No correlation was seen for measurements below 0.25 pg/µl. For these low quant values, variable profile qualities could be expected (Figure 5d). This was a reason why the methods previously compared contained at least 2 pg/µl, and data with less than 2 pg/µl were excluded from these comparisons to avoid incorrect conclusions or bias.



A. quants ≥ 20pg/ul (n=41 of 306) **B**. quants ≤ 20pg/ul (n=265 of 306)



C. quants ≤ 10pg/ul (n=241 of 306) **D**. quants ≤ 2pg/ul (n=148 of 306)

Figure 5. Correlation between DNA amounts and fraction of profiles for fingerprints samples with quant values ≥ 20pg/ul (**A**), ≤ 20pg/ul (**B**), ≤ 10pg/ul (**C**), and ≤ 2pg/ul (**D**) : X-axis: DNA amounts and Y-axis: fraction of DNA profiles obtained.

6.4 Single fingerprint analysis

The aforementioned comparative study between extraction and amplification protocols led to a protocol with a highest number of database eligible STR profiles, which is Zygem DNA extraction followed by STR PCR amplification using AmpF*l*STR® Identifiler® kit in 31 cycles. Thereby this protocol was used to perform extensive analysis on fingerprint samples to better understand their inherent challenges. The protocol also included swabbing prints with a portion of cotton swab moistened in 5% Triton X-100.

In this thorough study, DNA was recovered from the fingerprints on various days after their deposition. In addition, the impact of various substrates, and shedding of the individual on DNA recovery from fingerprints was investigated. Most importantly, a sectioning sampling approach was tested on items that were touched by three persons consecutively to create mixtures.

6.4.1 Visible deposition and fraction profile

Fingerprints on touched items can greatly vary in the density of deposited skin cells. To determine whether the shedding level, sparse or dense, could be used as a predictor of successful DNA amplification, DNA profiles were obtained and compared to the assigned shedding levels. In addition, linear regression was performed using the shedding score as the independent variable and percent of profile obtained as the outcome of interest.

Prior to donating fingerprints, volunteers were asked to refrain from washing their hands for at least 2 h. Fingerprints of left and right thumbs were separately deposited on microscope slides, visually examined under the stereomicroscope (Olympus® SZX-16) prior to swabbing and DNA extraction. Based on the density of skin cells within the fingerprint immersion quality scores 1-5 (sparse to dense shedding) were assigned to each fingerprint. Occasionally the scores 1 and 5 were assigned, but most fingerprints had the scores 3 or 4. Nevertheless, some individuals tend to leave more cells on the glass slides than others. Over 110 fingerprints were analyzed per person. Although, there were not very great differences, fingerprints from donors 10 and 21 tended to receive higher shedding scores than prints from donors 12 and 14 (Figure 6A). Following visual examination, fingerprints were swabbed, DNA was extracted using the Zygem procedure, and the samples were quantified and amplified using ID31. Figure 6B shows the range of percent profiles obtained for the same volunteers whose shedding scores were shown in Figure 6A. Great variation in profile completeness was obtained for each volunteer. Volunteers with higher propensity to shed did not necessarily produce higher quality DNA profiles. Prints from donors 14 and 21 tended to produce profiles that were more complete than those from donors 10 and 12, although donors 10 and 21 were the better shedders (Figures 6A and B).

The average percentage of DNA profile obtained increased between score 1 and 3 (from

43.6 to 66.2% profiles), while higher scoring did not further increase percent of DNA profile obtained. Using linear regression, the increase in the amount of DNA of 2.24 pg/µl ($p=0.20$) and in the percent of DNA profile of 4.2% ($p=4.8x10^{-4}$) per one unit in shedding score was observed. But, because of the great variability in profile qualities from different shedding scores recovered, the shedding score was not considered to be used as a reliable predictor for profiling success (Ostojic et al., 2014).

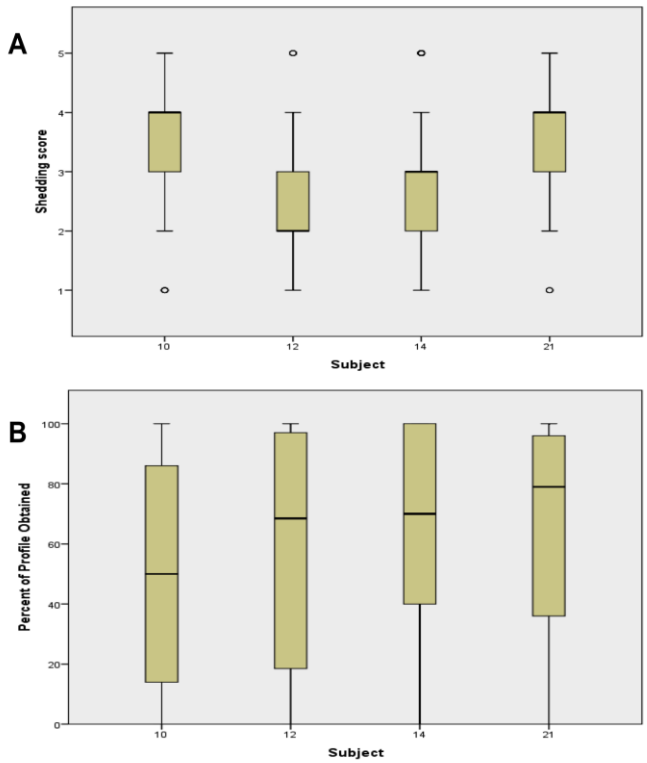


Figure 6. Single fingerprint analysis: (**A**) Distribution of shedding scores (1-5) for fingerprints and (**B**) distribution of percent profile obtained from fingerprints collected from volunteers 10, 12, 14, and 21. Shown are median (dark line), 25th and 75th percentile (bottom and top of box), minimum and maximum (T-bars), and outliers (circles).

6.4.2 Analysis of right versus left fingerprints

756 fingerprints with assigned shedding scores of 1 to 5 were compared to reveal if left and right hands differ in cell density deposition upon touching an object. A significant difference between shedding score of donors' right and left hand could not be found for any of the volunteers (Chi- square test showed p- values of >0.4 for all volunteers) (Ostojic et al., 2014).

In order to find out if this holds for the percentage of the profile, comparisons were done between 643 prints from three right handed volunteers (volunteers 1, 2, and 3) which were swabbed, extracted using Zygem and amplified with ID31. 25 out of 643 fingerprint samples were mixtures and were excluded from the comparisons. Mixtures are commonly observed with touched samples due to secondary transfer which happens by transferring DNA fragments from other people, by shaking their hands, or by handling previously touched objects (van Oorschot et al., 2010; Zoppis et al., 2014). The DNA profiles obtained from their left hands were more complete than from their right hands (Figure 7). However, t-tests showed that the difference between left and right hand for volunteer 1 was not significant ($p=0.206$), but was for the other two ($p=0.001$ for volunteer 2 and $p=0.020$ for volunteer 3, see Figure 7). T-tests for all fingerprints showed that the difference of profiles obtained from left and right thumbs was significant ($p<0.001$).

The study illustrated that less complete profiles could be expected from a hand more frequently used. This could be due to losing some cells and extracellular DNA that shed on various objects during the 2 h between washing and sampling.

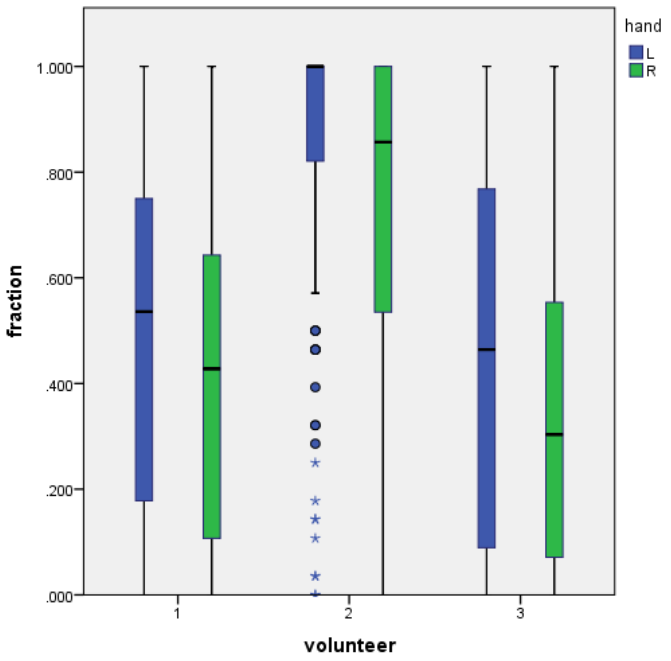


Figure 7. Single fingerprint analysis: Distribution of percent profile obtained from fingerprints collected from left (blue) and right (green) hand from volunteers 1, 2, and 3. Shown are median (dark line), 25th and 75th percentile (bottom and top of box), minimum and maximum (T-bars), and outliers (circles).

6.4.3 Time course

A time course study was performed to find out if the fingerprints provoke quality changes in STR-profiles over time. In addition, it was evaluated whether in-house conditions (fingerprints on microscope slides stored in open boxes which were exposed to dust) could affect the quality of the resulting STR data. The fingerprints were left in the laboratory space, at room temperature, and were not exposed to sunlight.

The prints were swabbed using 5% Triton X-100, DNA was isolated using Zygem and STR amplification was done with ID31. Of 643 fingerprints, 25 mixtures were found and excluded from data analysis.

Figure 8 shows the profile fractions obtained from 381 prints that were stored in clean, closed boxes for the following time periods: 1, 3, 10, 20, and 40 days.

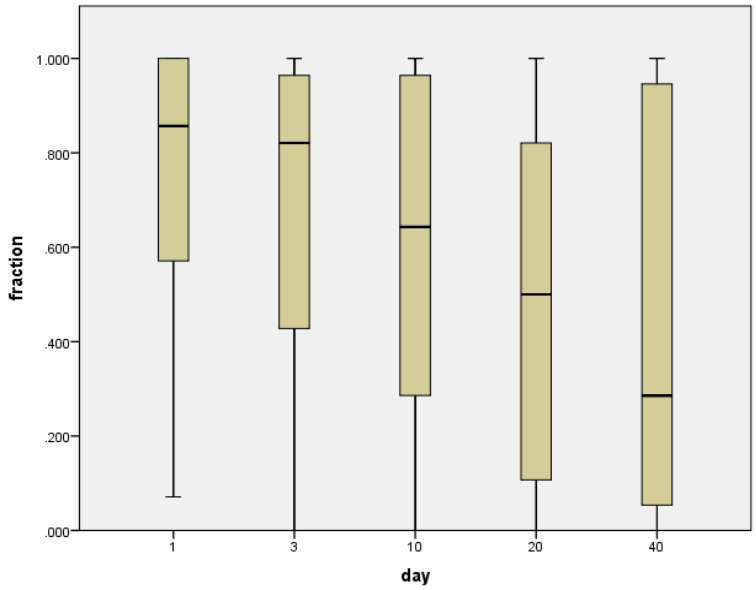
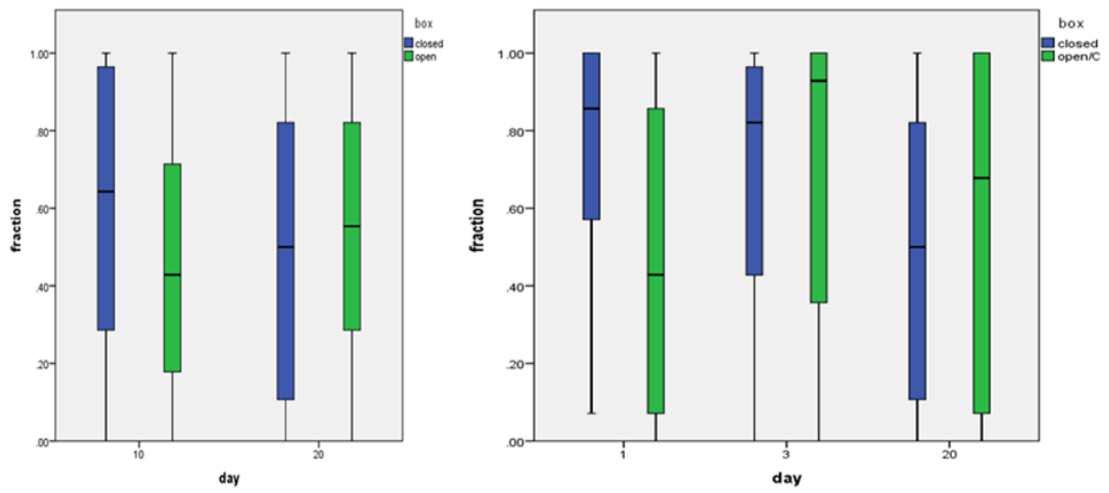


Figure 8. Time course study: Y-axis: completeness of the STR profiles as fraction; X-axis: 1 (n=66), 3 (n=65), 10 (n=67), 20 (n=147), and 40 (n=36) days. Fingerprints were protected from dust in closed boxes.

The fraction of profile obtained from all the prints varied greatly. However, over time, fingerprints result in smaller fractions of database eligible profiles. A great portion of fingerprints, more than a quarter, showed profiles that were at least 70% complete after 40 day storage, and even some full profiles were obtained. More profiles were database eligible the shorter storage time was. ANOVA analysis showed significance between these time points and t-tests specified that the decrease of the fraction of the profile was not significant between day 1 and day 3 ($p=0.24$), but the decrease was significant between day 1 and day 10 ($p=0.013$), as well as for longer time periods ($p<0.0001$).

An additional 82 fingerprints, stored in open boxes, were exposed for 10 and 20 days to in-house conditions of the laboratory area, e.g. dust. Figure 9 represents fingerprints stored in open and closed boxes for 10 and 20 days from three volunteers. Prints that were exposed 10 days to in-house conditions, e.g. dust, led to lower fraction profiles than the fingerprints that were protected (t-test: $p=0.033$). After 20 days, a difference between open and closed boxes could not be observed ($p=0.3$) (Figure 9A). The impact of dust and time on DNA degradation after 20 days was similar.



A. N=296 (volunteer 1, 2, and 3) **B.** N=433 (volunteer 1, 2, and 3)

Figure 9. Comparison of storage conditions: **A**: blue bars: storage in closed boxes; green bars: storage in open boxes (5 µl DNA template for STR amplification); X-axis: storage time in days: 10 (closed: n=67, open: n=40), 20 (closed: n=147, open: n=42); **B**: blue bars: storage in closed boxes; green bars: storage in open boxes, samples were consumed (6 µl DNA template for STR amplification); X-axis: storage time in days: 1 (closed: n=66, open: n=42), 3 (closed: n=65, open: n=52), and 20 (closed: n=147, open n=61); Y-axis: storage (for 9A and 9B): fraction of STR profiles.

For the tests above, the extracted DNA was quantified and 5 µl was used for PCR amplification, which was done in triplicate. When working with fingerprints, there is more of a problem in having too little than too much DNA; therefore, further tests were done skipping quantification, consuming the sample in amplification and the volume of DNA template was increased from 5 to 6 µl per PCR reaction. For another 155 fingerprints that were stored in open boxes, for the time periods 1, 3 and 20 days, the extracted DNA was not quantified. The comparison to storage in closed boxes revealed a difference existing between prints after one day of storage, which was not seen after three of the 20 days (Figure 9B). A small difference was seen for 20 day storage in open boxes using 5 or 6 µl for the STR amplification (Figure 9A and B, comparing green box-plot of day 20). These results indicate a minor difference between the protected storage in closed boxes and in open boxes in laboratories. Nevertheless, it should be noted that after almost three weeks of storage more than 25 percent of the profiles were

database eligible.

It can be concluded that touched items should be processed as soon as possible in order to get meaningful STR profiles. However, the study also demonstrated that it could be possible to obtain forensically usable profiles from touched evidence even if it was examined 40 days after a crime was committed.

6.4.4 Fingerprints on various substrates

When prioritizing touched evidence from a crime scene for STR analysis it is important to know which one might have the highest likelihood of success of obtaining a database eligible DNA profile. Therefore, to test the effect that various substrates may have on cellular material, fingerprints were placed on glass (microscope slide), metal (a cleaned US Quarter dollar, made of 91.67% Cu and 8.33% Ni), paper, and plastic (a sheet protector made of polypropylene). These are typical substrates of items that are commonly touched in households and offices.

Prior to fingerprint collection, the substrates were, except for paper, decontaminated using 10% bleach, followed by water, and 70% EtOH. In addition, all substrates were UV irradiated for 30 minutes in the NuAire biosafety cabinet. The fingerprints were swabbed three days after their deposition with 5% Triton X-100. Time course study indicated that three day storage could result in slightly less complete STR profiles; however, the difference was not found to be significant (Figure 8). DNA was extracted using Zygem followed by ID31 amplification without quantification. 6 μl of DNA extract was used for STR amplification, consuming the sample. There were 32 prints per substrate analyzed. From the total of 128 samples, five mixtures were detected and excluded from analysis. The completeness of the DNA profiles ranged widely. From each of these substrates, except metal, full profiles could be obtained. Over half of the fingerprints on glass slides led to database eligible profiles (≥70% complete). By comparing Figures 8 and 9 (3 days on a glass slide), no difference can be seen in the outcome for using 5 or 6 μl of DNA extract for STR amplification. Over a quarter of fingerprints on plastic led to database eligible profiles, but noticeably less from paper (Figure 10). Almost no profiles were obtained from metal (Figure 10). The best profiles were obtained from glass as substrate, followed by plastic and paper, and no profiles were obtained from metal (Figure 10).

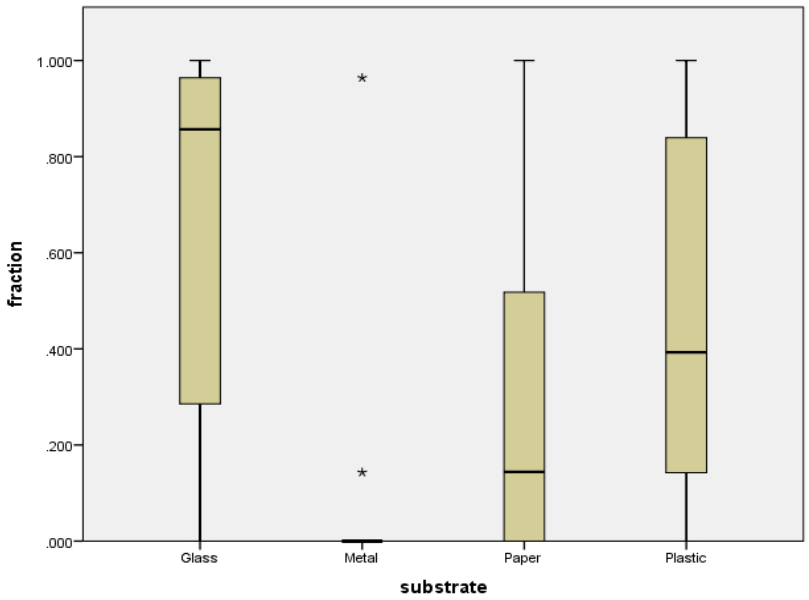


Figure 10. Fingerprints on various substrates: Y-axis: completeness of the STR profiles as fraction; X-axis: substrates: glass (n=32), metal (n=32), paper (n=32) and plastic (n=27). Median values of fraction STR profile for glass: 0.86; metal: 0.00; paper: 0.14; and plastic: 0.39.

Metal ions may cause DNA degradation by catalyzing the generation of hydroxyl radicals via the Fenton reaction, which can extensively damage DNA (Henle and Linn, 1997). In particular, copper and nickel, metals a US Quarter dollar are made of, bind and damage DNA resulting in no DNA profiles (Sagripanti et al., 1991; Spinello et al., 2013). Therefore, when prioritizing touched evidence for examination, glass would be the preferred substrate since it showed the highest fraction profiles and database eligible DNA profiles compared to the other types of substrates tested.

6.4.5 Using tape to collect prints and skin cells

Adhesive tapes are often found as evidence when used to commit a crime, such as to gag or immobilize victims (Zamir et al., 2000; Zech et al., 2012). Tapes can also be used to transfer prints from larger objects.

The study was performed to determine if tape lifting compromises DNA recovery from a fingerprint. Fingerprints were deposited directly on the tape and on glass microscope slides from which they were lifted on the following day. The fingerprint lifting tape

from SIRCHIE was selected for this study. Prior to that, the tape was tested in AmpF*l*STR® Identifiler® PCR Amplification and showed no PCR inhibition. Prints were swabbed under the stereomicroscope (Axiozoom .V16) using cotton threads moistened with 10 µl of 1:2 Zygem dilution in water held by manual tweezers (Dumont N5), followed by DNA extraction by Zygem and STR amplification by ID31. Of 86 fingerprint samples tested, 8 mixtures were detected. Mixtures were excluded from data analysis.

The comparison of the fraction profiles of fingerprints deposited directly on tape (n=37) and fingerprints deposited on glass slides that were lifted by tape (n=41) showed that both methods led to database eligible profiles (Butler, 2006). As shown in Figure 11, direct deposition on the tape resulted in more complete profiles; however, the difference was not significant (t-test: p=0.345).

In addition, areas of SIRCHIE tape can also be cut out to isolate single cells from the surrounding parts of the tape. However, this manipulation may be suitable when working with nucleated epithelial or sperm cells, but not for skin cells whose collection is preferable by swabbing due to extracellular DNA contributions (Gandarillas et al., 1999; Kita et al., 2008; Quinones and Daniel, 2012).

In conclusion, this study demonstrated that tape lifting the fingerprints may not compromise DNA recovery in comparison to amounts of DNA that are expected when the prints are swabbed directly of the tape.

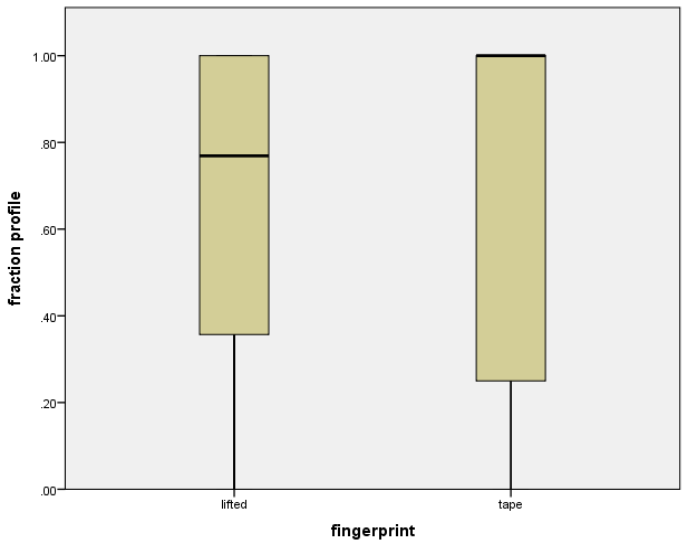


Figure 11. Using tape to collect fingerprints: Y-axis: completeness of the STR profiles as fraction, X-axis: fingerprints deposited on glass slide that were lifted by tape (n=41) and fingerprints deposited directly on tape (n=37).

6.4.6 Three- person mixtures

Routinely used methods for sampling touched evidence from a crime scene, by using a single swab to sample an entire surface, often generates mixtures if an item was touched by multiple persons. Deconvolutions may be possible if one person contributed most of DNA to the sample. This cell donor is also known as the major DNA donor. However, individual DNA profile(s) of other contributor(s) are more difficult to determine and results may not be informative. Mixtures could also happen due to a secondary transfer, where volunteers deposited not only their DNA but also DNA by touching other people or objects already held/touched by other individual(s) (Farmen, 2008; Fonnelop et al., 2015; Goray et al., 2010a; Goray et al., 2010b; Lowe et al., 2002; Zoppis et al., 2014). Mixtures may also arise due to possible contamination. However, negative extraction and amplification controls were tested with each test batch and none showed positive results. If any of the negative controls tested negative, but the associated samples appeared to be a mixture, they were excluded from data analysis.

It is most likely that interpretation of samples amplified from touched items can be improved by sectioning the evidence and handling them separately. The mathematical model of Ballantyne's et al. has shown that random isolation of multiple groups of cells

('binomial sampling') from the admixture of 1:1 donors could create separate cell sub-populations with differing constituent weight ratios that may lead to genotype identification of their donors (Ballantyne et al., 2013). This approach may simplify complex mixtures to two contributors where a major DNA donor could be easily determined, or may even lead to single source profiles from the same evidence. Finally, it could also lead to identifying more than one or ideally all contributors of a mixture.

For the three persons fingerprint mixture study, an empty beer (amber glass) bottle size 22 cm x 5 cm was used (Figure 12). The label was removed and the bottle was cleaned using 10% bleach, water, and 70% EtOH. The body of the bottle was etched into six equivalent sections. The size of the etched parts was approximately 6 cm x 6.3 cm, which is larger than a fingerprint. This sizing was chosen in order to increase the probability of obtaining enough cellular material to generate database STR profiles (≥70% complete). It was assumed that parts of the bottle may not been touched. Also, using larger area accounted for the fact that DNA from the palm of the hand sheds significantly less than from the fingers (Oleiwi et al., 2015).

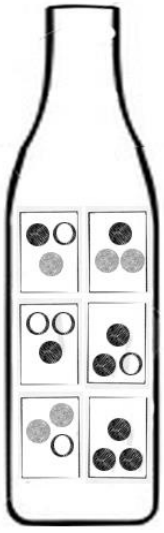


Figure 12. Three-person mixture: The trunk of a bottle was sectioned into six equal segments. The bottle was touched by three individuals. The sectioning sampling method is outlined. 216 samples of 36 bottles were analyzed.

The bottle was touched consecutively by three volunteers, each holding the bottle for 60 s. The order of volunteers touching the bottle was recorded and changed throughout the study. The bottle was examined under an Olympus SZX-16® stereomicroscope to observe the presence of the cells. The density of the cells varied between sections, also patterns of the fingerprints and palms were overlapping. Each section was swabbed entirely, generating six samples per bottle (Figure 12). The samples were processed separately using the Zygem extraction procedure followed by ID31. The results were analyzed following the High Sensitivity interpretation protocol for single source and mixed samples (Caragine et al., 2009). Briefly, the composite profile contained alleles that occurred in at least two out of the three amplifications and was used to classify a sample as a single source or a mixture of two or three donors. Samples with three or more repeating alleles in two loci were interpreted as mixtures. If at least 2 loci of the composite profile contained 3-4 alleles, the sample was considered as a two person mixture. If 5-6 repeating alleles are present in at least two loci, the sample was considered a three person mixture. Thirty-six bottles were tested. Six samples were generated per bottle and the six composite profiles together were used to determine the number of contributors in each sample. A total of 216 samples were generated in the mixture study.

The analysis showed that most of the samples of each bottle were at least two-person mixtures. Analysis of 6 of the composite profiles from each bottle revealed the presence of three persons for the majority of bottles tested. The third person on a bottle was revealed by listing the alleles at each locus for all 6 composite profiles, and determining the total number of different alleles at each locus. 5 or 6 alleles on at least two loci for the majority of bottles (26/36 bottles) were identified, therefore detecting three persons touching the bottles (Table 2). In addition, for eight of the bottles, a two person mixture was detected plus an indication of a third person (Table 2). These bottles didn't meet the NYC OCME guidelines to classify them as a three person mixtures. There were 5 or 6 alleles at only one locus which indicates the presence of third person. It was concluded that simultaneous analysis of all 6 samples per bottle lead to a good estimate of how many people touched the bottle.

Table 2. Number of contributors and different STR profiles per bottle.

# of contributors identified per bottle	# of bottles	STR profiles		# of bottles
3	26	3 different profiles	≥50%	0
2 with a hint of a third	8	2 different profiles	≥70%	5
			≥50-69%	3
2	2	1 profile	≥70%	19
			≥50-69%	6
		0 profile	-	3

As noted before, the percentage of profiles detected varied a great deal. In this study, only profiles that were ≥ 50% complete were considered: ≥ 50-69% for potential database comparison, and ≥ 70% for uploading to databases. As shown in Table 2, none of the bottles tested revealed DNA profiles of all three contributors that were considered usable, either for database comparison (50-69% complete) or for uploading to database (≥ 70% complete). Five out of 36 bottles (14%) showed two different profiles of ≥ 70% completeness, and an additional three bottles showed two different profiles, of which one profile was ≥ 50-69% complete, while the other was ≥ 70%. A single profile, ≥ 70% complete, was found on 19/36 bottles (53%) and an additional six bottles showed a single profile suitable for comparison (≥ 50-69% complete). Taken together, over 90% of the 36 bottles tested resulted in forensically usable STR profiles.

Table 3 represents a detailed outcome of the three-person mixture study, describing the outcome from each sample for all 36 bottles tested. Possible outcomes included: inconclusive, 2 or 3 person mixtures and single source. From all the samples (n=216) generated in this study, most were two-person mixtures (159). Of these, some could be used for deconvolution if the major contributor could clearly be determined. All single-source profiles (24) could be used and from the three-person mixtures (20), only a few led to informative profiles (Table 3).

Multiple STR profiles were obtained from the majority of bottles (Table 3). As found on many bottles, profiles of the same person were obtained in several of the samples, leading to increased confidence in these outcomes. This is specifically valuable if profiles were below 70% (bottle 2 and 16 in Table 3). Altogether 74 profiles (≥ 50%

complete) were obtained from 36 bottles, which equals an average of 2 profiles per bottle.

Furthermore, it was found that the last person touching the bottle was not always the major contributor. Considering the order of touching, it was found that the DNA of the last person who touched the bottle was detected in three series: A-B-C (bottles 1-6), B-C-A (bottles 19-24), and B-A-C (bottles 31-36), see Table 3. However, donor C contributed to most profiles in five of the six series, even when being the first person to have touched the bottle: A-B-C, B-A-C, C-B-A, and A-C-B. For the remaining series, it was person A, who contributed to most profiles, the last person who have touched the bottle: B-C-A. Furthermore, by counting the profiles per person (A: n=22, B: n=9, and C: n=43), it seems that person C shed most cellular material leading to most STR profiles, followed by person A and B. Therefore, it was concluded that the order of touching was not relevant in predicting whose DNA would be obtained, but more likely depended on prevalence of shedding and therefore amounts of cells and extracellular DNA left on an object upon touching it.

This sampling approach, which generates several samples per item, has led to the identification of individual contributors in mixed touched samples by providing database eligible profiles and/or profiles that could be used for direct comparisons.

Table 3. Detailed outcome of three-person mixture study.

Series of persons touching	Bottle #	Outcome				
		Inconclusive	2 persons mixture	3 persons mixture	Single source	Persons detected (% profile)*
A-B-C	1	2	4			C (67)
	2		4	2		A (61,50), **B** (93)
	3		6			**A** (82), **C** (70,70,80,80)
	4		6			**B** (80)
	5		4		2	**C** (100,90)
	6		4		2	**C** (100,93,87)
B-A-C	7		5		1	**C** (93)
	8		4	1	1	**A** (100,71,68)
	9	2	2		2	**C** (97,93,63)
	10		5		1	**A** (100,100,50), **B** (89)
	11		3		3	**C** (100,100,100,97)
	12		3	3		**A** (86), B (60)
C-A-B	13	1	4		1	**A** (100), C (57)
	14		5		1	**B** (100,53)
	15	1	4	1		
	16	2	4			C (57,50)
	17		3	1	2	**A** (100,79,79,64)
	18		5	1		**C** (87)
B-C-A	19		5	1		
	20		6			**A** (100,68), **B** (100)
	21		4		2	**A** (71), **B** (100)
	22		6			**A** (86,50)
	23		4	1	1	**C** (77)
	24		5	1		**C** (87)
A-C-B	25	1	5			**C** (87,83,73)
	26		6			C (60)
	27	1	4	1		C (63)
	28	1	5			**C** (83)
	29		5	1		A (57)
	30		6			**C** (80,73,60)
B-A-C	31		2	2	2	**C** (100,100,100)
	32	1	5			
	33		5		1	**A** (100)
	34	1	3	2		**C** (79)
	35		2	3	1	C (63)
	36		5		1	**B** (100), **C** (83,57)
Total		13	159	20	24	

* Letters in bold indicate at least one profile ≥70%

6.5 Comparison of micromanipulation devices: P.A.L.M. ®, Axiozoom .V16, and aureka® by using fingerprint samples, buccal, and sperm cells

Micromanipulation techniques can be used to separate individual cell from a cell mixture in order to generate DNA profiles of cell donors. In this study P.A.L.M.®, Axiozoom .V16 and aureka® were evaluated for their efficiency, sensitivity, and reproducibility in collecting single cells and fingerprints. This evaluation demonstrated the suitability of the different cellular materials per technique.

6.5.1 Protocols for DNA extraction and STR amplification of fingerprints, epithelial, and sperm cells

Fingerprint samples are considered the most challenging forensic samples (Balogh et al., 2003a; Ostojic et al., 2014; Wickenheiser, 2002). These samples were swabbed in their entirety using a moistened cotton swab or rubber cement sphere for partial fingerprints, followed by Zygem DNA extraction and ID31 amplification (Ostojic et al., 2014). Using the same protocol, DNA from buccal cells was extracted and analyzed. Conversely, this protocol was not able to release DNA from sperm cells, due to abundant disulfide bonds between integral membrane proteins in the outer membrane. Therefore, in-house NYC OCME protocol was used. This protocol employs DTT to reduce these bonds between thiol groups of membrane proteins (Horsman et al., 2005) and thus release cellular DNA. Furthermore, DNA extracted from sperm cells was not purified nor concentrated. Samples were amplified in ID31 in triplicate tests, and the data was analyzed following the High Sensitivity interpretation protocols for single source samples (Caragine et al., 2009).

6.5.2. Micromanipulation devices: P.A.L.M.®, Axiozoom .V16, and aureka® for collection of single cells and fingerprints

Micromanipulation includes the use of a microscope and physical or laser manipulations, often done in conjunction with cell staining to select the cells of interest from the background. Micromanipulations enable isolation of single cells and therefore separation of mixed cellular material prior to DNA extraction and STR amplification. Three instruments were tested for their efficiency to physically isolate single cells from other cells and debris and for profiling success.

The instruments that were tested included: (1) the P.A.L.M.® (Positioning and Ablation with Laser Microbeams), an inverted microscope, which utilizes laser energy to ablate the area around the cell and subsequently catapult it with dispersed UV laser light into the cap of 0.2 ml PCR flat-cap tube filled with 20 µl of extraction buffer, (2) the Axiozoom .V16, a high-resolution stereomicroscope, and (3) the aureka®, a robotic arm that can be used with the Axiozoom .V16 for micromanipulations. These instruments were tested on fingerprints, epithelial (buccal), and sperm cells for their efficiency to collect single cells.

When using P.A.L.M.®, cells were smeared onto a PEN membrane coated slide and visualized under the microscope. Computer software "P.A.L.M. Robo Software" was used to mark and memorize position of the cells on a PEN membrane slide, to adjust laser settings and control laser functions. Using a computer tool "Auto RoboLPC" the position of cells was marked and subsequently memorized into the software. Prior to cutting and catapulting cells of the membrane slide, P.A.L.M.® system was calibrated to adjust the laser setting. Calibration was done on areas of the same slide that have no cells in order to adjust laser energy and focus for the membrane. Using a tool "Auto RoboLPC", 10 circular elements of various sizes were selected, and then catapulted to estimate which settings enable successful catapulting. Calibration of the instrument is considered completed and successful if at least 8 elements, out of 10 selected, were completely cut and catapulted. Then, those successful settings functions were used to isolate cells from the smear. If not, the laser setting was readjusted by repeating the calibration. Changing the objective also requires resetting of the laser setting. Sperm cells were observed with 40 X objective, while buccal with 20 X.

Default settings for energy and focus were: cut: 35, LPC: 30; and cut: 46 and LPC: 2, respectively. Subsequently, by bringing the UV laser into focus, a narrow beam was generated that would cut the marked area on the membrane of a slide carrying the cell. Followed by a force of a quick pulse of diffused UV light, a marked cell was catapulted (against gravitational force) into a lid of a reaction tube. This was filled with extraction buffer and inversely positioned on the arm of the P.A.L.M.® microscope. A magnification up to 400 fold was used to collect single cells.

The Axiozoom .V16 is a high-resolution stereomicroscope (magnification up to 112 X), to which the aureka®, a motorized micromanipulator, could be connected. The

Axiozoom .V16 is equipped with a UV-light unit that contains several filters allowing adjustment for different surface materials and morphological assessment of nuclei. This microscope can also memorize positions of the stage, light and magnification until deleted (Bruck et al., 2011). The robotic arm aureka® was attached to the Axiozoom .V16 microscope. It can be moved in four directions: x-axis (left-right), y-axis (forward-backward), z-axis (up-down), and m-axis (diagonally). The attached tweezers can be opened and closed. The aureka® also memorizes positions for tweezers and the tungsten needle until it is turned off, and then a resetting is needed (Schneider et al., 2012). However, during its operation, it can lose its precision of the x, y, and z directions and should be turned off in order to reset the instrument. The Axiozoom stage was designed in-house at the NYC OCME laboratory to enable easier workflow at cell collection stage. It includes a platform that holds two microscope slides, one was used to make the spheres from Arabic gum/glycerol or rubber cement and the other contains the cells or cell mixtures (Figure 13). This platform can be rotated. The stage also contains a rack for 0.2 ml reaction tubes allowing the direct transfer of the cells (Figure 13).

Figure 13. Modification of the Axiozoom .V16 stage for easier manipulation from the slide.

In order to compare the P.A.L.M. ®, Axiozoom .V16 handled manually or with the robotic arm, and the aureka®, STR profiles were generated from 10 buccal cells (n=25 repeats). Figure 14 shows that the fraction profile using the P.A.L.M.® was low, while the Axiozoom v.16 operated manually or with the aureka® had values between 0.7 and

0.8, which were considered database eligible. These differences were significant (t-test, Table 4).

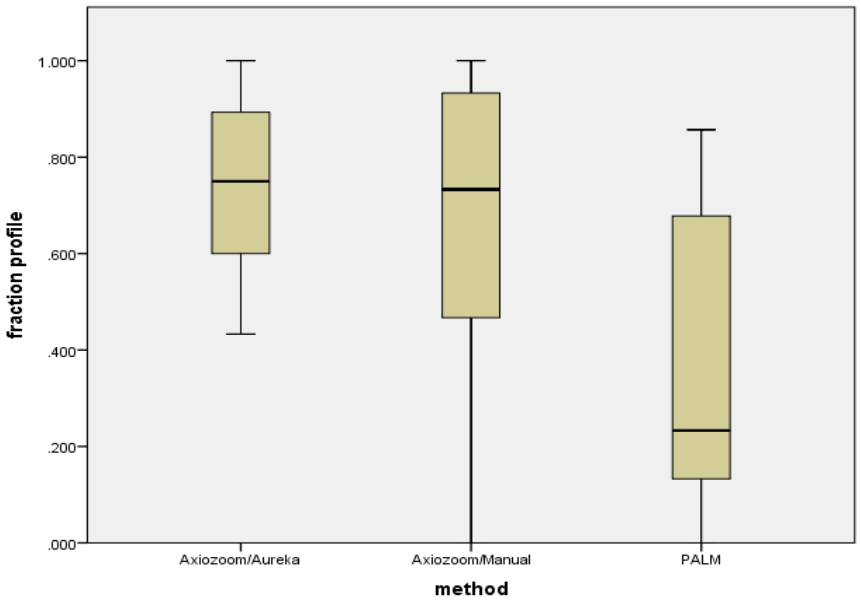


Figure 14. Comparison of P.A.L.M.®, Axiozoom handled manually and with aureka®: X-axis: instrument procedures: Axiozoom with aureka® and handled manually and with P.A.L.M.®), and Y-axis: fraction of STR profiles obtained from 10 buccal cells.

Table 4. Statistical comparisons between outcomes when using different instruments when isolating 10 buccal cells.

	p-value from t-test
P.A.L.M.® vs Axiozoom/aureka®	<0.0001
P.A.L.M.® vs Axiozoom/Manual	0.0004
Axiozoom/aureka® vs Manual	0.41

This outcome was supported with results from testing sperm cells. Ten and twenty sperm cells were selected, DNA extracted and STR profiles generated using ID31 (at least 9 times repeated, as shown in Figure 15). Ten sperm cells led to better fraction profiles than ten epithelial (buccal) cells. Most fraction profiles were higher than 70%.

Sperm cells are smaller than epithelial cells but they are compact and the DNA is protected by a rigid cell membrane that could make the collection easier. Twenty sperm cells led to fraction profiles higher than 70%. Again, the results using the P.A.L.M.® were not as good as using the Axiozoom .V16, operated manually or with the aureka® (Figure 15). These differences were significant (t-test Table 5).

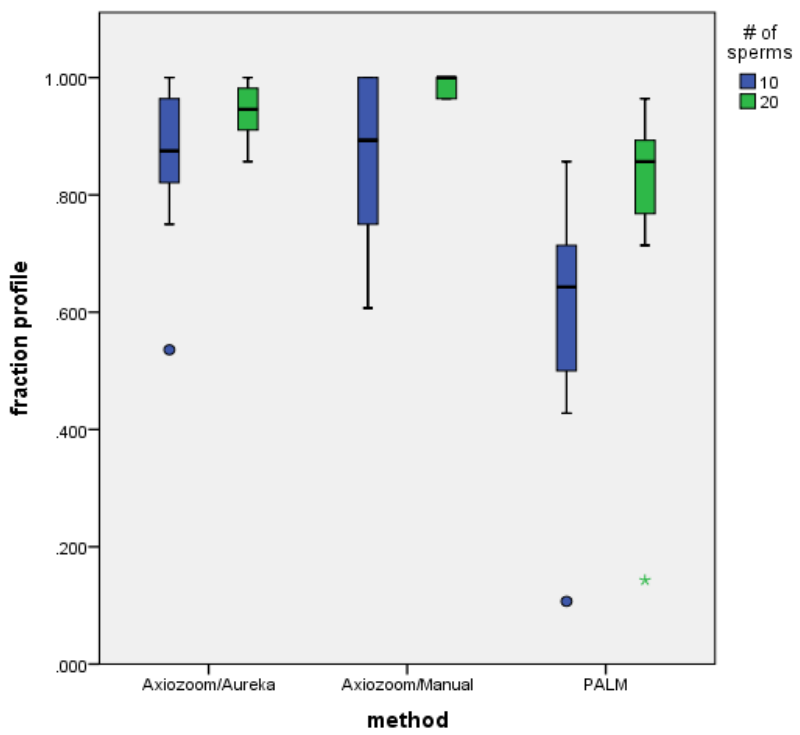


Figure 15. Comparison of P.A.L.M.®, Axiozoom .V16 handled manually and with aureka®: X-axis: instrument procedures: Axiozoom .V16 with aureka® and handled manually and with P.A.L.M.®), and Y-axis: fraction of STR profiles obtained from 10 (blue) and 20 sperm cells.

Table 5. Statistical comparisons between outcomes when using different instruments when isolating 10 and 20 sperm cells.

	p-value from t-test (10 sperm cells)	p-value from t-test (20 sperm cells)
P.A.L.M.® vs Axiozoom/aureka®	0.004	0.02
P.A.L.M.® vs Axiozoom/Manual	0.003	0.01
Axiozoom/aureka® vs Manual	0.82	0.01

	p-value from t-test (10 to 20 sperm cells)
P.A.L.M.®	0.052
Axiozoom/aureka®	0.0004
Axiozoom/Manual	0.015

Conclusions from these comparisons (Table 6) are that the P.A.L.M.® can be used for single cell picking (laser cutting and transfer into reaction tube) of epithelial (buccal) cells and sperm cells. The Axiozoom .V16 operated manually or with aureka®, can be used to swab fingerprints, either directly or off the tape, to cut tapes that contain prints or cells, and for single cell epithelial (buccal) and sperm cells lifting by using AG spheres. Manual swabbing of larger areas is preferred than using aureka®, since it is faster.

Table 6. Conclusions from comparisons of P.A.L.M.®, Axiozoom .V16 handled manually and with aureka® and their suitability for cell type collection.

Instrument	Cell type	Collection	
P.A.L.M. ®	Epithelial cells (buccal) Sperm cells	Picking (laser cutting)	Single cells
Axiozoom/Manual	Fingerprints (direct/tape) Epithelial cells (buccal) Sperm cells	Swabbing Cutting tape Picking	Larger/small areas single cells
Axiozoom/aureka®	Fingerprints (direct/tape) Epithelial cells (buccal) Sperm cells	Swabbing Cutting tape Picking	(Large) small area single cells

7. Discussion

Investigators often encounter evidence samples containing a trace amount of biological material, which may get gradually degraded during the course of handling, transportation and examination for forensic DNA analysis. The ability to obtain DNA profiles from trace, degraded, or mixed biological samples, such as in sexual assault cases or property crimes where objects are handled by multiple individuals, are major areas of challenge in the field of forensic DNA typing. Generating high-quality DNA profiles from the aforementioned types of samples that can be used in CODIS would have a tremendous potential in the investigation of a wide variety of criminal cases.

The NYC OCME laboratory has pioneered the High Sensitivity or LCN technique in the United States providing accurate and reliable forensic DNA testing results from evidence with trace amounts of DNA (Caragine et al., 2009). However, these samples show great variation in amounts of DNA and quality of STR profiles (van Oorschot et al., 2010), and numerous samples are often inconclusive due to DNA amounts below threshold for current identification. In addition, in routine workflow, touched samples mostly originate from evidence that is touched by multiple individuals, whose identify is frequently not able to be determined.

In order to process touched items more effectively, in this study various DNA extraction and STR amplification methods were compared to elucidate which method can maximize evidentiary value of touched objects and thus help with criminal investigations. These comparisons were done on fingerprint samples since they were considered to be the most difficult forensic samples and can be found on a wide range of crime scene items recovered from various criminal offenses. Fingerprints can yield little to no DNA or they can result in DNA profiles suitable for upload to forensic STR databases (Prinz et al., 2006). Challenges inherent to fingerprints were identified since the first DNA profiling was done on touched samples (van Oorschot and Jones, 1997).

A procedure, which can increase the probative forensic biology value of fingerprints, was investigated by testing: (i) fingerprint collection techniques, (ii) DNA extraction procedures, and (iii) STR amplification strategies, on over 700 fingerprints. Two swabbing solutions (water and Triton X-100), three extraction methods (one-tube, HighSens, and Zygem), and two STR amplification systems (AmpF*l*STR® Identifiler®

using either 28 or 31 cycles, and AmpF*l*STR® Identifiler® Plus using 32 cycles) were compared.

Comparisons of extraction methods and the impact on swabbing solutions were evaluated based on the amount of DNA that was yielded from the sample upon extraction. DNA was quantified using a real-time PCR method (Nicklas and Buel, 2003).

With respect to DNA concentrations obtained, HighSens and Zygem were significantly better than the one-tube extraction (Figure 3). Single measurements for DNA quantitation led to higher results than triplicate measurements, but the triplicate approach eliminated some outliers. Although the single versus triplicate approach to quantify DNA in the sample cannot improve DNA concentration of a sample, the median value of three measurements appears to be a better estimate of the true concentration than a single measurement (Ostojic et al., 2014). However, further experiments refrained from triplicate measurements due to a substantial portion of the DNA extract that was required.

Amounts of DNA recovered from touched samples could also be influenced by the swabbing solutions used to collect fingerprints. Thus, the impact of water and detergent were investigated. SDS was shown to improve DNA yields from touched items (Thomasma and Foran, 2012) and was routinely used by the NYC OCME laboratory to collect DNA from touched objects. Nevertheless, SDS was dismissed from this study due to its inhibitory effects on PCR amplification (Schrader et al., 2012), which required DNA purification upon extraction. Zygem and one-tube extraction methods were compared in this study and did not include a purification step to remove inhibitors. Thus Triton X-100 was used instead, which should not influence PCR (Radstrom et al., 2004). Triton X-100 more effectively collected all fingerprint material in comparison to water since sweat and sebaceous material may be present in the fingerprint impression besides cell flakes (Cooper-Dunn et al., 2017). However, Triton X-100 didn't lead to higher DNA concentrations and the qualities of STR profiles were not improved (Figure 3), even though substantial amounts of DNA in touched samples could be extracellular (Stanciu et al., 2015). Nevertheless, Triton X-100 was used as a swabbing solution due to faster and easier collection of cellular material and oil from the fingerprint than water.

Furthermore, the DNA extraction and STR amplification procedures were compared together to find out which protocol resulted in more complete STR profiles (Figure 4). Samples with concentrations less than 2pg/µl were excluded from these comparisons due to a lack of correlation between the amounts of DNA and STR profile completeness, where only 21 out of 274 samples resulted in profiles that were at least 70% complete. Greater than 70% complete profiles were considered usable for databases in the U.S such as CODIS (Butler, 2006). Therefore, samples with DNA concentrations ≥2pg/µl were compared for donor's profile obtained (i.e., possibly CODIS eligible). Negative controls were tested along with samples to detect any contamination of consumables and reagents. If controls were negative, and some samples appeared as mixtures (containing DNA from more than one individual) their occurrence was attributed to secondary DNA transfer. Secondary DNA transfer happens by transferring DNA fragments from other persons or objects touched prior to providing fingerprints for DNA testing (Breathnach et al., 2016; Cale et al., 2016; Fonnelop et al., 2015; Goray et al., 2010a; Goray et al., 2010b; Goray and van Oorschot, 2015; Lowe et al., 2002). It was well documented that previous activities of an individual (van Oorschot et al., 2014) and secretion of sebum may have an impact on picking and transferring foreign DNA onto touched objects (Zoppis et al., 2014), so the appearance of mixtures was not surprising. Mixtures were excluded from these comparisons. There was only ~6% mixture rate which was low compared to other studies where secondary transfer ranged from 10% to 85% (Barash et al., 2010; Cale et al., 2016; Daly et al., 2012; Djuric et al., 2008; Grisedale and van Daal, 2012; Helmus et al., 2016; Parsons et al., 2016; Pesaresi, 2003; Schneider et al., 2011; Sewell et al., 2008; van Oorschot et al., 2014). This could be explained by single fingerprint analysis, which was done in this study. Single prints are less likely to transfer detectable amounts of foreign DNA in comparison to an entire hand or examining larger areas on evidence.

The one-tube DNA extraction followed by ID31 amplification was considered a baseline for comparisons. It was found that the Zygem extraction significantly improved the outcome (Figure 4). The HighSens extraction decreased the percent of profile obtained, although 90% of the HighSens samples produced DNA concentrations ≥2pg/µl. This could be because the HighSens procedure included multiple steps of tube exchange, purification and concentration (Schiffner et al., 2005), which may shear DNA

molecules, and thus leave fewer DNA molecules suitable to generate STR profiles of at least 70% completeness.

The ID+ kit was designed to improve the amplification performance by providing tolerance to PCR inhibitors (Wang et al., 2012). The ID+ amplification following the one-tube extraction led to significantly better outcomes than ID31. However, this was not confirmed for HighSens and Zygem extractions. It could be suspected that the lack of impact of the ID+ amplification could be due to the cleaning of samples from inhibitors in the microcon stage during the High Sensitivity extraction, or by using the Zygem extraction which was already specifically optimized to overcome PCR inhibition.

The effect of the multiple amplification approach was also investigated. Triple amplification with the construction of a consensus profile (Cowen et al., 2011; Gill et al., 2000; Grisedale and van Daal, 2012) showed an increase (1 and 8%) in percent profile obtained over single amplification, which was not significant. Nevertheless, triplicate amplification is recommended for low template samples because of the increased occurrence of drop-in alleles, thus resulting in more reliable profiles (Benschop et al., 2011; Caragine et al., 2009; Cowen et al., 2011; Gill et al., 2000; Petricevic et al., 2010; Prinz et al., 2006). Conversely, if samples contain larger amounts of DNA, usually above 20 pg/µl, this DNA could hinder foreign DNA fragments and thus identification of drop-in. Therefore, these samples could be amplified only one time (Butler, 2004, 2007).

As a result, Zygem followed by ID31 was more effective than other procedures. Additional benefits of implementing this procedure in routine forensic work to process fingerprints and trace biological material is the ability to automate the procedure due to short and simple laboratory set up. Approximately 70% of the fingerprints processed with this protocol produced at least 70% complete DNA profiles. This was comparable to another study where 71% of the processed fingerprints led to database eligible profiles (Templeton and Linacre, 2014). The outcome of the comparisons was in compliance with the findings of Templeton et al. who indicated that fewer steps in the DNA testing process can significantly improve profiling results from touched DNA samples, by circumventing routinely used multiple step/tube extraction protocols which

can lead to subsequent DNA loss by irreversible binding of DNA on plastic (Templeton and Linacre, 2014).

The tendency to leave behind genetic material through contact was shown to genetically differ among individuals (Farmen, 2008; Lowe et al., 2002) and depends on the specificity of each individual's skin. DNA traces have been assumed to originate from the keratinocytes shed off the upper epidermal layers (Gandarillas et al., 1999), and depending on the degree of skin's propensity to leave DNA traces, individuals could be classified as good and bad shedders (Farmen, 2008; Lowe et al., 2002). There are a number of factors that may affect STR profiling results which included: individual, the area of contact, the moisture level of the hands, etc. (van Oorschot and Jones, 1997; Wickenheiser, 2002). A complete DNA profile could be obtained from a good shedder (Farmen, 2008) even if an individual touching an object has recently washed their hands (Lowe et al., 2002). Conversely, Phipps and Petricevic demonstrated that none of their volunteers produced a full DNA profile after touching an object with their thoroughly washed hands (Phipps and Petricevic, 2007). Thereby, in this study fingerprints were collected from volunteers who refrained from washing their hands for at least 2 hours. This could mimic real forensic scenarios and offer some time for cells and oil to shed from hands. Also, a 2-hour period after washing hands was previously reported as sufficient time to provide enough cellular deposits in the fingerprint to generate full DNA profiles (Templeton and Linacre, 2014). In this study correlation between shedding level, DNA amounts and quality of STR profiles was investigated. Fingerprints were visually examined for their count of cell flakes, assigned a shedding score, and evaluated for the quality of STR profiles obtained. Linear regression found correlation between shedding score and quality of profiles, although for each observed volunteer large variability in quality of STR profiles existed (Figure 6). Good quality profiles were obtained from samples that had high but also low shedding scores and poor profiles were obtained from samples that had low but also high shedding scores. Therefore, it was concluded that a shedding score alone cannot be used as a reliable predictor for profile quality. This could be because many deposited cells of a fingerprint may not be nucleated (Gandarillas et al., 1999), or may not carry extracellular DNA (Quinones and Daniel, 2012). This study is consistent with another finding that the number of cells in touch samples was uncorrelated to the total DNA yield (Stanciu et

al., 2015).

In routine workflow, numerous items could be received for examination and due to limited resources and capacity of the laboratory, many items can sit for days, weeks or even months prior to their forensic examination. The impact of time on STR profile qualities was investigated. It was visually recognized that fingerprints age and degrade over time (De Alcaraz-Fossoul et al., 2016). The aging of fingerprints was confirmed in this study by evaluating STR profile quality obtained from fingerprint samples for several time points. Although a portion of database eligible profiles decreased over time, successful STR profiles can be generated 24 h after deposition. This aligned with previous findings (Djuric et al., 2008). A slight decrease was noticed after three days, but was not significant. Time periods exceeding 10 days were significantly decreased, however successful STR profiles were obtained 40 days after deposition (Ostojic and Wurmbach, 2017), which was in concordance with an earlier study (Raymond et al., 2009). Also, influence of the laboratory environment was tested by storing fingerprints in open boxes where they are exposed to air and dust. There was no difference found. This could be due to relatively pristine laboratory conditions, such as filtered air. The boxes were also stored without direct sun exposure, or any other environmental conditions that usually could affect real forensic samples (Bruskov et al., 2002; Lindahl and Andersson, 1972).

Fingerprints from the left and right hand of each tested individual were separately collected and processed in this study. In order to learn more about fingerprints, left and right hands were analyzed separately by comparing profile qualities obtained from their deposited fingerprints. Three right-handed individuals were tested. The left, non-dominant, hands resulted in slightly more complete profiles, which was not significant. This could be possibly due to abrasion that happens to the more frequently used dominant hand, while the non-dominant hand may still carry material suitable for DNA testing. Conversely, the dominant hand is also more frequently used to touch the face or the nose when sneezing, thereby transferring DNA (Wickenheiser, 2002). Therefore, no conclusion was drawn.

Understanding whether the evidentiary material of evidence affects the quality of DNA is important when prioritizing evidence for examination. Examining item with a higher likelihood to obtain database eligible profile could speed up the process of forensic

investigations. Thus, most commonly used material (glass, paper, plastic and metal- US Quarter dollar) in households and offices were tested. Glass led to the best STR profile quality, followed by plastic and paper. Almost no profiles were obtained from metal. This could be due to the possibility that Cu- and Ni- ions provoke DNA degradation (Henle and Linn, 1997) and the metals bind and damage DNA (Sagripanti et al., 1991). Difficulty of working with various metals and alloys was also documented by others. Cu- and Zn- ions present in brass (often found in firearms) have inhibitory effects and affect PCR yields (Templeton and Linacre, 2014). Similar to the findings here, previous studies reported best DNA recovery from glass, when compared to metal and wood (Pesaresi, 2003). It was also shown that DNA could be recovered from paper (Balogh et al., 2003b; Parsons et al., 2016; Sewell et al., 2008; Soltyszewski et al., 2015). The substrates tested had rather smooth surfaces. However, the impact of substrate surfaces (textured and jagged) on DNA recovery was noticed by others, indicating that wood and fabric could lead to higher DNA yields than from glass (Daly et al., 2012). These findings signify that more research is needed in order to obtain more insight and consistent results.

Touched items are mostly handled by multiple individuals and thus carry DNA from more than one individual, generating biological mixtures. Most challenging in forensic biology is obtaining forensically useful STR profiles from biological mixtures, especially when containing small or trace amounts of cellular material (Gill et al., 2015; Haned et al., 2012). Sometimes it is possible to deconvolute only a major DNA donor, whose DNA is prevailing in the sample, but if similar amounts of donors' DNA are present, the resulting STR profiles cannot be deconvoluted to their individual contributors (Schneider et al., 2011). It was recognized that the sub-division of evidence into zones of potential contact and sub-sampling them accordingly may maximize their evidentiary value (Ballantyne et al., 2013; Barash et al., 2010; Wickenheiser, 2002). Also, targeted sampling of areas on evidence that was most likely touched, rather than sampling the entire evidence may lead to better DNA profiling results (Parsons et al., 2016). This approach was utilized in this study by separately sampling six equally sized areas on a body of a beer bottle that was previously touched by three volunteers consecutively. The size of the sampled areas were larger than a single fingerprint, since some parts of the section could remain untouched, or carry DNA deposited from the

palm which were shown to shed significantly less DNA than from two fingers (Oleiwi et al., 2015). However, the bigger the section was, the higher the chance of having a mixture that cannot be resolved. Conversely, with smaller sections, the chances of encountering too little material for usable results are higher.

In this study 36 bottles were tested generating over 200 samples. Over 84% of samples were two-person mixtures and single source. However, the number of different alleles from 6 composite profiles from 6 sampled areas on the same bottle revealed that a bottle was touched by three individuals (Ostojic and Wurmbach, 2017). Thereby, it was confirmed that the sectioned sampling approach decreased the complexity of mixtures by reducing the number of contributors for most of the samples. It also provided accurate estimates of actual number of contributors. Furthermore, despite the finding that the transfer of cells happens upon initial contact and does not depend on length of time holding the object (van Oorschot and Jones, 1997), the last person touching the object wasn't always leaving the most cells and the most DNA, as was expected (Lowe et al., 2002; van Oorschot and Jones, 1997; Wickenheiser, 2002).

90% of the bottles resulted in an informative STR profile, and 75% produced profiles that were ≥ 70% complete. Many bottles revealed several profiles that belonged to the same person, increasing the confidence in the results. Also, this approach led to the identification of two different STR profiles on the same bottle. 8 out of 36 bottles identified two different cell donors.

Another approach to minimize the appearance of the mixture could be achieved by direct visualization of cells under the microscope, lifting targeted cells and subsequently transferring them into a test tube. Here, various micromanipulation techniques were evaluated for their ability to pick individual sperm or epithelial (buccal) cells and thus separate cells from various donors prior to DNA extraction and STR amplification.

The instruments tested included: (i) the P.A.L.M.® (Positioning and Ablation with Laser Microbeams), (ii) manual manipulations performed under the Axiozoom .V16 microscope, (iii) and robotic manipulations with aureka®, which was integrated onto the Axiozoom .V16. These instruments were tested on fingerprints, epithelial (buccal), and sperm cells for their efficiency to aid in collecting single cells to successfully and robustly produce database eligible profiles that were at least 70% complete (Ostojic et al., 2014).

Laser microdissection using the P.A.L.M.® system can successfully isolate single cells smeared on a membrane slide. Therefore, isolation of cells directly from the evidence material using P.A.L.M.® was not possible (Di Martino et al., 2004; Elliott et al., 2003; Han et al., 2014; Murray et al., 2007; Sanders et al., 2006; Vandewoestyne and Deforce, 2010). Conversely, direct cell isolation was enabled with manual or robotic manipulations. Thus, commercially available microcarriers were tested for single cell collection (Bruck et al., 2011; Schneider et al., 2012). The microcarriers from SoloHill did not have enough chemical to lift cells, and Cytodex™3 showed PCR inhibition. Water-soluble glue was found suitable for transfer of cellular material from touched items (Hanson and Ballantyne, 2013). Consequently, various glues were evaluated and it was found that small spheres made from Arabic gum mixed with glycerol (AG spheres) were suitable for single cell collection and rubber cement for swabbing small areas. Both materials showed no PCR inhibition. These spheres were operated by hand or with the robotic arm, aureka®, using a tungsten needle or tweezers, respectively. AG spheres were synthetized in this study and were found as a promising method in forensic testing for single cell collection. The advantage of using AG spheres over synthetic glues, is because AG is organic, sugar based, simply prepared, and is dissolved immediately upon contacting buffer solution, enabling complete transfer of collected material into a test tube.

Means to isolate individual cells (Anoruo et al., 2007; Bruck et al., 2011; Elliott et al., 2003; Schneider et al., 2011) are preferably applied only on nucleated cells, such as spermatozoid or epithelial cells, but not on skin cells despite the study which demonstrated the ability to obtain full STR profiles from fifty skin flakes (Hanson and Ballantyne, 2013). Skin cells were collected by swabbing a fingerprint to account for nuclear and extracellular DNA contributions (Quinones and Daniel, 2012) and numbers of keratinized cells that may be non-nucleated (Kita et al., 2008).

When comparing P.A.L.M. ® and Axiozoom .V16 operated manually or with the robotic arm aureka® it was found that 10 buccal or sperm cells were sufficient to generate database eligible STR profiles. This number was lower than previously published (Anoruo et al., 2007; Axler-DiPerte, 2011; Meredith et al., 2012; Vandewoestyne and Deforce, 2010).

The fraction of profiles obtained from 10 buccal cells was significantly lower for the P.A.L.M.® than for the Axiozoom .V16 (aureka® or manually). Using the Axiozoom®.V16 for cell collection, most of the STR profiles were database eligible (>70%). A similar outcome was obtained for 10 sperm cells, but their fraction of profiles was higher for all instruments tested. This may be due to the sperm cell size and more compact cell membranes that make sperm cells easier to be collected and transferred into the test tube. This comparison led to finding that aureka® manipulations under Axiozoom.V16 produced the most consistent and reproducible results. This method is more labor intensive and time consuming than manual methods and requires training of laboratory personnel; however there is no impact of human error such as in manual manipulations. On the other hand, P.A.L.M.® is fast and easy to operate; however using this inverted type of microscope where laser ablated cells are catapulted off the slide against gravitation may not always allow cells to reach the extraction buffer and thus lead to inconsistent results.

Previously described sectioned sampling approaches used on a bottle, which reduced the complexity of samples leading to more easily resolved 2 people mixtures, could also be applied on other types of case evidence. For example, generating multiple samples of the same evidence, each containing 10 nucleated cells, could be a promising method to decipher DNA profiles of perpetrators where cells of the same kind from a great number of individuals are encountered, such as in a gang rape. These generated samples could contain different ratios of cell donors, and lead to identification of some if not all donors' DNA profiles.

Finally, as tapes are widely used as a sampling method in forensic techniques (Barash et al., 2010; Verdon et al., 2014; Zech et al., 2012), they were also tested for DNA recovery from fingerprints. Tapes can be very suitable for transfer of biological material if evidence is too large to put it under the microscope. The fraction of profiles from fingerprints deposited directly on tape were compared to fingerprints deposited on glass slides and lifted by tape. The results revealed no significant difference. Manual swabbing of the fingerprints in their entirety was preferred although single cell isolation from the tape was also possible by using micro-blades operated manually or robotically. Excising cells from a tape is suitable when biological stains are collected from fabric to reduce transfer of inhibitors into the test tube (Gunnarsson et al., 2010; Kopka et al.,

2011). Conversely, the P.A.L.M.® was not found compatible with tapes since the laser was not cutting through the tape. Among evaluated tapes, SIRCHIE tape, which is widely used in forensics, did not cause PCR inhibition and was successfully used for the collection of fingerprints for DNA profiling.

8. Conclusions

Touched items are often submitted as evidence from various crimes, which required further improvements to existing protocols to enhance their evidentiary value. This study demonstrated that it is difficult to obtain full STR profiles from single fingerprints reliably, but improvements were possible. Among the protocols compared, the most favorable was using Zygem for DNA extraction followed by ID31 amplification. This protocol was able to generate database eligible STR profiles from the majority of tested fingerprints samples. It could also be applied to other biological materials, apart from semen. Due to its simplicity, it may be easily automated into a routine workflow, which consequently may expedite criminal investigations.

Resources of forensic biology laboratories could be maximized if evidence is prioritized for a forensic examination, testing items that are more likely to yield database eligible STR profiles. Therefore, the impact of the most commonly used substrate types on DNA recovery was examined. It was shown that glass led to the best STR profiles qualities, followed by plastic and paper. No profiles were obtained from metal (a Quarter Dollar), assuming that metal ions may have an effect on DNA degradation (Henle and Linn, 1997). This study also demonstrated that time and environment have an impact on the qualities and quantities of resulting STR profiles from fingerprints. The effect of time was substantial, indicating that evidence should be processed for touched DNA as soon as possible in order to maximize the results. Nevertheless, if an item of evidence sits for days prior to examination it still may be possible to produce database eligible profiles even 40 days after fingerprint deposition. This could be important if the crime scene evidence is discovered long after the commission of the crime.

Furthermore, touched evidence frequently carries DNA from multiple individuals whose DNA profiles cannot be resolved by currently applied methodologies. To address this problem, this study examined a sectioned sampling approach on the body of a beer bottle touched by three individuals. This approach reduced the complexity of the sample, by generating two-person mixtures and single source DNA profiles from the majority of tested samples. This sampling of evidence could be particularly useful for

cases when similar amounts of donors' DNA are present, or great numbers of donors are encountered in the sample.

Further, to approach mixed samples, various instruments (P.A.L.M.®, manual or robotic manipulations with aureka® under Axiozoom .V16) were compared and revealed different abilities to lift single cells off an object. It was found that database eligible STR profiles can be obtained from only 10 sperm or 10 buccal cells. Using P.A.L.M.®, collection of cells was efficient, but unreliable due to inconsistency of the catapult process. Manual manipulations are fast but rely on individual skill, while use of robotic micromanipulations by aureka® can produce more consistent results by removing variables introduced by operators inaccuracy. By applying the sectioned sampling approach, and collecting several times 10 nucleated cells per sample, contributors in mixed samples could be revealed, due to less complex and easily deconvoluted mixtures. These cell manipulations could be applied on stains from multiple bleeders or semen samples of several semen donors. Ultimately, this may resolve cases which are major obstacles for currently applied methodologies of testing in forensic biology.

9. Literature

Abu Al-Soud, W., Radstrom, P., 2000, Effects of amplification facilitators on diagnostic PCR in the presence of blood, feces, and meat. J Clin Microbiol 38, 4463-4470.

Akane, A., Matsubara, K., Nakamura, H., Takahashi, S., Kimura, K., 1994, Identification of the heme compound copurified with deoxyribonucleic acid (DNA) from bloodstains, a major inhibitor of polymerase chain reaction (PCR) amplification. J Forensic Sci 39, 362-372.

Alaeddini, R., Walsh, S.J., Abbas, A., 2010, Forensic implications of genetic analyses from degraded DNA--a review. Forensic Sci Int Genet 4, 148-157.

Alessandrini, F., Cecati, M., Pesaresi, M., Turchi, C., Carle, F., Tagliabracci, A., 2003, 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, 586-592.

Anoruo, B., van Oorschot, R., Mitchell, J., Howells, D., 2007, Isolating cells from non-sperm cellular mixtures using the PALM microlaser micro dissection system. Forensic Sci Int 173, 93-96.

Axler-DiPerte, G.O., S. Singh, A. Caragine, T Prinz, M. Budimlija, Z. M. , 2011, Comparison and optimization of DNA recovery from sperm vs. epithelial cells using laser capture microdissection technology and an immunofluorescent staining system. Forensic Science International: Genetics Supplement Series 3, 224-225.

Ballantyne, J., Hanson, E.K., Perlin, M.W., 2013, DNA mixture genotyping by probabilistic computer interpretation of binomially-sampled laser captured cell populations: combining quantitative data for greater identification information. Sci Justice 53, 103-114.

Balogh, M.K., Burger, J., Bender, K., Schneider, P.M., Alt, K.W., 2003a, Fingerprints from fingerprints. Int. Congr. Ser. 1239, 953-957.

Balogh, M.K., Burger, J., Bender, K., Schneider, P.M., Alt, K.W., 2003b, STR genotyping and mtDNA sequencing of latent fingerprint on paper. Forensic Sci. Int. 137, 188-195.

Barash, M., Reshef, A., Brauner, P., 2010, The use of adhesive tape for recovery of DNA from crime scene items. J Forensic Sci 55, 1058-1064.

Benschop, C.C., Haned, H., Jeurissen, L., Gill, P.D., Sijen, T., 2015, The effect of varying the number of contributors on likelihood ratios for complex DNA mixtures. Forensic Sci Int Genet 19, 92-99.

Benschop, C.C., van der Beek, C.P., Meiland, H.C., van Gorp, A.G., Westen, A.A., Sijen, T., 2011, Low template STR typing: effect of replicate number and consensus method on genotyping reliability and DNA database search results. Forensic Sci. Int. Genet. 5, 316-328.

Bickley, J., Short, J.K., McDowell, D.G., Parkes, H.C., 1996, Polymerase chain reaction (PCR) detection of Listeria monocytogenes in diluted milk and reversal of PCR inhibition caused by calcium ions. Lett Appl Microbiol 22, 153-158.

Biesecker, L.G., Bailey-Wilson, J.E., Ballantyne, J., Baum, H., Bieber, F.R., Brenner, C., Budowle, B., Butler, J.M., Carmody, G., Conneally, P.M., Duceman, B., Eisenberg, A., Forman, L., Kidd, K.K., Leclair, B., Niezgoda, S., Parsons, T.J., Pugh, E., Shaler, R., Sherry, S.T., Sozer, A., Walsh, A., 2005, Epidemiology.

DNA identifications after the 9/11 World Trade Center attack. Science 310, 1122-1123.
Bille, T., Bright, J.A., Buckleton, J., 2013, Application of random match probability calculations to mixed STR profiles. J Forensic Sci 58, 474-485.
Bille, T.W., Weitz, S.M., Coble, M.D., Buckleton, J., Bright, J.A., 2014, Comparison of the performance of different models for the interpretation of low level mixed DNA profiles. Electrophoresis 35, 3125-3133.
Bragulla, H.H., Homberger, D.G., 2009, Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia. J Anat 214, 516-559.
Brauns, T.C., Goos, M., 2005, Micromanipulation of single cells from tissue imprints is an alternative to laser-assisted microdissection. J Cutan Pathol 32, 461-466.
Breathnach, M., Williams, L., McKenna, L., Moore, E., 2016, Probability of detection of DNA deposited by habitual wearer and/or the second individual who touched the garment. Forensic Sci Int Genet 20, 53-60.
Bright, J.A., Petricevic, S.F., 2004, Recovery of trace DNA and its application to DNA profiling of shoe insoles. Forensic Sci Int 145, 7-12.
Bruck, S., Evers, H., Heidorn, F., Muller, U., Kilper, R., Verhoff, M.A., 2011, Single cells for forensic DNA analysis--from evidence material to test tube. J Forensic Sci 56, 176-180.
Bruskov, V.I., Malakhova, L.V., Masalimov, Z.K., Chernikov, A.V., 2002, Heat-induced formation of reactive oxygen species and 8-oxoguanine, a biomarker of damage to DNA. Nucleic Acids Res 30, 1354-1363.
Buckleton, J.S., Curran, J.M., Gill, P., 2007, Towards understanding the effect of uncertainty in the number of contributors to DNA stains. Forensic Sci Int Genet 1, 20-28.
Budimlija, Z.M., Caragine, T.A., 2012, Interpretation guidelines for multilocus STR forensic profiles from low template DNA samples. Methods Mol Biol 830, 199-211.
Budowle, B., Eisenberg, A.J., van Daal, A., 2009a, Validity of low copy number typing and applications to forensic science. Croat Med J 50, 207-217.
Budowle, B., Moretti, T.R., Baumstark, A.L., Defenbaugh, D.A., Keys, K.M., 1999, Population data on the thirteen CODIS core short tandem repeat loci in African Americans, U.S. Caucasians, Hispanics, Bahamians, Jamaicans, and Trinidadians. J Forensic Sci 44, 1277-1286.
Budowle, B., Onorato, A.J., Callaghan, T.F., Della Manna, A., Gross, A.M., Guerrieri, R.A., Luttman, J.C., McClure, D.L., 2009b, Mixture interpretation: defining the relevant features for guidelines for the assessment of mixed DNA profiles in forensic casework. J Forensic Sci 54, 810-821.
Burton, M.P., Schneider, B.G., Brown, R., Escamilla-Ponce, N., Gulley, M.L., 1998, Comparison of histologic stains for use in PCR analysis of microdissected, paraffin-embedded tissues. Biotechniques 24, 86-92.
Bus, M.M., Allen, M., 2014, Collecting and preserving biological samples from challenging environments for DNA analysis. Biopreserv Biobank 12, 17-22.
Butler, J.M., 2004, Short tandem repeat analysis for human identity testing. Curr Protoc Hum Genet Chapter 14, Unit 14 18.
Butler, J.M., 2005, Constructing STR multiplex assays. Methods Mol Biol 297, 53-66.
Butler, J.M., 2006, Genetics and genomics of core short tandem repeat loci used in human identity testing. J Forensic Sci 51, 253-265.

Butler, J.M., 2007, Short tandem repeat typing technologies used in human identity testing. Biotechniques 43, ii-v.
Butler, J.M., Shen, Y., McCord, B.R., 2003, The development of reduced size STR amplicons as tools for analysis of degraded DNA. J Forensic Sci 48, 1054-1064.
Cale, C.M., Earll, M.E., Latham, K.E., Bush, G.L., 2016, Could Secondary DNA Transfer Falsely Place Someone at the Scene of a Crime? J Forensic Sci 61, 196-203.
Caragine, T., Mikulasovich, R., Tamariz, J., Bajda, E., Sebestyen, J., Baum, H., Prinz, M., 2009, Validation of testing and interpretation protocols for low template DNA samples using AmpFlSTR Identifiler. Croat. Med. J. 50, 250-267.
Chellappan, S., Jasmin, C., Basheer, S.M., Kishore, A., Elyas, K.K., Bhat, S.G., Chandrasekaran, M., 2011, Characterization of an extracellular alkaline serine protease from marine Engyodontium album BTMFS10. J Ind Microbiol Biotechnol 38, 743-752.
Cho, S., Yu, H.J., Han, J., Kim, Y., Lee, J., Lee, S.D., 2014, Forensic application of SNP-based resequencing array for individual identification. Forensic Sci Int Genet 13, 45-52.
Clark, J.M., 1988, Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases. Nucleic Acids Res 16, 9677-9686.
Clayton, T.M., Whitaker, J.P., Sparkes, R., Gill, P., 1998, Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic Sci Int 91, 55-70.
Collins, P.J., Hennessy, L.K., Leibelt, C.S., Roby, R.K., Reeder, D.J., Foxall, P.A., 2004, Developmental validation of a single-tube amplification of the 13 CODIS STR loci, D2S1338, D19S433, and amelogenin: the AmpFlSTR Identifiler PCR Amplification Kit. J Forensic Sci 49, 1265-1277.
Cooper-Dunn, A., Jones, O., Bond, J.W., 2017, The Effect of Varying the Composition of Fingerprint Sweat Deposits on the Corrosion of Brass and Fingerprint Visibility. J Forensic Sci.
Cooper, S., McGovern, C., Bright, J.A., Taylor, D., Buckleton, J., 2015, Investigating a common approach to DNA profile interpretation using probabilistic software. Forensic Sci Int Genet 16, 121-131.
Corach, D., Sala, A., Penacino, G., Iannucci, N., Bernardi, P., Doretti, M., Fondebrider, L., Ginarte, A., Inchaurregui, A., Somigliana, C., Turner, S., Hagelberg, E., 1997, Additional approaches to DNA typing of skeletal remains: the search for "missing" persons killed during the last dictatorship in Argentina. Electrophoresis 18, 1608-1612.
Cowell, R., 2016, Combining allele frequency uncertainty and population substructure corrections in forensic DNA calculations. Forensic Sci Int Genet 23, 210-216.
Cowell, R.G., Lauritzen, S.L., Mortera, J., 2007, Identification and separation of DNA mixtures using peak area information. Forensic Sci Int 166, 28-34.
Cowen, S., Debenham, P., Dixon, A., Kutranov, S., Thomson, J., Way, K., 2011, An investigation of the robustness of the consensus method of interpreting low-template DNA profiles. Forensic Sci. Int. Genet. 5, 400-406.
D'Aquila, R.T., Bechtel, L.J., Videler, J.A., Eron, J.J., Gorczyca, P., Kaplan, J.C., 1991, Maximizing sensitivity and specificity of PCR by pre-amplification heating. Nucleic Acids Res 19, 3749.

Daly, D.J., Murphy, C., McDermott, S.D., 2012, The transfer of touch DNA from hands to glass, fabric and wood. Forensic Sci Int Genet 6, 41-46.
De Alcaraz-Fossoul, J., Mestres Patris, C., Barrot Feixat, C., McGarr, L., Brandelli, D., Stow, K., Gené Badia, M., 2016, Latent Fingermark Aging Patterns (Part I): Minutiae Count as One Indicator of Degradation Journal of Forensic Sciences 61, 322-333.
Di Martino, D., Giuffre, G., Staiti, N., Simone, A., Le Donne, M., Saravo, L., 2004, Single sperm cell isolation by laser microdissection. Forensic Sci Int 146 Suppl, S151-153.
Djuric, M., Valjen, T., Stanojevic, A., Stojkovic, O., 2008, DNA typing from handled items. Forensic Sci Int genet Supplement Series 1, 411-412.
Dolan, S.M., Saraiya, D.S., Donkervoort, S., Rogel, K., Lieber, C., Sozer, A., 2009, The emerging role of genetics professionals in forensic kinship DNA identification after a mass fatality: lessons learned from Hurricane Katrina volunteers. Genet Med 11, 414-417.
Dror, I.E., Hampikian, G., 2011, Subjectivity and bias in forensic DNA mixture interpretation. Sci Justice 51, 204-208.
Dumache, R., Ciocan, V., Muresan, C., Enache, A., 2016, Molecular DNA Analysis in Forensic Identification. Clin Lab 62, 245-248.
Edwards, M.C., Gibbs, R.A., 1994, Multiplex PCR: advantages, development, and applications. PCR Methods Appl 3, S65-75.
Ellegren, H., 2004, Microsatellites: simple sequences with complex evolution. Nat Rev Genet 5, 435-445.
Elliott, K., Hill, D.S., Lambert, C., Burroughes, T.R., Gill, P., 2003, Use of laser microdissection greatly improves the recovery of DNA from sperm on microscope slides. Forensic Sci Int 137, 28-36.
Ensenberger, M.G., Lenz, K.A., Matthies, L.K., Hadinoto, G.M., Schienman, J.E., Przech, A.J., Morganti, M.W., Renstrom, D.T., Baker, V.M., Gawrys, K.M., Hoogendoorn, M., Steffen, C.R., Martin, P., Alonso, A., Olson, H.R., Sprecher, C.J., Storts, D.R., 2016, Developmental validation of the PowerPlex((R)) Fusion 6C System. Forensic Sci Int Genet 21, 134-144.
Espina, V., Wulfkuhle, J.D., Calvert, V.S., VanMeter, A., Zhou, W., Coukos, G., Geho, D.H., Petricoin, E.F., 3rd, Liotta, L.A., 2006, Laser-capture microdissection. Nat Protoc 1, 586-603.
Farmen, R.K.J., R. Cortez, P. Froyland, E. S., 2008, Assessment of individual shedder status and implication for secondary DNA transfer. Forensic Science International: Genetics Supplement Series 1, 415-417.
Flores, S., Sun, J., King, J., Budowle, B., 2014, Internal validation of the GlobalFiler Express PCR Amplification Kit for the direct amplification of reference DNA samples on a high-throughput automated workflow. Forensic Sci Int Genet 10, 33-39.
Fonnelop, A.E., Egeland, T., Gill, P., 2015, Secondary and subsequent DNA transfer during criminal investigation. Forensic Sci Int Genet 17, 155-162.
Forster, L., Thomson, J., Kutranov, S., 2008, Direct comparison of post-28-cycle PCR purification and modified capillary electrophoresis methods with the 34-cycle "low copy number" (LCN) method for analysis of trace forensic DNA samples. Forensic Sci Int Genet 2, 318-328.

Gaines, M.L., Wojtkiewicz, P.W., Valentine, J.A., Brown, C.L., 2002, Reduced volume PCR amplification reactions using the AmpFlSTR Profiler Plus kit. J Forensic Sci 47, 1224-1237.
Gandarillas, A., Goldsmith, L.A., Gschmeissner, S., Leigh, I.M., Watt, F.M., 1999, Evidence that apoptosis and terminal differentiation of epidermal keratinocytes are distinct processes. Exp. Dermatol. 8, 71-79.
Gill, P., 2001, Application of low copy number DNA profiling. Croat. Med. J. 42, 229-232.
Gill, P., 2002, Role of short tandem repeat DNA in forensic casework in the UK--past, present, and future perspectives. Biotechniques 32, 366-368, 370, 372, passim.
Gill, P., Brenner, C.H., Buckleton, J.S., Carracedo, A., Krawczak, M., Mayr, W.R., Morling, N., Prinz, M., Schneider, P.M., Weir, B.S., 2006a, DNA commission of the International Society of Forensic Genetics: Recommendations on the interpretation of mixtures. Forensic Sci Int 160, 90-101.
Gill, P., Fereday, L., Morling, N., Schneider, P.M., 2006b, The evolution of DNA databases--recommendations for new European STR loci. Forensic Sci Int 156, 242-244.
Gill, P., Haned, H., 2013, A new methodological framework to interpret complex DNA profiles using likelihood ratios. Forensic Sci Int Genet 7, 251-263.
Gill, P., Haned, H., Bleka, O., Hansson, O., Dorum, G., Egeland, T., 2015, Genotyping and interpretation of STR-DNA: Low-template, mixtures and database matches-Twenty years of research and development. Forensic Sci Int Genet 18, 100-117.
Gill, P., Jeffreys, A.J., Werrett, D.J., 1985, Forensic application of DNA 'fingerprints'. Nature 318, 577-579.
Gill, P., Whitaker, J., Flaxman, C., Brown, N., Buckleton, J., 2000, An investigation of the rigor of interpretation rules for STRs derived from less than 100 pg of DNA. Forensic Sci. Int. 112, 17-40.
Goray, M., Eken, E., Mitchell, R.J., van Oorschot, R.A., 2010a, Secondary DNA transfer of biological substances under varying test conditions. Forensic Sci Int Genet 4, 62-67.
Goray, M., Mitchell, R.J., van Oorschot, R.A., 2010b, Investigation of secondary DNA transfer of skin cells under controlled test conditions. Leg Med (Tokyo) 12, 117-120.
Goray, M., van Oorschot, R.A., 2015, The complexities of DNA transfer during a social setting. Leg Med (Tokyo) 17, 82-91.
Grisedale, K.S., van Daal, A., 2012, Comparison of STR profiling from low template DNA extracts with and without the consensus profiling method. Investig Genet 3, 14.
Gunnarsson, J., Eriksson, E., Ansell, R., 2010, Success Rate of a Forensic Tape-lift method for DNA recovery. Problems of Forensic Sciences 83, 243-254.
Haines, A.M., Tobe, S.S., Kobus, H., Linacre, A., 2013, Detection of DNA within fingermarks. Forensic Science International: Genetics Supplement Series 290-291.
Hameed, I., 2014, Genetic Variation and DNA markers in forensic analysis. African Journal of Biotechnology 13, 3122-3136.
Han, J.P., Yang, F., Xu, C., Wei, Y.L., Zhao, X.C., Hu, L., Ye, J., Li, C.X., 2014, A new strategy for sperm isolation and STR typing from multi-donor sperm mixtures. Forensic Sci Int Genet 13, 239-246.

Haned, H., Pene, L., Lobry, J.R., Dufour, A.B., Pontier, D., 2011a, Estimating the number of contributors to forensic DNA mixtures: does maximum likelihood perform better than maximum allele count? J Forensic Sci 56, 23-28.
Haned, H., Pene, L., Sauvage, F., Pontier, D., 2011b, The predictive value of the maximum likelihood estimator of the number of contributors to a DNA mixture. Forensic Sci Int Genet 5, 281-284.
Haned, H., Slooten, K., Gill, P., 2012, Exploratory data analysis for the interpretation of low template DNA mixtures. Forensic Sci Int Genet 6, 762-774.
Hanson, E.K., Ballantyne, J., 2013, "Getting blood from a stone": ultrasensitive forensic DNA profiling of microscopic bio-particles recovered from "touch DNA" evidence. Methods Mol Biol 1039, 3-17.
Hardjasa, A., Ling, M., Ma, K., Yu, H., 2010, Investigating the effect of DMSO on PCR fidelity using a restriction digest-based method. Journal of Experimental Microbiology and Immunology 14, 161-164.
Hares, D.R., 2012, Expanding the CODIS core loci in the United States. Forensic Sci Int Genet 6, e52-54.
Helmus, J., Bajanowski, T., Poetsch, M., 2016, DNA transfer-a never ending story. A study on scenarios involving a second person as carrier. Int J Legal Med 130, 121-125.
Henle, E.S., Linn, S., 1997, Formation, prevention, and repair of DNA damage by iron/hydrogen peroxide. J Biol Chem 272, 19095-19098.
Horsman-Hall, K.M., Orihuela, Y., Karczynski, S.L., Davis, A.L., Ban, J.D., Greenspoon, S.A., 2009, Development of STR profiles from firearms and fired cartridge cases. Forensic Sci Int Genet 3, 242-250.
Horsman, K.M., Barker, S.L., Ferrance, J.P., Forrest, K.A., Koen, K.A., Landers, J.P., 2005, Separation of sperm and epithelial cells in a microfabricated device: potential application to forensic analysis of sexual assault evidence. Anal Chem 77, 742-749.
Hoss, M., Jaruga, P., Zastawny, T.H., Dizdaroglu, M., Paabo, S., 1996, DNA damage and DNA sequence retrieval from ancient tissues. Nucleic Acids Res 24, 1304-1307.
Huffine, E., Crews, J., Kennedy, B., Bomberger, K., Zinbo, A., 2001, Mass identification of persons missing from the break-up of the former Yugoslavia: structure, function, and role of the International Commission on Missing Persons. Croat Med J 42, 271-275.
Hunt, J.L., Finkelstein, S.D., 2004, Microdissection techniques for molecular testing in surgical pathology. Arch Pathol Lab Med 128, 1372-1378.
Ip, S.C., Lin, S.W., Li, C., Lai, K.M., 2014, Forensic DNA typing strategy of degraded DNA on discarded cigarette ends using the AmpFlSTR(R) Identifiler(R), Identifiler(R) Plus and MiniFiler PCR amplification kits. Sci Justice 54, 311-315.
Jain AK, A, R., 2015, Bridging the gap: from biometrics to forensics. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 370.
Jeffreys, A.J., Wilson, V., Thein, S.L., 1985a, Hypervariable 'minisatellite' regions in human DNA. Nature 314, 67-73.
Jeffreys, A.J., Wilson, V., Thein, S.L., 1985b, Individual-specific 'fingerprints' of human DNA. Nature 316, 76-79.

Jobling, M.A., Gill, P., 2004, Encoded evidence: DNA in forensic analysis. Nat Rev Genet 5, 739-751.
Katsanis, S.H., Wagner, J.K., 2013, Characterization of the standard and recommended CODIS markers. J Forensic Sci 58 Suppl 1, S169-172.
Kehr, J., 2003, Single cell technology. Curr Opin Plant Biol 6, 617-621.
Kimpton, C., Fisher, D., Watson, S., Adams, M., Urquhart, A., Lygo, J., Gill, P., 1994, Evaluation of an automated DNA profiling system employing multiplex amplification of four tetrameric STR loci. Int J Legal Med 106, 302-311.
Kita, T., Yamaguchi, H., Yokoyama, M., Tanaka, T., Tanaka, N., 2008, Morphological study of fragmented DNA on touched objects. Forensic Sci Int Genet 3, 32-36.
Klein, C.A., Schmidt-Kittler, O., Schardt, J.A., Pantel, K., Speicher, M.R., Riethmuller, G., 1999, Comparative genomic hybridization, loss of heterozygosity, and DNA sequence analysis of single cells. Proc Natl Acad Sci U S A 96, 4494-4499.
Kloosterman, A.D., Kersbergen, P., 2003, Efficacy and limits of genotyping low copy number (LCN) DNA samples by multiplex PCR of STR loci. J Soc Biol 197, 351-359.
Kopka, J., Leder, M., Jaureguiberry, S.M., Brem, G., Boselli, G.O., 2011, New optimized DNA extraction protocol for fingerprints deposited on a special self-adhesive security seal and other latent samples used for human identification. J Forensic Sci 56, 1235-1240.
Kreader, C., 1996, Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Envirtonmental Microbiology 62, 1102-1106.
Krenke, B.E., Tereba, A., Anderson, S.J., Buel, E., Culhane, S., Finis, C.J., Tomsey, C.S., Zachetti, J.M., Masibay, A., Rabbach, D.R., Amiott, E.A., Sprecher, C.J., 2002, Validation of a 16-locus fluorescent multiplex system. J Forensic Sci 47, 773-785.
Ladd, C., Lee, H.C., Yang, N., Bieber, F.R., 2001, Interpretation of complex forensic DNA mixtures. Croat Med J 42, 244-246.
Larkin, A., Harbison, S., 1999, An improved method for STR analysis of bloodstained denim. Int J Legal Med 112, 388-390.
Li, L., Lin, Y., Liu, Y., Zhu, R., Zhao, Z., Que, T., 2015, A case of false mother included with 46 autosomal STR markers. Investig Genet 6, 9.
Lindahl, T., Andersson, A., 1972, Rate of chain breakage at apurinic sites in double-stranded deoxyribonucleic acid. Biochemistry 11, 3618-3623.
Lowe, A., Murray, C., Whitaker, J., Tully, G., Gill, P., 2002, The propensity of individuals to deposit DNA and secondary transfer of low level DNA from individuals to inert surfaces. Forensic Sci. Int. 129, 25-34.
Lygo, J.E., Johnson, P.E., Holdaway, D.J., Woodroffe, S., Whitaker, J.P., Clayton, T.M., Kimpton, C.P., Gill, P., 1994, The validation of short tandem repeat (STR) loci for use in forensic casework. Int J Legal Med 107, 77-89.
Martin, P., de Simon, L.F., Luque, G., Farfan, M.J., Alonso, A., 2014, Improving DNA data exchange: validation studies on a single 6 dye STR kit with 24 loci. Forensic Sci Int Genet 13, 68-78.
Martin, P.D., Schmitter, H., Schneider, P.M., 2001, A brief history of the formation of DNA databases in forensic science within Europe. Forensic Sci Int 119, 225-231.

Mayrand, P.E., 1992, The use of fluorescence detection and internal lane standards to size PCR products automatically. Applied Theoretical Electrophoresis 3, 1-11.
Mehta, B., Daniel, R., Phillips, C., McNevin, D., 2017, Forensically relevant SNaPshot(R) assays for human DNA SNP analysis: a review. Int J Legal Med 131, 21-37.
Meredith, M., Bright, J.A., Cockerton, S., Vintiner, S., 2012, Development of a one-tube extraction and amplification method for DNA analysis of sperm and epithelial cells recovered from forensic samples by laser microdissection. Forensic Sci Int Genet 6, 91-96.
Mighell, A.J., Markham, A.F., Robinson, P.A., 1997, Alu sequences. FEBS Lett 417, 1-5.
Mitchell, A.A., Tamariz, J., O'Connell, K., Ducasse, N., Budimlija, Z., Prinz, M., Caragine, T., 2012, Validation of a DNA mixture statistics tool incorporating allelic drop-out and drop-in. Forensic Sci Int Genet 6, 749-761.
Montinaro, F., Boschi, I., Trombetta, F., Merigioli, S., Anagnostou, P., Battaggia, C., Capocasa, M., Crivellaro, F., Destro Bisol, G., Coia, V., 2012, Using forensic microsatellites to decipher the genetic structure of linguistic and geographic isolates: A survey in the eastern Italian Alps. Forensic Sci Int Genet 6, 827-833.
Moretti, T.R., Baumstark, A.L., Defenbaugh, D.A., Keys, K.M., Brown, A.L., Budowle, B., 2001a, Validation of STR typing by capillary electrophoresis. J Forensic Sci 46, 661-676.
Moretti, T.R., Baumstark, A.L., Defenbaugh, D.A., Keys, K.M., Smerick, J.B., Budowle, B., 2001b, Validation of short tandem repeats (STRs) for forensic usage: performance testing of fluorescent multiplex STR systems and analysis of authentic and simulated forensic samples. J Forensic Sci 46, 647-660.
Mulero, J.J., Chang, C.W., Lagace, R.E., Wang, D.Y., Bas, J.L., McMahon, T.P., Hennessy, L.K., 2008, Development and validation of the AmpFlSTR MiniFiler PCR Amplification Kit: a MiniSTR multiplex for the analysis of degraded and/or PCR inhibited DNA. J Forensic Sci 53, 838-852.
Murray, C., McAlister, C., Elliott, K., 2007, Identification and isolation of male cells using fluorescence in situ hybridisation and laser microdissection, for use in the investigation of sexual assault. Forensic Sci Int Genet 1, 247-252.
Nicklas, J.A., Buel, E., 2003, Development of an Alu-based, real-time PCR method for quantitation of human DNA in forensic samples. J. Forensic Sci. 48, 936-944.
Nishino, T., Morikawa, K., 2002, Structure and function of nucleases in DNA repair: shape, grip and blade of the DNA scissors. Oncogene 21, 9022-9032.
Oleiwi, A.A., Morris, M.R., Schmerer, W.M., Sutton, R., 2015, The relative DNA-shedding propensity of the palm and finger surfaces. Sci Justice 55, 329-334.
Onori, N., Onofriri, V., Alessandrini, F., Buscemi, L., Pesaresi, M., Turchi, C., Tagliabracci, A., 2006, Post-mortem DNA damage: A comparative study of STRs and SNPs typing efficiency in simulated forensic samples. International Congress Series 1288, 510-512.
Oostdik, K., Lenz, K., Nye, J., Schelling, K., Yet, D., Bruski, S., Strong, J., Buchanan, C., Sutton, J., Linner, J., Frazier, N., Young, H., Matthies, L., Sage, A., Hahn, J., Wells, R., Williams, N., Price, M., Koehler, J., Staples, M., Swango, K.L., Hill, C., Oyerly, K., Duke, W., Katzilierakis, L., Ensenberger, M.G., Bourdeau, J.M., Sprecher, C.J., Krenke, B., Storts, D.R., 2014, Developmental validation of the PowerPlex((R)) Fusion System for analysis of casework and reference samples:

A 24-locus multiplex for new database standards. Forensic Sci Int Genet 12, 69-76.
Opel, K.L., Chung, D., McCord, B.R., 2010, A study of PCR inhibition mechanisms using real time PCR. J Forensic Sci 55, 25-33.
Opel, K.L., Chung, D.T., Drabek, J., Tatarek, N.E., Jantz, L.M., McCord, B.R., 2006, The application of miniplex primer sets in the analysis of degraded DNA from human skeletal remains. J Forensic Sci 51, 351-356.
Ostojic, L., Klempner, S.A., Patel, R.A., Mitchell, A.A., Axler-DiPerte, G.L., Wurmbach, E., 2014, Qualitative and quantitative assessment of single fingerprints in forensic DNA analysis. Electrophoresis 35, 3165-3172.
Ostojic, L., Wurmbach, E., 2017, Analysis of fingerprint samples, testing various conditions, for forensic DNA identification. Sci Justice 57, 35-40.
Paoletti, D.R., Doom, T.E., Krane, C.M., Raymer, M.L., Krane, D.E., 2005, Empirical analysis of the STR profiles resulting from conceptual mixtures. J Forensic Sci 50, 1361-1366.
Parsons, L., Sharfe, G., Vintiner, S., 2016, DNA Analysis and Document Examination: The Impact of Each Technique on Respective Analyses. J Forensic Sci 61, 26-34.
Pesaresi, M.B., L. Alessandrini, F. Cecati, M. Tagliabracci A., 2003, Qualitative and quantitative analysis of DNA recovered from fingerprints. International Congress Series 1239, 947– 951.
Petricevic, S., Whitaker, J., Buckleton, J., Vintiner, S., Patel, J., Simon, P., Ferraby, H., Hermiz, W., Russell, A., 2010, Validation and development of interpretation guidelines for low copy number (LCN) DNA profiling in New Zealand using the AmpFlSTR SGM Plus multiplex. Forensic Sci Int Genet 4, 305-310.
Phipps, M., Petricevic, S., 2007, The tendency of individuals to transfer DNA to handled items. Forensic Sci. Int. 168, 162-168.
Polley, D., Mickiewicz, P., Vaughn, M., Miller, T., Warburton, R., Komonski, D., Kantautas, C., Reid, B., Frappier, R., Newman, J., 2006, An Investigation of DNA Recovery from Firearms and Cartridge Cases. Canadian Society of Forensic Science Journal 39, 217-228.
Prieto, L., Haned, H., Mosquera, A., Crespillo, M., Aleman, M., Aler, M., Alvarez, F., Baeza-Richer, C., Dominguez, A., Doutremepuich, C., Farfan, M.J., Fenger-Gron, M., Garcia-Ganivet, J.M., Gonzalez-Moya, E., Hombreiro, L., Lareu, M.V., Martinez-Jarreta, B., Merigioli, S., Milans Del Bosch, P., Morling, N., Munoz-Nieto, M., Ortega-Gonzalez, E., Pedrosa, S., Perez, R., Solis, C., Yurrebaso, I., Gill, P., 2014, Euroforgen-NoE collaborative exercise on LRmix to demonstrate standardization of the interpretation of complex DNA profiles. Forensic Sci Int Genet 9, 47-54.
Prinz, M., Schiffner, L.A., Sebestyen, J.A., Bajda, E., Tamariz, J., Shaler, R.C., Baum, H., Caragine, T., 2006, Maximization of STR DNA typing success for touched objects. Int. Congr. Ser. 1288, 651-653.
Puch-Solis, R., Pope, S., Evett, I., 2010, Calculating likelihood ratios for a mixed DNA profile when a contribution from a genetic relative of a suspect is proposed. Sci Justice 50, 205-209.
Quinones, I., Daniel, B., 2012, Cell free DNA as a component of forensic evidence recovered from touched surfaces. Forensic Sci. Int. Genet. 6, 26-30.

Radstrom, P., Knutsson, R., Wolffs, P., Lovenklev, M., Lofstrom, C., 2004, Pre-PCR processing: strategies to generate PCR-compatible samples. Mol. Biotechnol. 26, 133-146.
Ralser, M., Querfurth, R., Warnatz, H.J., Lehrach, H., Yaspo, M.L., Krobitsch, S., 2006, An efficient and economic enhancer mix for PCR. Biochem Biophys Res Commun 347, 747-751.
Raymond, J.J., van Oorschot, R.A., Gunn, P.R., Walsh, S.J., Roux, C., 2009, Trace evidence characteristics of DNA: A preliminary investigation of the persistence of DNA at crime scenes. Forensic Sci Int Genet 4, 26-33.
Roeder, A.D., Elsmore, P., Greenhalgh, M., McDonald, A., 2009, Maximizing DNA profiling success from sub-optimal quantities of DNA: a staged approach. Forensic Sci. Int. Genet. 3, 128-137.
Roewer, L., 2013, DNA fingerprinting in forensics: past, present, future. Investig Genet 4, 22.
Romanini, C.F.M.R.C., M. L. Vullo, C., 2011, A comparison of AmpFlSTR Identifiler™ Kit versus AmpFlSTR Identifiler Plus™ Kit in challenging bone samples by using normal and increased PCR cycle number. Forensic Science International: Genetics Supplement Series 3, 514-515.
Rossen, L., Norskov, P., Holmstrom, K., Rasmussen, O.F., 1992, Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 17, 37-45.
Rutty, G.N., 2002, An investigation into the transference and survivability of human DNA following simulated manual strangulation with consideration of the problem of third party contamination. Int J Legal Med 116, 170-173.
Sagripanti, J.L., Goering, P.L., Lamanna, A., 1991, Interaction of copper with DNA and antagonism by other metals. Toxicol Appl Pharmacol 110, 477-485.
Sanchez, J.J., Phillips, C., Borsting, C., Balogh, K., Bogus, M., Fondevila, M., Harrison, C.D., Musgrave-Brown, E., Salas, A., Syndercombe-Court, D., Schneider, P.M., Carracedo, A., Morling, N., 2006, A multiplex assay with 52 single nucleotide polymorphisms for human identification. Electrophoresis 27, 1713-1724.
Sanders, C.T., Sanchez, N., Ballantyne, J., Peterson, D.A., 2006, Laser microdissection separation of pure spermatozoa from epithelial cells for short tandem repeat analysis. J Forensic Sci 51, 748-757.
Schiffner, L.A., Bajda, E.J., Prinz, M., Sebestyen, J., Shaler, R., Caragine, T.A., 2005, Optimization of a simple, automatable extraction method to recover sufficient DNA from low copy number DNA samples for generation of short tandem repeat profiles. Croat Med J 46, 578-586.
Schmid, C.W., 1996, Alu: structure, origin, evolution, significance and function of one-tenth of human DNA. Prog Nucleic Acid Res Mol Biol 53, 283-319.
Schneider, C., Muller, U., Kilper, R., Siebertz, B., 2012, Low copy number DNA profiling from isolated sperm using the aureka(R)-micromanipulation system. Forensic Sci Int Genet 6, 461-465.
Schneider, H., Sommerer, T., Rand, S., Wiegand, P., 2011, Hot flakes in cold cases. Int. J. Legal Med. 125, 543-548.
Schrader, C., Schielke, A., Ellerbroek, L., Johne, R., 2012, PCR inhibitors - occurrence, properties and removal. J Appl Microbiol 113, 1014-1026.

Sewell, J., Quinones, I., Ames, C., Multaney, B., Curtis, S., Seeboruth, H., Moore, S., Daniel, B., 2008, Recovery of DNA and fingerprints from touched documents. Forensic Sci Int Genet 2, 281-285.
Shrader, C., 2012, PCR inhibitors- occurence, properties and removal. J. Appl. Microbiol. 113, 1014-1026.
Shutler, G.G., Gagnon, P., Verret, G., Kalyn, H., Korkosh, S., Johnston, E., Halverson, J., 1999, Removal of a PCR inhibitor and resolution of DNA STR types in mixed human-canine stains from a five year old case. J Forensic Sci 44, 623-626.
Simpson, C.L., Patel, D.M., Green, K.J., 2011, Deconstructing the skin: cytoarchitectural determinants of epidermal morphogenesis. Nat Rev Mol Cell Biol 12, 565-580.
Sinha, R.P., Hader, D.P., 2002, UV-induced DNA damage and repair: a review. Photochem Photobiol Sci 1, 225-236.
Smith, S., Morin, P.A., 2005, Optimal storage conditions for highly dilute DNA samples: a role for trehalose as a preserving agent. J Forensic Sci 50, 1101-1108.
Snabes, M.C., Chong, S.S., Subramanian, S.B., Kristjansson, K., DiSepio, D., Hughes, M.R., 1994, Preimplantation single-cell analysis of multiple genetic loci by whole-genome amplification. Proc Natl Acad Sci U S A 91, 6181-6185.
Sobrino, B., Brion, M., Carracedo, A., 2005, SNPs in forensic genetics: a review on SNP typing methodologies. Forensic Sci Int 154, 181-194.
Sobrino, B., Carracedo, A., 2005, SNP typing in forensic genetics: a review. Methods Mol Biol 297, 107-126.
Soltyszewski, I., Szeremeta, M., Skawronska, M., Niemcunowicz-Janica, A., Pepinski, W., 2015, Typeability of DNA in Touch Traces Deposited on Paper and Optical Data Discs. Adv Clin Exp Med 24, 437-440.
Spinello, A., Terenzi, A., Barone, G., 2013, Metal complex-DNA binding: Insights from molecular dynamics and DFT/MM calculations. J Inorg Biochem 124, 63-69.
Stanciu, C.E., Kwon, Y.J., Ehrhardt, C.J., 2016, Forward-scatter and side-scatter dataset for epithelial cells from touch samples analyzed by flow cytometry. Data Brief 6, 416-418.
Stanciu, C.E., Philpott, M.K., Kwon, Y.J., Bustamante, E.E., Ehrhardt, C.J., 2015, Optical characterization of epidermal cells and their relationship to DNA recovery from touch samples. F1000Res 4, 1360.
Stouder, S.L., 2001, Trace Evidence Scrapings:
A Valuable Source of DNA? Forensic Science Communiactions 4.
Taberler, P., Griffin, Sally, Goossens, Benoit, Questiau, Sophie, Manceau, Valerie, Escaravange, Nathalie, Waits, Lisette P., Bouver, Jean, 1996, Reliable Genotyping of Samples with Very Low DNA Quantities Using PCR. Nucleic Acids Reseaarch 24, 3189-3194.
Templeton, J.E., Linacre, A., 2014, DNA profiles from fingermarks. Biotechniques 57, 259-266.
Teoule, R., Cadet, J., 1978, Radiation-induced degradation of the base component in DNA and related substances--final products. Mol Biol Biochem Biophys 27, 171-203.
Tewari, A., Sarkany, R.P., Young, A.R., 2012, UVA1 induces cyclobutane pyrimidine dimers but not 6-4 photoproducts in human skin in vivo. J Invest Dermatol 132, 394-400.

Thomasma, S.M., Foran, D.R., 2012, The Influence of Swabbing Solutions on DNA Recovery from Touch Samples(,). J. Forensic Sci. 58, 465-469.
Thomasma, S.M., Foran, D.R., 2013, The influence of swabbing solutions on DNA recovery from touch samples. J Forensic Sci 58, 465-469.
Trauring, M., 1963, Automatic comparison of finger-ridge patterns. Nature 197, 938-940.
Van Hoofstat, D.E., Deforce, D.L., Hubert De Pauw, I.P., Van den Eeckhout, E.G., 1999, DNA typing of fingerprints using capillary electrophoresis: effect of dactyloscopic powders. Electrophoresis 20, 2870-2876.
van Oorschot, R.A., Ballantyne, K.N., Mitchell, R.J., 2010, Forensic trace DNA: a review. Investig. Genet. 1, 14.
van Oorschot, R.A., Glavich, G., Mitchell, R.J., 2014, Persistence of DNA deposited by the original user on objects after subsequent use by a second person. Forensic Sci Int Genet 8, 219-225.
van Oorschot, R.A., Jones, M.K., 1997, DNA fingerprints from fingerprints. Nature 387, 767.
Vandewoestyne, M., Deforce, D., 2010, Laser capture microdissection in forensic research: a review. Int J Legal Med 124, 513-521.
Vandewoestyne, M., Van Hoofstat, D., Franssen, A., Van Nieuwerburgh, F., Deforce, D., 2013, Presence and potential of cell free DNA in different types of forensic samples. Forensic Sci Int Genet 7, 316-320.
Verdon, T.J., Mitchell, R.J., van Oorschot, R.A., 2014, Evaluation of tapelifting as a collection method for touch DNA. Forensic Sci Int Genet 8, 179-186.
Verheij, S., Harteveld, J., Sijen, T., 2012, A protocol for direct and rapid multiplex PCR amplification on forensically relevant samples. Forensic Sci Int Genet 6, 167-175.
Verzeletti, A., Cortellini, V., Cerri, A., Pretto, S., Ferrari, F.D., 2013, PowerPlex1 Fusion kit: A 23 plex autosomal STR kit for human identity testing Forensic Science International: Genetics Supplement Series 4, 344-345.
Walch, A., Komminoth, P., Hutzler, P., Aubele, M., Hofler, H., Werner, M., 2000, Microdissection of tissue sections: application to the molecular genetic characterisation of premalignant lesions. Pathobiology 68, 9-17.
Walters, K.A., Roberts, Michaelk S., 2002, Dermatological and Transdermal Formulations. Marcel Dekker, Inc., 120 p.
Wang, D.Y., Chang, C.W., Lagace, R.E., Calandro, L.M., Hennessy, L.K., 2012, Developmental validation of the AmpFlSTR(R) Identifiler(R) Plus PCR Amplification Kit: an established multiplex assay with improved performance. J. Forensic Sci. 57, 453-465.
Wang, Q., Wang, X., Yu, Z., Yuan, X., Jiao, K., 2011, Spectroscopic and Electrochemical Studies on The Binding Mechanism of DNA With an Anthraquinone Biological Dye, Nuclear Fast Red International Journal of Electrochemical Science 6, 5470-5481.
Watson, R.J., Blackwell, B., 2000, Purification and characterization of a common soil component which inhibits the polymerase chain reaction. Can J Microbiol 46, 633-642.
Watt, F.M., 2014, Mammalian skin cell biology: at the interface between laboratory and clinic. Science 346, 937-940.

Webb, L.G., Egan, S.E., Turbett, G.R., 2001, Recovery of DNA for forensic analysis from lip cosmetics. J Forensic Sci 46, 1474-1479.
Welch, L.A., Gill, P., Phillips, C., Ansell, R., Morling, N., Parson, W., Palo, J.U., Bastisch, I., 2012, European Network of Forensic Science Institutes (ENFSI): Evaluation of new commercial STR multiplexes that include the European Standard Set (ESS) of markers. Forensic Sci Int Genet 6, 819-826.
Westen, A.A., Nagel, J.H., Benschop, C.C., Weiler, N.E., de Jong, B.J., Sijen, T., 2009, Higher capillary electrophoresis injection settings as an efficient approach to increase the sensitivity of STR typing. J. Forensic Sci. 54, 591-598.
Wickenheiser, R.A., 2002, Trace DNA: a review, discussion of theory, and application of the transfer of trace quantities of DNA through skin contact. J Forensic Sci 47, 442-450.
Wiegand, P., Kleiber, M., 1997, DNA typing of epithelial cells after strangulation. Int J Legal Med 110, 181-183.
Wilson, I.G., 1997, Inhibition and facilitation of nucleic acid amplification. Appl Environ Microbiol 63, 3741-3751.
Yao, H.B., Wang, C.C., Wang, J., Tao, X., Shang, L., Wen, S.Q., Du, Q., Deng, Q., Xu, B., Huang, Y., Wang, H.D., Li, S., Bin, C., Ma, L., Jin, L., Krause, J., Li, H., 2017, Genetic structure of Tibetan populations in Gansu revealed by forensic STR loci. Sci Rep 7, 41195.
Young, C.C., Burghoff, R.L., Keim, L.G., Minak-Bernero, V., Lute, J.R., Hinton, S.M., 1993, Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. Appl Environ Microbiol 59, 1972-1974.
Zaken, N., Motro, U., Berdugo, R., Sapir, L.E., Zamir, A., 2013, Can brothers share the same STR profile? Forensic Sci Int Genet 7, 494-498.
Zamir, A., Springer, E., Glattstein, B., 2000, Fingerprints and DNA: STR typing of DNA extracted from adhesive tape after processing for fingerprints. J Forensic Sci 45, 687-688.
Zech, W.D., Malik, N., Thali, M., 2012, Applicability of DNA analysis on adhesive tape in forensic casework. J Forensic Sci 57, 1036-1041.
Zhu, B., Furuki, T., Okuda, T., Sakurai, M., 2007, Natural DNA mixed with trehalose persists in B-form double-stranding even in the dry state. J Phys Chem B 111, 5542-5544.
Zoppis, S., Muciaccia, B., D'Alessio, A., Ziparo, E., Vecchiotti, C., Filippini, A., 2014, DNA fingerprinting secondary transfer from different skin areas: Morphological and genetic studies. Forensic Sci Int Genet 11, 137-143.

10. Biography of author

Lana Ostojic was born on February 22, 1981. in Vukovar, Croatia. Upon graduating from gymnasium "Jovan Jovanovic Zmaj" Novi Sad, Serbia, Lana entered the University of Belgrade, Faculty of Biology, and attended curriculum in Molecular biology and physiology. In 2006 Lana got accepted at the Università Degli Studi di Milano, Italy, to complete her diploma thesis project with Professor Marco Muzzi-Falconi. She defended her diploma thesis: "Characterizations of the role of Exo1 in the rapid checkpoint activation after UV radiation in budding yeast cells blocked in G2" at the University of Belgrade, Faculty of Biology, and was awarded a maximum grade of 10. She graduated as a molecular biologist and physiologist, in the field of academic expertise of Genetic engineering and biotechnology, with the average grade of 9.23 of maximum 10. In 2007, Lana moved to New York City, where she was hired as a Criminalist level II in the Forensic Biology department at the Office of Chief Medical Examiner. In 2011, she received an NIJ grant award as a co-principal investigator and the same year entered the Ph.D. program at the University of Belgrade, Faculty of Biology. The grant was successfully finalized in 2016, and generated few publications and public presentations at the biggest forensic science conferences in the United States, an AAFS and NEAFS. Lana is currently working and living in the New York City. She is promoted to the Criminalist level IV, where she supervises, and mentors lower level Criminalists, oversees DNA testing process, and testifies in the court of law.

10. Biografija autora

Lana Ostojić je rođena 22. februara 1981 godine u Vukovaru, Republika Hrvatska. Po završetku gimnazije "Jovan Jovanović Zmaj" u Novom Sadu, Republika Srbija, upisuje Biološki fakultet Univerziteta u Beogradu, smer Molekularna biologija i fiziologija. Godine 2006. primljena je na Università Degli Studi di Milano, u Milanu, Republika Italija, gde je uradila eksperimentalni deo diplomskog rada u klasi profesora Marco Muzzi-Falconi. Diplomski rad pod nazivom: "Characterizations of the role of Exo1 in the rapid checkpoint activation after UV radiation in budding yeast cells blocked in G2" je odbranjen na Biološkom fakultetu u Beogradu sa ocenom 10. Lana je diplomirala u zvanju molekularni biolog i fiziolog, na usmerenju Genetičkog inženjerstva i biotehnologije, sa prosečnom ocenom 9.23. Godine 2007. počela je da radi u Njujorku, Sjedinjenje američke države, u New York City Office of Chief Medical Examiner, u departmanu za forenzičku biologiju, gde i danas radi u zvanju Criminalist level IV. Supervizor je za procedure i rezultate DNK testiranja, uključena je u organizaciju rada laboratorije i veštači na sudu.

Godine 2011. kao ko-aplikant je dobila grant američkog Nacionalnog instituta za pravdu, i iste godine je upisala doktorske studije na Biološkom fakultetu Univerziteta u Beogradu. Dobijeni grant je uspešno završen 2016. godine i u okviru njega je produkovano nekoliko naučnih publikacija, koje su predstavljene u načnim časopisima i na najvećim skupovima iz oblasti forenzičkih nauka u Sjedinjenim američkim državama, AAFS i NEAFS.

Прилог 1.

Изјава о ауторству

Име и презиме аутора Лана Остојић

Број индекса М3004/2011

Изјављујем

да је докторска дисертација под насловом

Квалитативна и квантитавна анализа хуманих биолошких трагова минималних граничних количина у форензичким анализама ДНК

- резултат сопственог истраживачког рада;
- да дисертација у целини ни у деловима није била предложена за стицање друге дипломе према студијским програмима других високошколских установа;
- да су резултати коректно наведени и
- да нисам кршио/ла ауторска права и користио/ла интелектуалну својину других лица.

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У Београду, _____

_____

Прилог 2.

Изјава о истоветности штампане и електронске верзије докторског рада

Име и презиме аутора Лана Остојић

Број индекса М3004/2011

Студијски програм Молекуларна биологија

Наслов рада Квалитативна и квантитавна анализа хуманих биолошких трагова минималних граничних количина у форензичким анализама ДНК

Ментор др Душан Кецкаревић

Изјављујем да је штампана верзија мог докторског рада истоветна електронској верзији коју сам предао/ла ради похрањена у **Дигиталном репозиторијуму Универзитета у Београду.**

Дозвољавам да се објаве моји лични подаци везани за добијање академског назива доктора наука, као што су име и презиме, година и место рођења и датум одбране рада.

Ови лични подаци могу се објавити на мрежним страницама дигиталне библиотеке, у електронском каталогу и у публикацијама Универзитета у Београду.

Потпис докторанта

У Београду, _____

_____

Прилог 3.

Изјава о коришћењу

Овлашћујем Универзитетску библиотеку „Светозар Марковић“ да у Дигитални репозиторијум Универзитета у Београду унесе моју докторску дисертацију под насловом:

Квалитативна и квантитавна анализа хуманих биолошких трагова минималних граничних количина у форензичким анализама ДНК

која је моје ауторско дело.

Дисертацију са свим прилозима предао/ла сам у електронском формату погодном за трајно архивирање.

Моју докторску дисертацију похрањену у Дигиталном репозиторијуму Универзитета у Београду и доступну у отвореном приступу могу да користе сви који поштују одредбе садржане у одабраном типу лиценце Креативне заједнице (Creative Commons) за коју сам се одлучио/ла.

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У Београду, _____

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