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BOSTON UNIVERSITY

SCHOOL OF MEDICINE

Thesis

OPTIMIZING THE ISOLATION AND ANALYSIS OF EXOGENOUS TRACE DNA FROM FINGERNAIL EVIDENCE

by

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B.S., Drake University, 2018

Submitted in partial fulfillment of the

requirements for the degree of

Master of Science

2020

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ACKNOWLEDGMENTS

I would like to thank Dr. Robin Cotton for her support, enthusiasm, and contributions throughout the entirety of this process. I would also like to thank my thesis committee, Dr. Sandra Haddad and Ms. Caitlin Izzo for their time and feedback. Additionally, I would like to thank the faculty and staff of the Boston University Biomedical Forensic Sciences program for their guidance and support over the last two years.

To my parents and sisters, I am forever grateful for your endless support, encouragement, and love. I am also thankful for the support from and friendships made through my time in the BMFS program.

OPTIMIZING THE ISOLATION AND ANALYSIS OF EXOGENOUS TRACE DNA FROM FINGERNAIL EVIDENCE

MARY CORRINE NAGLE

ABSTRACT

Fingernail evidence is often collected in criminal cases of violent and/or sexual assault. In acts of aggression and self-defense, foreign deoxyribonucleic acid (DNA) can be transferred from the perpetrator to beneath the surface of the fingernail of the victim. It is possible to recover this foreign, exogenous DNA from the victim's fingernails and potentially identify the perpetrator via DNA analysis. When attempting to recover this DNA from fingernail clippings, a couple of problems can occur. Often times, not enough exogenous DNA gets trapped underneath fingernails, so there is not usually much DNA to work with for recovery. The other major problem is the presence of endogenous DNA from the fingernail donor. Not only is there donor DNA in the fingernail itself, but the donor's own DNA can build up underneath their nails simply by rubbing their face or combing their fingers through their hair. This means that there can be more donor DNA present that can mask the presence of the foreign DNA and cloud the results.

In an attempt to improve the recovery of foreign DNA and produce a reportable, informative profile, a time course study was developed. Typically, when using *forensic*GEM as an extraction method, the samples would incubate for 15 minutes before going through protease inactivation. For this study, the extraction period was broken up into four 5-minute periods of incubating, for a total of 20 minutes, before inactivating the protease. This was done to pinpoint the time period at which more foreign DNA is being

extracted from the surface of the nail before endogenous DNA is extracted in excess and clouds or even hides the presence of foreign DNA altogether. Female fingernail clippings were spiked with neat male saliva to observe the ratio of male to female DNA during quantitation and on the electropherograms.

The quantitation results depicted a strong presence of male DNA through the entirety of the time course, and female DNA did not appear to be extracted in greater levels until the 15 minutes of incubation. The resulting profiles exhibited the male saliva profile as the major contributor for most of the samples, especially at the 5- and 10-minute markers. In 50% of the profiles, a minor female contributor could be identified as the nail donor. One sample produced a single, male profile for each time point with no indication of a female donor present in the extract; another sample produced a profile with a male major contributor with only 3 to 6 loci having additional detectable alleles of a minor contributor at each time point. These alleles could not be conclusively attributed to the female nail donor, but she could not be excluded.

These preliminary results indicate that a shortening of the *forensic*GEM extraction period could be beneficial for improving the recovery ratio of exogenous to endogenous DNA from fingernail evidence.

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LIST OF ABBREVIATIONS

AT	Analytical threshold
CA	California
CFS	Centre of Forensic Sciences
DI	Deionized
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
E-cell	Epithelial cell
HL	High level
Hi-Di	Highly deionized
µL	Microliter
mg	Milligram
mL	Milliliter
mM	Millimolar
mtDNA	Mitochondrial DNA
ng	Nanogram
NY	New York
nuDNA	Nuclear DNA
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
proK	Proteinase kinase
qPCR	Quantitative PCR

RFU	Relative fluorescence units
STR	Short tandem repeat
TE	Tris-EDTA
WA	Washington

1. INTRODUCTION

1.1 Fingernails in a Forensic Context

Fingernail evidence often comes across a deoxyribonucleic acid (DNA) analyst's workbench in a forensic laboratory. Fingernail clippings are collected as evidence in assault and homicide cases so that a DNA analyst can attempt to identify the profile of a perpetrator. DNA that comes from fingernail evidence can be endogenous DNA, exogenous DNA, or a mix of the two.

Endogenous DNA can be found in the nail itself due to a process called keratinization. The formation and hardening of the nail plate leaves cellular DNA fragments in the keratin structure of a nail (1,2). However, endogenous DNA can also be non-nail DNA that builds up under the nail in the hyponychium. An area that is a space as much as it is a surface, the hyponychium is the region between the end of the fingertip and the edge of the fingernail. This can then turn into a catch-all for microbes, dirt, and in the forensic sense, evidence (2).

The foreign DNA detected in fingernail evidence is exogenous DNA. This type of DNA typically makes its way into the hyponychium through physical contact with another person by scratching them or through contact with that person's skin, blood, saliva, or semen (3). In a forensic setting, exogenous DNA may be found in combination with endogenous DNA in cases where acts of aggression, as well as self-defense, are reported or suspected. During these physical altercations, the perpetrator's DNA can end up in the victim's hyponychium (3,4). According to several studies, there is roughly between 6% and 24% chance of finding exogenous DNA profiles beneath fingernails depending on the

population (5–8). DNA analysis of nail clippings and the associated debris is a useful approach as it can become an evidential link of there being contact between the victim and the suspect; this can be very helpful during a criminal investigation because the analysis could provide a possible source of the assailant's DNA if there is no other viable source of DNA present in the case (4,5).

In an unusual case report reviewed by Song et al., however, a suspect was found with the victim's DNA under his fingernails, which eventually led to his conviction (9). During a death investigation in China, forensic scientists inspected the corpse of a young 20-year-old woman and deduced her death was caused by mechanical asphyxia by strangulation. Some of the evidence that led to this conclusion was the discovery of abrasions and contusions with several crescent-shaped finger marks. After a thorough, yet rapid, investigation, the former boyfriend to the deceased was included as the likely suspect. With the evidence of the fingernail markings around the victim's neck, fingernail clippings were collected from the deceased, and two days after the incident, the former boyfriend's fingernails were collected as well. Unfortunately, no traces of the suspect were found on the woman's fingernails. The former boyfriend, though, was found with the victim's DNA under his fingernails; this strong evidence led to his confession and conviction. After this case review, Song et al. recommend the recovery of fingernail clippings in cases of violent crimes, even when there is a time lapse between offense and examination (9).

DNA analysis of fingernail evidence is not always used to identify a suspect from the victim's nails. Fingernails can be used as an alternate source to blood, muscle, bone, or

teeth for DNA identification of unknown or decomposed bodies. A 2008 study done by Allouche et al. evaluated the use of nails as an alternative genetic identification source for decomposed cadavers (10). They found that regardless of lysis time, post-mortem interval, and the environment the body was found in, full DNA profiles were still able to be obtained. Despite fingernails providing a comparable amount of DNA to post-mortem blood or muscle (11), being easy to collect and store at room temperature, and being extracted relatively quickly, they are rarely used for everyday or urgent genetic identifications. The use of fingernails as a source of genetic identification will not likely replace blood if it is collected within three weeks post-mortem for a DNA analysis. After about two months, muscle can no longer be used as a source of genetic identification so sampling of bone and teeth would typically be done. But the process of doing so is invasive, time consuming, and results in less DNA. However, nails are more advantageous and could soon replace muscle, bone, and teeth as DNA sources since results can be obtained much easier, faster, and produce a higher quantity of DNA (10).

1.2 Fingernail Anatomy

To understand how to get DNA from a fingernail for forensic purposes, one must first understand the anatomy of the fingernail unit. The nail itself can be divided into five substructures; for a human specifically, those are the nail matrix or root, nail bed, nail plate, eponychium or cuticle, and the hyponychium (Figure 1). The nail matrix grows into and permanently becomes the nail plate. The standard appearance and growth of this product is dependent on the integrity of the surrounding tissues and the phalangeal bone, which contribute to the nail unit (2,12). The growth of the nail starts in the root with germinal

matrix cells that differentiate into the formation of the nail plate. In the transition from the root to the nail plate, the epithelial cells are fully keratinized and compacted. These cells form the smooth upper surface of the nail plate that linearly align microfilaments in the same direction as the growth pattern. The microfilaments, or longitudinal ridges, can vary in number and change with age (1,2). These ridges are specific enough to be used for forensic identification and the distinction between twins. In 1990, Diaz et al. conducted a monozygotic twin study to determine if the ridge patterns that appear on fingernails could be used for forensic identification. With the use of microscopic examination, it was determined that ridge patterns on fingernails are readily distinguishable from one another, making them unique and can contribute to personal identification (13).

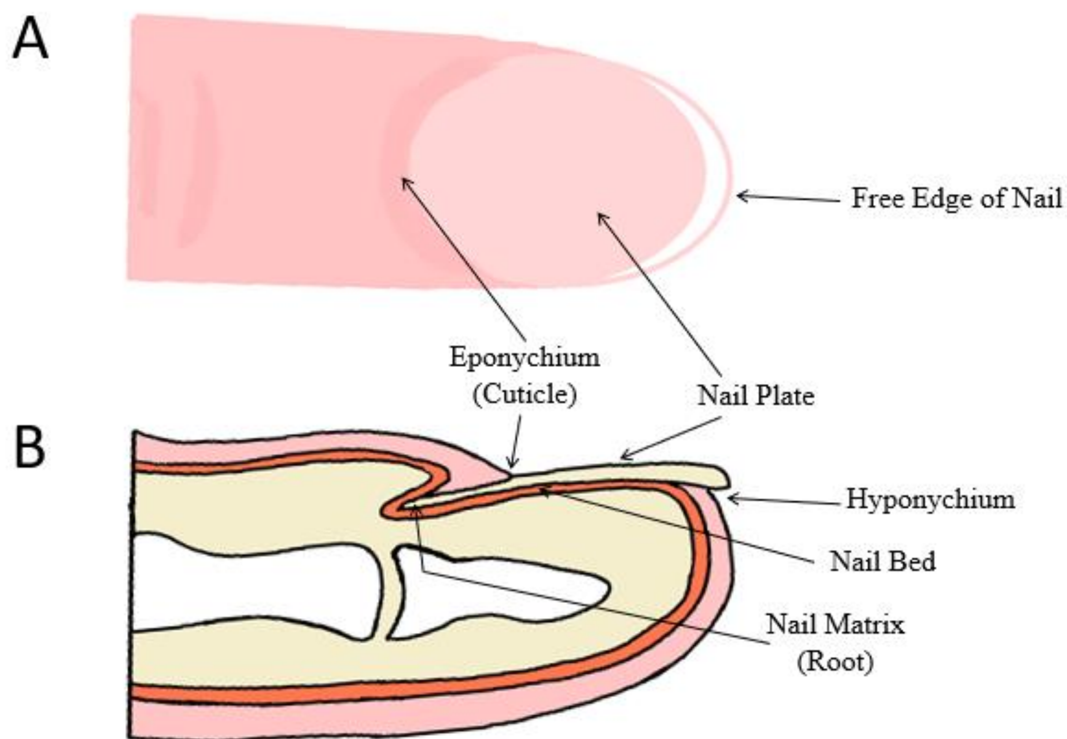

Figure 1. Anatomical Structure of Nail. (A) Plane view of Fingernail. (B) Sagittal section of fingertip. Adapted from Saito et al. (12).

1.2.1 Keratinization: The Protein and the Process

As the keratinization process occurs, the epithelial cells that make up the nail plate undergo cell death, and with that, DNA degradation can be expected (1). Keratin is the intracellular protein mesh that the other elements of the nail plate fit into. This highly ordered structure of proteins explains the high degree of chemical and physical resistance that is not characteristic of the skin. The keratin proteins in hair and nails are held together by a strong, extensive folding made up of a disulfide-bonded molecular structure. The disulfide bonds come from the amino acid cysteine, which is found in hair, skin, and nails. The geometry of these disulfide bonds slightly differs in structure from what is found in the skin, making them sturdier (2). Nails have a sturdier structure because they have more

cysteine molecules than skin or hair. The abundance of this amino acid leads to greater inter-chain disulfide bonding and thus a stronger and harder structure (14). This bond formation coupled with the protein molecule folding results in a lesser degree of hydration than is seen in the skin (2), and it creates a hydrophobic nature in the proteins which in return slows down microbial degradation (1).

A variety of complicated factors affect the quality and quantity of DNA, and these factors are related to the biological purpose and formation process of the source of DNA. Tissues such as blood and muscle produce high levels of DNA while other tissues, like bone, nail, and hair, do not because of the manner in which these tissues are formed. There are some tissues that are specifically derived from living progenitor cells, similar to stem cells, that undergo cell death; these tissues are called keratinous tissues and include hair, nail, horns, feathers and scales. Since cell death is a part of the formation process of these keratinous tissues, it leads to low level and heavily fragmented DNA. There is variation in the amount of DNA that can be recovered from a nail based on the location the DNA comes from. The root of the nail contains high quality DNA but other non-root components, like the nail plate and hyponychium that are usually collected in a forensic setting, tend to have poor quality DNA results in comparison. That's not to say polymerase chain reaction (PCR) amplifiable DNA cannot be obtained, though (1).

Almost all sources of nail DNA that are not hundreds of years old and does not originate from the root of the nail can still be amplified (1,15). Numerous studies have shown that under the appropriate conditions, mitochondrial DNA (mtDNA) can survive up to decades, even centuries, within the unit of a hair or nail (16–19). However, forensic

laboratories typically use nuclear DNA (nuDNA) for genetic identification. While nuDNA is harder to obtain from dated sources, there have been a few studies done that confirmed short fragments of nuDNA can occasionally be recovered from hair. Short fragments of nuDNA have yet to be successfully recovered from dated sources of nail but mtDNA has been able to be recovered (17). The key reason DNA in keratinous tissues is able to be found even after hundreds of years is because of the robustness of the hard surface.

Other factors that affect the hair and nail structure can vary with age, time of year, and nutritional habits. Nail growth does require a sufficient amount of energy, so it makes sense that growth, keratinocyte germination, and cell keratinization vary with one's food intake. However, there is still much to be learned about nails as a source of DNA, specifically since the literature focuses mostly on hair and simply lumps other keratinous tissues into the same category with reasonable assumptions of similarities to the hair (1).

1.3 Prevalence of Exogenous DNA

The evidential value of the presence of foreign DNA beneath fingernails as it relates to a violent crime and/or sexual assault relies to some extent on the knowledge of background levels of foreign DNA observed beneath fingernails in the general population. Many studies have sought out to determine just how often foreign DNA will be found under the fingernails of the general public. A study done in 2007 by Cook and Dixon (8) set out to gather data that would help determine if foreign DNA could accumulate and remain in the hyponychium during an assault. This study gathered 200 fingernail swabs from 100 volunteers. Almost 70% of the samples resulted in a full or partial single donor profile, and only 13% of the samples were found with mixed DNA profiles. However, only 6% of the

samples containing more than one contributor were considered to actually be reportable for a database search with a mixture ratio less than 20:1. This demonstrates that a low level of foreign DNA is found under the fingernails of the general population. If the background level of the general population is significantly exceeded by the amount of exogenous DNA recovered in instances of violent or sexual crimes, it is more likely that intimate contact explains this observation as opposed to the DNA existing from previous contact between the suspect and victim. This assumes that the two individuals are not in close proximity due to cohabitation, work, or other situations. In this case, relatively high values of foreign DNA will therefore have greater evidential value in court. However, it is important to remember that previous consensual activities could be responsible for the mixed DNA results. The probability of finding a reportable mixed DNA profile decreases with the more time that passes following the assault because foreign DNA only persists under the fingernail for a limited amount of time before being dislodged or purged by daily activities. The persistence of foreign DNA under the nail is dependent on a range of factors and habits of the sample donor, so it is very likely to see a great variation between different individuals (8). For example, subconscious activity, like rubbing the face, scalp, eyes, or mouth, can significantly contribute to the donor's concentration of endogenous cells under their nails (20), changing the ratio of endogenous to exogenous DNA present and decreasing the chances of a foreign profile being found. Thus, a reportable mixed DNA profile is more likely to be found when the sample gets collected in the first 24 hours after the offense. The conclusion of this study by Cook and Dixon (8) is that only 6% of the general population will exhibit a mixed DNA profile when sampling fingernails.

A question that arose from the results found by Cook and Dixon (8) was: does cohabitation provide a greater opportunity for DNA transfer? So, in 2009, Malsom et al. (21) conducted a small study on 12 cohabitating couples to address this question. Each couple collected a set of swabs three times every five days, using one wet swab per hand. At each sampling, they filled out a questionnaire that outlined the activities done throughout the day. This study found that of 144 samples, 61% produced full or partial single donor profiles, 2% did not produce any DNA profile, and the remaining 37% of samples produced mixed DNA profiles. Of the samples that exhibited DNA from more than one donor, only 17% of the total samples were classed as reportable 2- or 3- person mixtures, and all but one of those samples had foreign DNA that matched those of the donors' partners. Of the possible variables noted in the questionnaire, only two were found to be statistically significant: time spent together and nail biting. Significantly higher incidences of mixed DNA profiles were produced among the couples that spent longer amounts of time together in the evening. Nail biting had the inverse effect on mixed DNA profiles. This sample set was small, so nail biting may not have as great of significance since only two of the donors had this habit. This study also sought to improve the results from female donors by using Yfiler testing to isolate the presence of a male DNA profile. A total of 54 female fingernail samples were processed, and without Yfiler, 87% of samples did not exhibit the presence of male DNA. Using Yfiler, 63% of the samples displayed a single source or mixed male DNA profiles with 1 to 13 alleles. Only one sample had a full profile of 17 alleles. The takeaway results of this study are that 17% of cohabiting couples exhibited foreign DNA from their partner under their fingernails in addition to their own,

and Yfiler is a useful tool for improving the observation of male DNA on female donor samples (21).

In 2011, Flanagan and McAlister (22) conducted a follow-up study to the two previously stated studies to answer two main questions: 1) Does female DNA always transfer after digital penetration of the vagina, and 2) in this scenario, what is the time period during which the foreign DNA will persist under a male's fingernails at detectable and informative levels. The first part of the study included eight volunteer couples that were sent home with a kit and instructions for fingernail swab collection. Samples from the males' right-hand fingernails were collected shortly after vaginal penetration. DNA quantitation results of these swabs found that there was a great deal of DNA transfer from the female vagina to under male fingernails, ranging from 7 to 18 nanograms (ng) per microliter (μL; also seen as ng/μL) of DNA for each couple. Full female profiles without any evidence of the donor male's profile were produced for 15 of the 16 samples collected for the transfer study. The one other sample produced a full female profile with only three male alleles at low levels. So, it is clear that female DNA is transferred after digital penetration of the vagina. For the persistence study, there were four additional volunteer couples who were also given collection kits, questionnaires, and instructions. This time however, the couples were given a different time interval (6, 12, or 18 hours) for collection of the fingernail swabs after the digital penetration. Between 0 and 6 hours, full female profiles were obtained from all swabs collected. This means that in the short term, female DNA always transfers and persists. In three-fourths of the samples taken at 12 hours, full female or informative male/female profiles were again obtained; similarly, at 18 hours,

informative male/female profiles were produced for the majority of the samples, just at a lower level. Based on these values, it could be expected that the quality of female profiles would continue to decline with each 6-hour lapse. A review of the questionnaire responses from the persistence study found that handwashing had a significant effect on the persistence of female DNA under male fingernails. In broader terms, water immersion, including hand and dish washing, had significant effects on the distribution of mixtures at both 12 and 18 hours. The final results of this study are that female DNA is transferred to underneath fingernails after digital penetration and will persist and can produce full DNA profiles for at least 6 hours and informative profiles can be produced up to 18 hours, even with hand or dish washing (22).

The first study conducted to determine if there is a correlation between the type of physical contact and the ability to obtain a foreign DNA profile was completed by Dowlman et al. in 2010 (23). Their results provide a clear link between intimate contact and the likelihood of obtaining high level (HL) mixed DNA profiles. For the purpose of this paper, a high-level mixed DNA profile is considered to be a 4 to 1 ratio of male to female. They analyzed fingernail swabs from each hand of 40 volunteers to compare the DNA profiles to the activities the individuals partook in 48 hours prior to sample collection. The donors completed a survey concerning their recent activities and forms of contact with other people in those 48 hours. Of the 80 samples collected, the majority, 59%, did not produce mixed profiles. The rest of the samples did result in mixture profiles, but only 9% of those had high levels of the non-donor DNA in the mixture; the remainder of the profiles were low-level mixtures. A statistical analysis was performed on the results from one hand

of each volunteer. It found that the only factor to significantly affect the amount of foreign DNA in the mixed profile was whether contact was intimate or not. The odds of a HL mixture being obtained from intimate contact was 14 times that of no intimate contact taking place. The majority of the donors washed their hands up to 7 times a day, some washing more than 13 times; this can play a factor in level of mixture, but high- and low-level mixtures were still able to be obtained from those who washed their hands as frequently as 13 times a day. Somewhat surprisingly, neither nail length nor frequency of nail cutting or biting seemed to have an effect on whether mixed or donor DNA profiles were obtained. This study (23) did result in a much higher percent of mixed DNA profiles being obtained overall (41%) in comparison to Cook and Dixon's (8) results of 13%. This could be due to the sampling method of each study. This study used a wet swab, then a dry mini-pointed swab (23), but Cook and Dixon (8) used only one wet swab. However, the sampling method may not be a factor at all. A new study conducted earlier this year (2020) by Hedman et al. found that the double-swabbing technique does not result in a substantial increase of DNA, and one wet swab is sufficient enough for DNA recovery (24). A study that looked into the recovery of saliva from skin found that swabbing first with a wet swab followed by a dry swab resulted in improved recovery of DNA when compared to a singular swab method (25). This is, therefore, one possible reason for a higher proportion of mixture profiles found by Dowlman et al. (23). The absence of a HL profile does not necessarily mean intimate contact did not occur. Mixed profiles of any sort were not produced from half of the samples taken where the individuals reported having intimate contact. This study did not utilize Yfiler for analyzing potentially mixed samples, so it is

possible that the profiles reported as single donor profiles could have had a low, undetectable male donor in the mixture that could have been picked up by using Yfiler. The type of intimate contact that takes place could have a role in the resulting DNA analysis. It is reasonable to expect that samples taken from individuals that participated in digital insertion of the oral, vaginal, or anal orifices would be more likely to pick up more cellular material than those who had only touched other parts of the body. The extent of this type of information wasn't collected in this study. Further studies would need to take place to account for these actions as a potential explanation for foreign DNA build-up underneath the fingernail (23). The final results of the study are consistent with previous studies that concluded foreign DNA under fingernails of the general population is low (8,21,23). It is also clear that observing HL DNA mixtures under fingernails is a relatively rare occurrence and it is not correlated with casual, everyday activities (23).

In 2012, Matte et al. (7) completed a series of studies and case reviews. Their data also shows that there is value in processing fingernail evidence when the case history suggests the possibility of close physical, intimate contact. The first of their studies was a review of casework in which 265 samples from 137 cases were obtained where contact between the perpetrator and victim was reported as a possibility. Of these samples, 57% of them contained only donor DNA, and 10% resulted in insufficient or no DNA at all to proceed to amplification. The remaining 33% contained a measurable amount of foreign DNA of varying quality. However, the authors made no determination of the suitability of the profiles that were detected. While it is unclear as to if and how many cases the findings

of foreign DNA profiles led to a suspect and/or conviction, the data still shows the prevalence and persistence of foreign DNA underneath fingernails (7).

After the casework review, Matte et al. (7) conducted three collective studies using Centre of Forensic Sciences (CFS) employees and university students in Toronto, Ontario, Canada to represent the general population with a total of 178 samples. The first of the three studies utilized the CFS employees. The authors found that extracting directly from a pooling of cuttings of the wooden scrapers used for collection from one hand resulted in a higher, more consistent yield, 86%, of samples with a sufficient amount of DNA to proceed to amplification, whereas swabbing of the scrapers only yielded 36%. The second part of the series gathered 50 samples from cohabitating university students to see if foreign DNA would appear under normal circumstances of simply living with roommates. Seven of the samples contained foreign DNA with partial profiles. None of the profiles had sufficient data to be attributed to a household member. This data suggests that it is unlikely to have foreign DNA transfer to underneath one's fingernails from casual contact when sharing a household environment and the common items within it. This also supports the inference that for fingernails to retain foreign DNA, intimate, close physical contact with someone or presence of a foreign body fluid is required. The third part of the study series was conducted on 50 random students, totaling 100 samples. A sufficient amount of DNA for profiling was found in 87 of the samples, and 23 of those contained detectable foreign DNA. This detectable foreign DNA ranged from only one locus to a full profile, but the majority, 14, of the 23 samples with foreign DNA profiles contained 4 or less loci. This

means that only 9% of this population sample had mixed DNA profiles from nails with reportable and informative data on the foreign contributor.

Lastly, Matte et al. (7) conducted a scratch study with 3 segments and a total of 30 samples. Data from all three data sets showed that, despite a thorough cleaning of the control samples, 4 out of the 30 scrapings still had detectable foreign DNA. From the 30 samples collected immediately after scratching, 11 contained a foreign source of DNA. However, 3 of the 11 came from the subset of less vigorous scratching and contained only 1 or 2 loci. Two of the three could not exclude the scratching partner, but the third could be excluded and attributed to the participant's husband. The husband's DNA remained under the participant's fingernails even after scratching someone else. The remaining eight samples with foreign DNA came from the subsets of data where the participants scratched more vigorously. These profiles had better quality and between three and nine loci were able to be detected. Six of these samples contained five or more loci that could not be excluded as coming from the scratching partner. Six hours later, only two of the 30 samples had detectable foreign DNA, both of which came from the more vigorously scratched group. One produced six loci of detectable foreign DNA, and this sample had also been detected immediately after scratching. The other only had one locus detected, but was not previously detected in the immediately after scratching subset. Overall, the entirety of the Matte et al. (7) studies demonstrate there is value in processing fingernail evidence in the cases that report the possibility of close contact between the victim and perpetrator. It also shows that the more recent the physical contact takes place, the more likely it is for foreign

DNA to be detectable underneath fingernails (7). The data reported in these studies also support the work done in previously described studies (8,21,23).

1.3.1 Value of Fingernail Evidence in Criminal Case Reviews

There have been many case reviews of violent or sexual assaults in which acts of aggression or self-defense were suspected or reported, leading to the collection of fingernail clippings, scrapings, and swabs. In 2003, Piccinini et al. (6) conducted a five-year study in which a total of 179 autopsies from homicide cases were conducted at the Institute of Legal Medicine in Milan between 1996 and 2000. Of those, 66 cases had fingernail clippings that were stored. A court order for their examination was made in 31 of the cases. No foreign profiles were detected in 17 of the 31 cases and 3 more were found to be inconclusive. Eleven samples produced detectable levels of foreign DNA, and seven of those matched the suspect. The remaining four cases did not have a suspect at the time for comparison but still produced a reportable foreign profile. The routine collection of fingernail evidence should be performed during the investigation of violent crimes because it can provide great value to the case and the reconstruction of the events of the crime (6). DNA, foreign or endogenous, from fingernail evidence is important for different reasons in various cases. It can be used to confirm other DNA evidence that was present and for comparison to a known suspect or searched in a database if no other DNA evidence was developed in the case.

Another example of the importance of fingernail evidence collection comes from a case where nail debris was recovered from the suspect two days after the sexual assault of a young woman. The suspect denied the assault, claimed he had washed his hands

numerous times since the reported incident, and said he had never been in contact with the woman. A DNA analysis of his fingernail debris produced alleles specific to the victim at all loci. This, combined with his contradicting statements, led to a conviction. Foreign DNA trapped in the hyponychium has shown to be persistent, and this case provides an additional example of how important the analysis of fingernail evidence can be despite a time lapse or claims of multiple hand washings between the offense and exam (26).

1.4 Collection Methods for Fingernail Evidence

There are several approaches used when collecting and analyzing fingernail evidence for the purpose of recovering exogenous DNA. These approaches were compared in a study done by Hebda et al. in 2014 (27). The three methods are swabbing under the nail, scraping under the nail, or clipping the nail for direct extraction. After collection, the exogenous DNA is isolated by organic extraction or through the use of a commercial DNA extraction kit, and the results can be analyzed using autosomal or Y-chromosome STRs. As summarized in the previous paragraphs, general population or mock evidence samples do not always produce mixed profiles. When it does, the low percentage of samples that have foreign alleles present is low. As a result, there have not been many attempts to optimize the DNA retrieval and analysis methods until the Hebda et al. (27) study in 2014 at Michigan State University's forensic biology laboratory. The fingernail clippings were provided by female volunteers, and the exogenous DNA was from a single male donor. These authors compared soaking, swabbing, and scraping methods, which produced varying results. The soaking method produced a significantly greater yield (96%) of exogenous DNA than swabbing (61%) and scraping (31%). These same samples

underwent autosomal STR analysis. The soaked and swabbed samples both generally contained all of the exogenous alleles, but they also contained endogenous DNA from the nail. The majority of the soaked samples produced a major or roughly equal profile of nail DNA to exogenous DNA. Oppositely, the swabbed samples produced major profiles of the exogenous DNA donor. The scraped samples also mostly produced exogenous DNA profiles, but there was significantly more dropout seen in both scraped and swabbed samples as well. All of the swabbed samples produced complete, single source Y-STR profiles with the scraped samples having some allelic dropout. Even though autosomal STR analysis produced strong profiles of the nail donor's DNA for the soaked samples, exogenous DNA was often seen to be present but was substantially weaker and, in some cases, not detectable. However, Y-STR analysis of those same samples did produce several full profiles. In 69% of the Y-STR loci, there were detectable alleles present that were consistent with the known exogenous DNA profile. This study found that the ideal method for collecting fingernail evidence in order to maximize exogenous DNA recovery is to obtain clippings. However, some living victims may not want their fingernails clipped, so the double swabbing method is the next best alternative (27).

Some of the common sampling methods used for the removal of foreign DNA on fingernail evidence are swabbing the underside of the fingernail clippings or soaking the fingernail in various buffers like a phosphate-buffered saline (PBS) solution, digestion buffer/proteinase kinase (proK) solution (27), or strong denaturants (15). However, the digestion buffer/proK and strong denaturant methods are not always ideal for extracting foreign DNA because they can release more endogenous DNA from the fingernails along

with the exogenous DNA. This can then increase the chance of obtaining a DNA mixture where the foreign DNA is overwhelmed by the endogenous DNA because there is no way to limit the amount of nail donor DNA being extracted. So in 2019, Hayden and Wallin (28) developed a study to compare three sampling methods in order to determine which is best for minimizing the amount of endogenous DNA removed and maximize the recovery of foreign DNA. The three methods they compared were swabbing, PBS soak, and PrepFiler® lysis buffer soak. Sweat from one male donor was pipetted onto the underside of fingernail clippings from one female donor. During quantitation, the highest average amount of male DNA came from the swab samples, closely followed by the lysis buffer soak samples, and lastly the PBS soak samples. As expected, the swab samples had the highest ratio of male to female DNA, but the lysis buffer soak samples had the lowest ratio of male to female DNA and PBS soak samples were in between the two. It was then determined that the low ratio of male to female DNA for the lysis buffer was caused by DNA degradation. Degradation only took place in the lysis buffer soak samples on the female DNA, but the mechanism remains unknown as to why. Hayden and Wallin (28) thus concluded that the best method of sampling for the recovery of exogenous DNA from fingernails while also minimizing the amount of endogenous DNA is swabbing (28).

1.5 Extraction Methods

There are numerous commercial kits that are used for DNA extraction in forensic laboratories; Chelex and QIAGEN are commonly used, and a variety of these have been applied to fingernails. As examples, the following methods were incorporated in the recent papers referenced above in the section discussing sampling procedures from fingernail evidence. These extraction procedures include an organic extraction by using the phenol-chloroform method and the QIAGEN method (27) and PrepFiler® (28). Some of these methods have also been automated through the use of a robot. In 2012, Phillips et al. (29) conducted a study to compare Chelex, column-based QIAGEN, and QIAcube—a QIAGEN automated robot using the same kit—as DNA extraction methods for how well they performed when using blood and buccal samples. Chelex is often a favored method because it is quick and does not require many tube transfers or use toxic solvents; however, this method does not allow for the removal of inhibitors, which can cause problems later on in the analysis process. QIAGEN is also a commonly used method of extraction because it is robust and is able to remove inhibitors from the sample all while maintaining a HL yield of quality DNA. The results from this study by Phillips et al. found no statistical difference between the results of the three methods, but as predicted, the automated QIAcube robot made processing of the samples easier and faster without introducing the possibility of DNA contamination (29).

In the previously discussed study by Hebda et al. (27), the quantitation results of the commercial QIAGEN kit, QIAamp® DNA Investigator Kit, showed that a single elution recovered a significantly greater amount of exogenous DNA than an organic

extraction did. Furthermore, DNA could also be successfully recovered from up to four 20 μL elutions, so if low amounts of DNA were recovered from the first elution, up to three more could be done to obtain greater levels of DNA (27).

A study done by Hanley (30) in 2018 compared three extraction methods to test how well each method recovered DNA and reduced loss. The three methods she compared were QIAGEN BioRobot EZ1, ZyGEM/Acrosolv, and the manual QIAGEN column-extraction method. She determined that the ZyGEM/Acrosolv method extracted the most DNA while reducing loss. This method outperformed the other methods across the board for both semen and saliva extractions, although, the EZ1 produced better results with semen while QIAGEN produced high recoveries of DNA with saliva. It was also found that the ZyGEM/Acrosolv method was 8 to 10 times faster to run, easier to use, more cost effective, and had better reproducibility (30).

1.6 Hypothesis

The EA1 protease in *forensic*GEM has fewer cut sites in the main nail protein component, keratin, than the commonly used protease, Proteinase K (31). We hypothesize that *forensic*GEM may be able to release exogenous DNA present on fingernails without rapid concomitant release of endogenous DNA from the nail donor. Methods and results are presented in the following sections.

2. MATERIALS AND METHODS

2.1 Sample Collection

In 2017, fingernail clippings from ten anonymous volunteer females were collected by a previous student in compliance with Boston University School of Medicine Institutional Review Board protocol #H-26187. Three of these donors were randomly selected for use in this present study. The fingernail clippings were stored in a single glassine envelope that was placed inside a manila envelope and labeled with the volunteer's donor number, date of collection, and sex. These samples were stored at room temperature before their use in this study and continued to be stored at room temperature throughout the study. By observation, the fingernail samples chosen appeared to be at least rudimentarily cleaned and lacked the presence of nail polish. Reference buccal swab profiles were also available for use in this study. An anonymous male donor provided a liquid saliva sample that was stored at 4ºC, then later at -30ºC.

2.2 Sample Preparation

Between five and ten fingernail clippings were chosen from each of the three donor samples based on weight. Sterilized tweezers were used to pick up and place each fingernail from the storage envelopes and weigh boats. The tweezers were sterilized with 10% bleach followed by 70% ethanol every time there was contact with a new nail clipping, even if the nails were coming from the same donor. Each fingernail sample was placed in a weigh boat on a Denver Instrument Analytical Semi-Micro Balance (Bohemia, NY) with a target weight of 4 to 10 milligrams (mg). A new weigh boat was used for each of the nails being weighed. There were enough clippings to choose from that if it was not within the desired

window of weight, the nail was placed back in the envelope and a new one was picked out and weighed. The fingernail clippings were individually stored in empty 0.5 mL PCR tubes that were labeled 1 through 25.

2.3 Epithelial Cell Preparation

An epithelial cell (e-cell) preparation was prepared using a protocol adapted from Ziegler (32). Neat saliva (roughly 2 milliliters (mL)) from a male donor was collected in a 2 mL collection tube and vortexed at medium speed to mix thoroughly. The saliva was then split evenly into two collection tubes with approximately 1 mL of saliva in each and briefly vortexed again. The sample was then centrifuged at 800 relative centrifugal fields (rcf) for 3 minutes. If a pellet was readily visible, the supernatant was removed and 2 mL of PBS buffer was added to re-suspend the pellet. The tubes were vortexed and centrifuged at 800 rcf for 3 minutes again. This time, the supernatant was removed and the pellet was re-suspended in approximately 2 mL of 10 millimolar (mM) Tris and 0.1 mM ethylenediaminetetraacetic acid (Tris-EDTA, also known as TE) buffer. The removal of the supernatant, resuspension of the pellet in TE buffer, and centrifugation of the tubes was repeated one more time. The remaining pellets were each re-suspended in 100 microliters (µL) of TE buffer, vortexed, and then recombined. The emptied tube was washed with 50 µL of TE buffer, which was also recombined into the full tube.

Two microliters of the epithelial cell preparation were used for a serial dilution quantitation run to determine how many nanograms of DNA were in a microliter of saliva. This quantitative value was used to choose the volume of saliva that would be pipetted onto the pre-weighed fingernail clippings.

2.4 Exogenous Recovery

2.4.1 Preparation of Saliva-Spiked Nails

Each of the previously mentioned twenty-five nails were spiked with the saliva that underwent e-cell preparation to assess the recovery of exogenous DNA when utilizing modified *forensic*GEM® extraction methods. Four microliters of saliva were aliquoted onto the undersurface of each of the 25 nail samples. The clippings air-dried overnight in individual weigh boats and then were placed back into the empty 0.5 mL PCR tubes until extraction.

Eight aliquots of 4 µL of saliva were prepared for e-cell control samples and dried in PCR tubes. Four samples were obtained from before being aliquoted onto the nails and another four samples were aliquoted after. All of these samples were stored at room temperature until extraction, quantitation, amplification, and capillary electrophoresis were carried out.

2.4.2 Use of *forensic*GEM®

*Forensic*GEM® was chosen as the extraction method because it is a reliable and simple procedure for DNA extraction that produces high yields of DNA for various forensic samples (33). This is because it utilizes a thermally stable enzyme, EA1, that has a relatively broad pH optimum. EA1 has at least 80% DNA recovery when the extraction occurs at a pH between 6 and 7.5. This enzyme is also functional at high temperatures; significant activity loss does not occur until it reaches 95°C (34).

2.4.3 First Modification of *forensic*GEM® Extraction

Two saliva-spiked nails and a control e-cell sample were extracted through a time course experiment adapted from the *forensic*GEM® Saliva protocol (35). A master mix for a 200 µL reaction was made using Buffer Blue. The master mix consisted of 20 µL of 10X Buffer Blue, 2 µL of *forensic*GEM enzyme, and 178 µL of deionized (DI) water to create a final volume of 200 µL per sample. The volume of the DI water and enzyme added were such that the final concentration of the 10X Buffer Blue was 1X. Each saliva-spiked fingernail clipping was placed in individual 0.5 mL PCR tubes and 200 µL of the Buffer Blue *forensic*GEM master mix was added to cover the entire clipping. The samples were mixed by pulse-vortexing for about 10 seconds. The tubes were briefly spun down to remove any liquid from the lid of the tube. Next, the samples were placed in the Veriti® 60-Well Thermal Cycler (Applied Biosystems, Foster City, CA) to incubate for 5 minutes at 75ºC to allow for *forensic*GEM protease activity, and then cooled down to 5ºC. The samples were removed from the thermocycler, and a set of 2 µL duplicate samples of the supernatant were taken from each and stored in individual tubes of an 8-tube strip; the strip tubes for each sample were stored in the refrigerator at 20ºC between runs on the thermocycler. The e-cell fractions were not in duplicate. This process of being run on the thermocycler at 75ºC for 5 minutes, cooling to 5ºC, and a set of 2 µL of supernatant being pulled off and set aside in the strip tubes was repeated three more times for a total of 20 minutes in the thermocycler at 75ºC. By the end, each of the eight tubes in the strip tube was filled with duplicate samples from the four runs in the thermocycler; these were carried straight through to quantitation. The 0.5 mL PCR tubes still containing the fingernail

clippings went back into the thermocycler one last time to incubate at 95ºC for 5 minutes to inactivate the protease. After this, they were removed again and briefly centrifuged. The nail was then discarded with sterilized tweezers, and the DNA samples were stored in the freezer at -30ºC until quantitation.

2.4.4 Second Modification of *forensic*GEM® Extraction

The second set of two saliva-spiked nails were extracted with essentially the same protocol except for a few adjustments. Before pulling off each set of 2 µL of supernatant to store in the strip tubes, the samples were vortexed and briefly spun down in the centrifuge. The other major change was that after the total of 20 minutes in the thermocycler, the process was repeated a fourth time with a final incubation at 75ºC for 20 minutes instead of 5 minutes, prior to the 5 minutes at 95ºC for protease inactivation. Another set of 2 µL fractions were pulled off for this round to be carried to quantitation. The remainder of the process was maintained.

The e-cell control sample for this extraction was run under the normal protocol for *forensic*GEM dried saliva samples with a few changes (35). The dried saliva was initially re-hydrated in 20 µL of Buffer Blue mixed with 178 µL DI water, instead of only DI water, for 15 minutes. Before it was put in the thermocycler, the 2 µL of *forensic*GEM enzyme was added, and the remainder of the protocol was appropriately followed.

2.4.5 Third Modification of *forensic*GEM® Extraction

The third modification of the extraction protocol was done with four saliva-spiked nails and two e-cell control samples. The fingernail samples followed essentially the same

steps that were stated in *2.4.3* except the additional incubation for 20 minutes at 75ºC was eliminated. The other change made was that when 2 µL fractions were being pulled off in duplicate for quantitation, an additional 4 µL was pulled off each time into a separate 8-tube strip tube for STR amplification (not in duplicate). The e-cell control samples were run under the same modifications made to the *forensic*GEM dried saliva sample protocol stated in *2.4.3* (35).

2.5 Quantitation

All samples were quantified with the Quantifiler® Duo DNA Quantification Kit (Applied Biosystems, Foster City, CA) by following the manufacturer's protocol and using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) (36). Using an external, validated calibration curve made by Grgicak et al., the sample concentrations were able to be determined (37). Quantitation of the saliva-spiked nail samples for exogenous DNA recovery was performed in duplicate.

2.6 Amplification

As discussed above, select samples were chosen for amplification. Sample extracts were amplified by following the manufacturer's protocol when using the GlobalFiler™ Express PCR Amplification Kit (Applied Biosystems, Foster City, CA) (38). The target DNA mass was between 0.5 ng and 1.0 ng for all of the samples. No dilutions were necessary. The amplification occurred in a Veriti® 60-Well Thermal Cycler (Applied Biosystems, Foster City, CA) which has the ramp rate set to 9600 emulation mode, starting with a hold for 1 minute at 95ºC, then 94ºC for 10 seconds, and 59ºC for 90 seconds for 30

cycles, followed by a 10-minute time delay file at 60ºC, and lastly a final hold in a soak at 4ºC.

2.7 Capillary Electrophoresis and STR Profile Analysis

According to the manufacturer's protocol, a master mix containing GeneScan™ 600 LIZ™ Size Standard (Applied Biosystems, Carlsbad, CA) and highly deionized (Hi-Di) formamide (Applied Biosystems, Foster City, CA) was prepared (39). Ten microliters of this master mix were dispensed into the appropriate wells of a 96-well MicroAmp reaction plate, and 10 μL of Hi-Di was added to any remaining empty wells of the same column to prevent damage to the capillaries. Next, 1 μL of the necessary allelic ladder was added to the appropriate wells followed by 1 μL of the amplicons in their respective wells. All amplified samples were injected for 5 seconds at 3 kilovolts on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) and Performance-Optimized Polymer 4 was used during capillary electrophoresis. GeneMapper® ID-X v. 1.1.1 software (Applied Biosystems, Foster City, CA) was utilized to analyze the resulting electropherograms. The analytical threshold (AT) was set to 30 relative fluorescence units (RFU) and the stutter filter was turned off for profile analysis. Peak height is based on the RFU, which is determined by the fluorescence given off by each allele fragment that is then read and collected by the 3130 Genetic Analyzer's charge-coupled device camera. The average percent of the nail donor DNA present in the sample was calculated by first dividing the sum of the RFUs from the nail donor's allele peak heights by the total sum of all peak heights. This was done for all loci that had no allele sharing. Those values were then averaged together for each time-point and the standard deviation was calculated.

2.8 Statistical Methods

Statistics were calculated by Microsoft® Excel 2016 (Microsoft, Redmond, WA).

3. RESULTS AND DISCUSSION

3.1 Exogenous Recovery of DNA

3.1.1 Extraction of Saliva-Spiked DNA Set One

Two fingernails were spiked with 4 μL of male saliva and dried overnight. The nails were extracted using the *forensic*GEM® protocol for saliva samples described in section *2.4.2*. A control of the male saliva was also prepared and dried overnight.

Looking at the e-cell control (Figure 2), the male DNA concentration is slightly greater than the total human DNA in solution indicating that the control sample only has male DNA present. With each 5-minute enzyme digestion period, the DNA concentration slightly increases for both male and total human DNA. In the last 5-minute period at 95ºC used to inactivate the protease, the concentration of DNA increases two-fold from the value at the 20-minute time point. The 25-minute marker represents the five minutes spent at 95ºC for protease inactivation. The male DNA concentration was 0.23 ng/μL at 5 minutes, at 20 minutes it was 0.30 ng/μL, and after protease inactivation, the final concentration was 0.59 ng/μL. The human DNA concentration was 0.19 ng/μL at 5 minutes, at 20 minutes it was 0.25 ng/μL, and after protease inactivation, the final concentration was 0.53 ng/μL. This data is depicted in Figure 2.

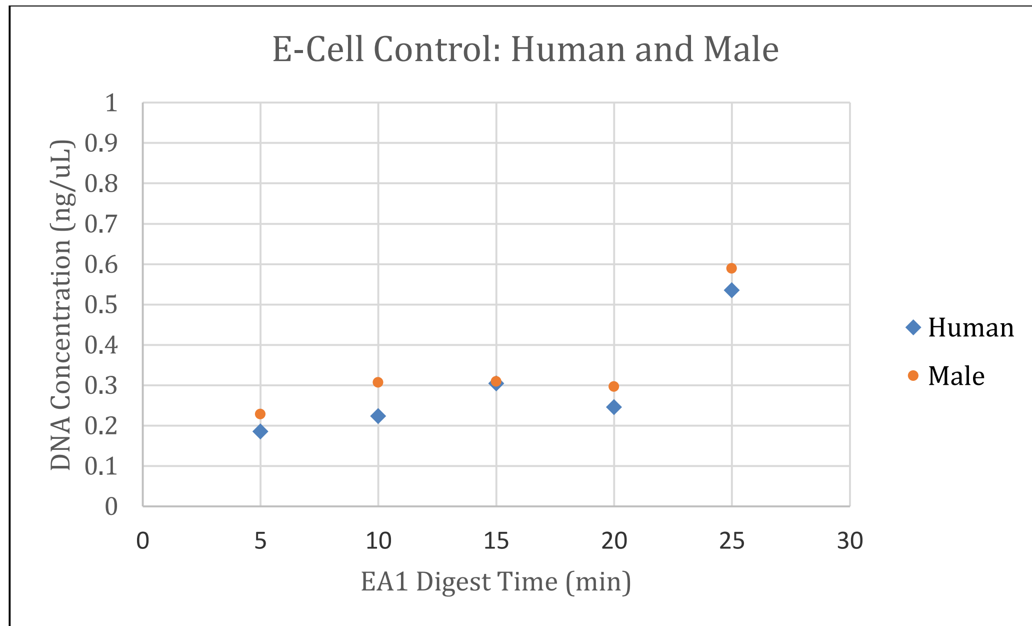

Figure 2. E-Cell Control: Time Course. E-cell control DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95℃.

The *forensic*GEM time course extraction data for the two saliva-spiked nails was gathered in duplicate except for the sample reported at 25 minutes for protease inactivation; the data reported is the average value of the duplicates. The two nails selected for this had similar weights but came from two different donors. This selection was done to limit the weight of the nail as a possible factor for DNA recovery and to be able to compare the recovery rate between donors. The first nail was designated "Nail 5" and the other "Nail 14." The concentration for male and total human DNA increased from the initial to the final time interval. There was a spike in concentration seen between the 5- and 10-minute marks; however, after 10 minutes, the concentrations began to decrease with a slight spike after protease inactivation for Nail 5, except Nail 14's male DNA continued the slight decrease trend during protease inactivation. At 5 minutes, the average value of total human

DNA for Nail 5 was 0.28 ng/µL and the male DNA was 0.27 ng/µL. The values spiked to 0.77 ng/µL and 0.84 ng/µL respectively. The final concentrations of total human and male DNA after protease inactivation were 0.88 ng/µL and 0.74 ng/µL respectively. For Nail 14, the average total human DNA at 5 minutes was 0.27 ng/µL and the male DNA was 0.22 ng/µL. The values spiked to 0.58 ng/µL and 0.67 ng/µL respectively. The final concentrations of total human and male DNA after protease inactivation were 0.82 ng/µL and 0.48 ng/µL respectively. The male and total human DNA concentrations were relatively close in value throughout the extraction time course, which means that even with the presence of the female fingernail clipping, the majority of the DNA being extracted was male DNA. For the first 10 minutes, more male DNA was extracted than total human DNA, but between 10 and 15 minutes, more total human DNA was extracted. This flip in concentration implies that in the first 10 minutes of extraction, the majority of the DNA extracted is male DNA, but after 10 minutes, more female nail donor DNA began to be extracted. These results are depicted in Figures 3 and 4.

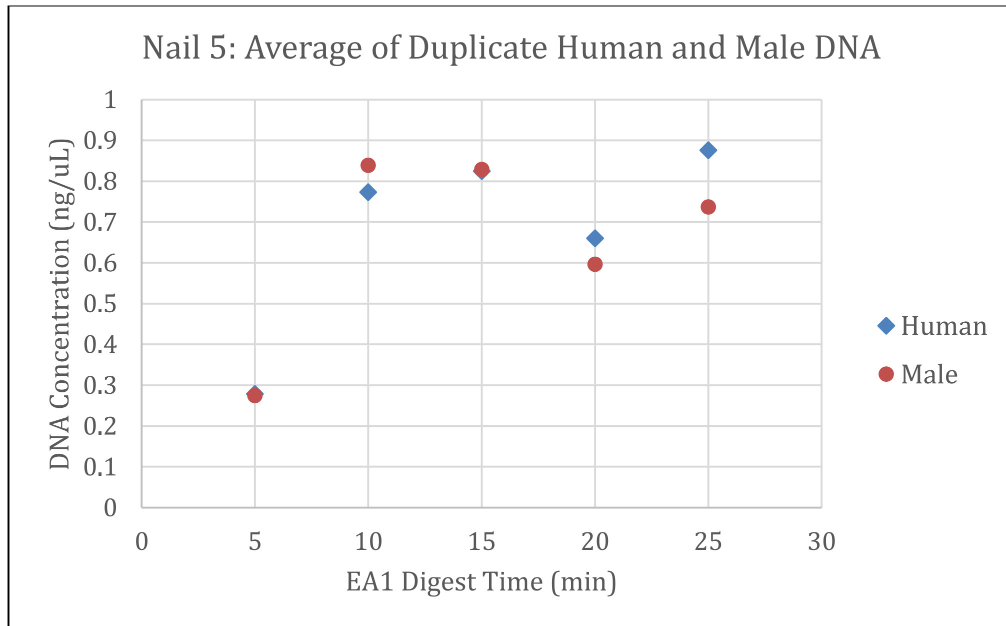

Figure 3. Nail 5: Time Course. Nail 5 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 5.

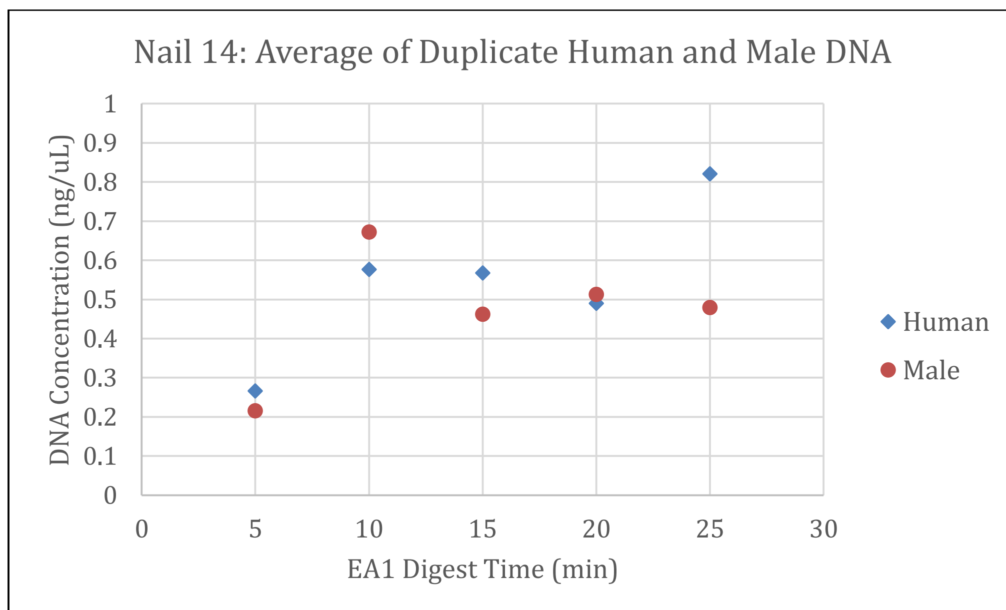

Figure 4. Nail 14: Time Course. Nail 14 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 14.

The nail sample DNA concentrations were compared against the dried control sample to get an understanding of how much more DNA was being extracted from the saliva-spiked nails. In the first 5 minutes, the amount of total human and male DNA was pretty much the same for both nails and the control. At 10 minutes and beyond, though, the control's DNA concentration remained relatively the same throughout the time course. The DNA for both fingernail samples, however, was 2 to 3 times the concentration of the control. This suggests that both the male DNA from the saliva and the female DNA from the fingernails was being digested. Data for both control saliva and nails is shown in Figures 5 and 6.

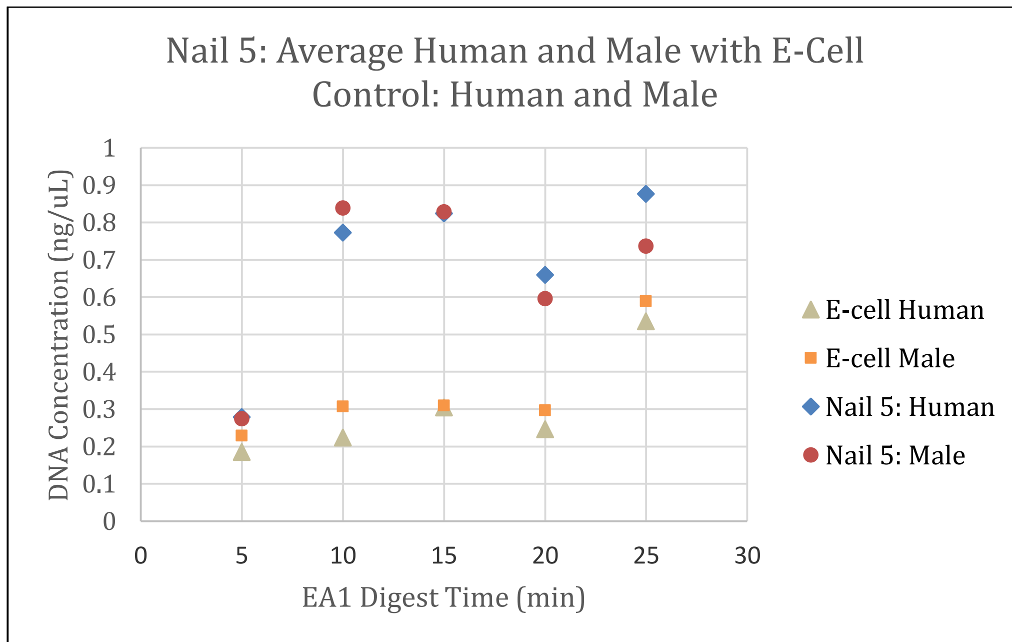

Figure 5. Nail 5: Time Course Compared to E-Cell Control. The values plotted for Nail 5 are the averages of the samples taken in duplicate. This is a comparison of the concentrations of DNA between the saliva-spiked nail sample and e-cell control.

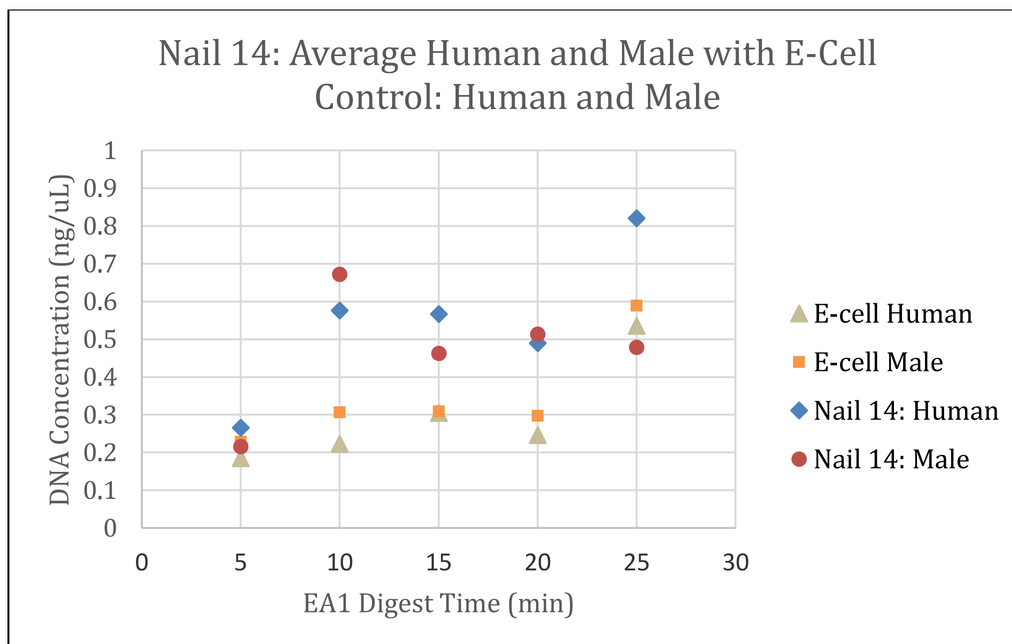

Figure 6. Nail 14: Time Course Compared to E-Cell Control. The values plotted for Nail 14 are the averages of the samples taken in duplicate. This is a comparison of the concentrations of DNA between the saliva-spiked nail sample and e-cell control.

3.1.2 Extraction of Saliva-Spiked DNA Set Two

Another two nails of similar weight from different donors were chosen and prepared in the same manner as previously stated and extracted with the same method; with one notable change. An additional 20-minute incubation at 75ºC was added before inactivating the protease. These samples were again collected in duplicate and the reported values are the averages. The nails were designated "Nail 3" and "Nail 16." The additional 20-minute incubation was done to see if a greater amount of DNA would be extracted. However, the additional 20-minute incubation did not increase the DNA concentration recovered from Nail 3 or Nail 16, as shown in Figures 7 and 8. Therefore, the additional incubation period was deemed unnecessary and removed from the protocol for the

subsequent set of samples. The 45-minute marker represents the five minutes spent at 95ºC for protease inactivation.

The control sample was extracted using the normal *forensic*GEM saliva protocol because the time course was shown to not have much of an impact on the amount of DNA extracted from beginning to end. The e-cell control's total human DNA concentration was 0.64 ng/µL and the male concentration was 0.77 ng/µL.

The data obtained from the extraction for Nail 3 resulted in the male DNA being slightly greater than the total human DNA through the entirety of the time course. The total human DNA concentration at 5 minutes was 0.79 ng/µL and the concentration after protease inactivation was 0.73 ng/µL. These results are depicted in Figure 7. The results for Nail 3 differ from the trends seen in the previous nails. This is because no DNA extraction produces the same results every time, even if the sample were run twice. The male and total human DNA concentrations were relatively close in value throughout the extraction time course, which means that even with the presence of the female fingernail clipping, the majority of the DNA being extracted was male DNA. This is supported by the male saliva e-cell controls' data. The extra 20 minutes of incubation did not have much of an effect on increasing the concentration of DNA. This is likely because the protease has a brief window for activity and it had reached its limit.

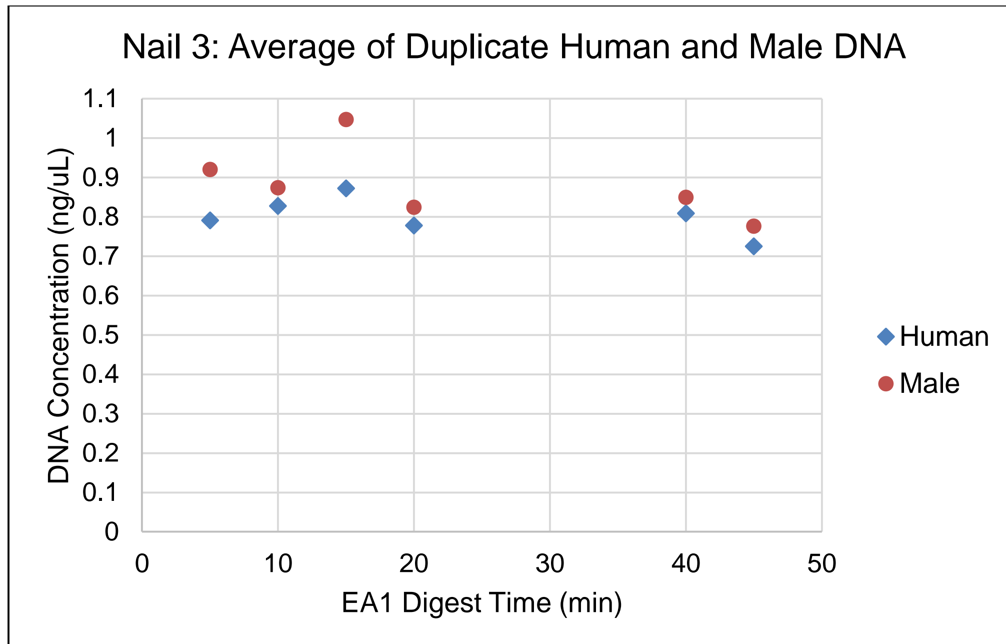

Figure 7. Nail 3: Time Course. Nail 3 DNA concentration recovery over time course of 40 minutes with 45-minute marker representing the protease inactivation at 95℃. The values plotted are the average of the samples taken in duplicate for sample Nail 3.

The extraction results for Nail 16 differ from those of Nail 3. The total human DNA was greater than the male DNA throughout the entirety of the time course, meaning more nail donor DNA was likely being extracted than what has been previously seen from other nails. The total human DNA concentrations were relatively consistent, but variation in the male DNA concentrations was observed. The concentration for the total human concentration at 5 minutes was 0.96 ng/µL and the final concentration was 0.98 ng/µL. For the male DNA, the concentration at 5 minutes was 0.58 ng/µL and the final concentration was 0.82 ng/µL. The results are depicted in Figure 8.

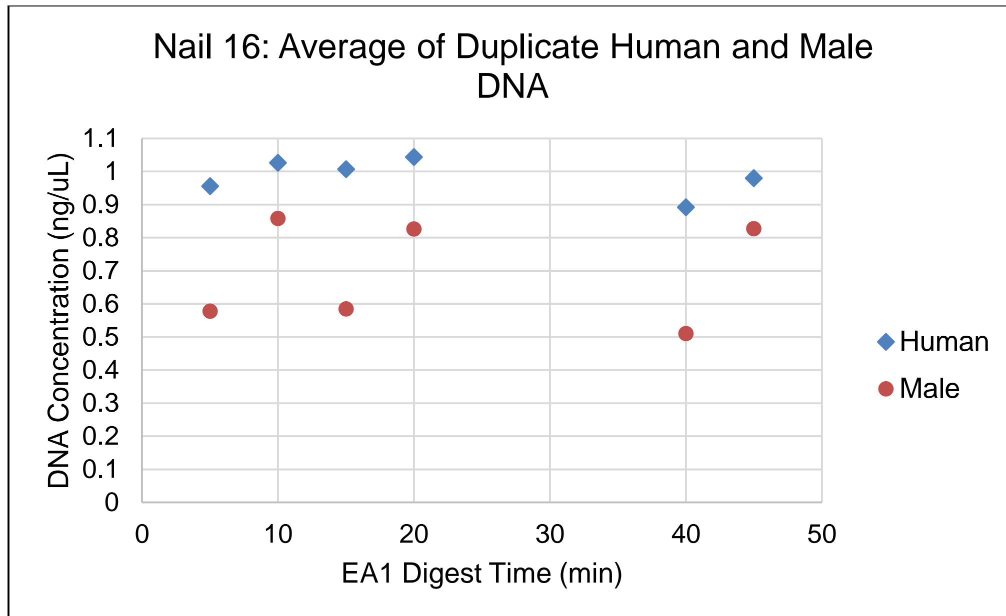

Figure 8. Nail 16: Time Course. Nail 16 DNA concentration recovery over time course of 40 minutes with 45-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 16.

Overall, the final concentrations of both the total human DNA and male DNA are relatively similar to or greater than the e-cell control meaning most of the DNA being extracted came from the male saliva, although some female DNA from the nail may also have been released by the protease.

3.1.3 Extraction of Saliva-Spiked DNA Set Three

Four nails were chosen and prepared in the same manner as previously stated in *3.1.1* and extracted with the approximately the same method as in *3.1.2*; the notable change to the method was the removal of the extra 20-minute incubation at 75ºC and an additional 4 µL pulled off and set aside for amplification. The samples for quantitation were also collected in duplicate but the reported values are the averages; the samples for amplification were not in duplicate. The nails were designated “Nail 4,” “Nail 7,” “Nail 18,” and “Nail 20.” Nails 4 and 7 came from the same donor, and Nails 18 and 20 came from another. The 25-minute marker again represents the 5 minutes spent at 95ºC for protease inactivation.

The two control samples were extracted using the normal *forensic*GEM saliva protocol. The total human DNA concentration for the two e-cell controls was 0.43 ng/µL and 0.57 ng/µL and the male concentration was 0.53 ng/µL and 0.51 ng/µL.

The results from each saliva-spiked nail sample all differ from one another and fairly represent how each sample and extraction can produce varying results, even if they have the same amount of foreign DNA, weigh roughly the same, or come from the same nail donor.

The concentrations of both total human and male DNA for Nail 4 were relatively equal. This means that most of the DNA is likely coming from the male saliva. At the first 5 minutes, the total human DNA was 0.77 ng/µL and the male DNA was 0.69 ng/µL. The final concentrations after protease inactivation spiked and were 0.91 ng/µL and 0.92 ng/µL respectively. Interestingly, the DNA concentrations of both total human DNA and male DNA decreased from the first time point of 5 minutes through the 20-minute time point, then the concentration spiked after protease inactivation. The results of the extraction for Nail 4 can be viewed in Figure 9.

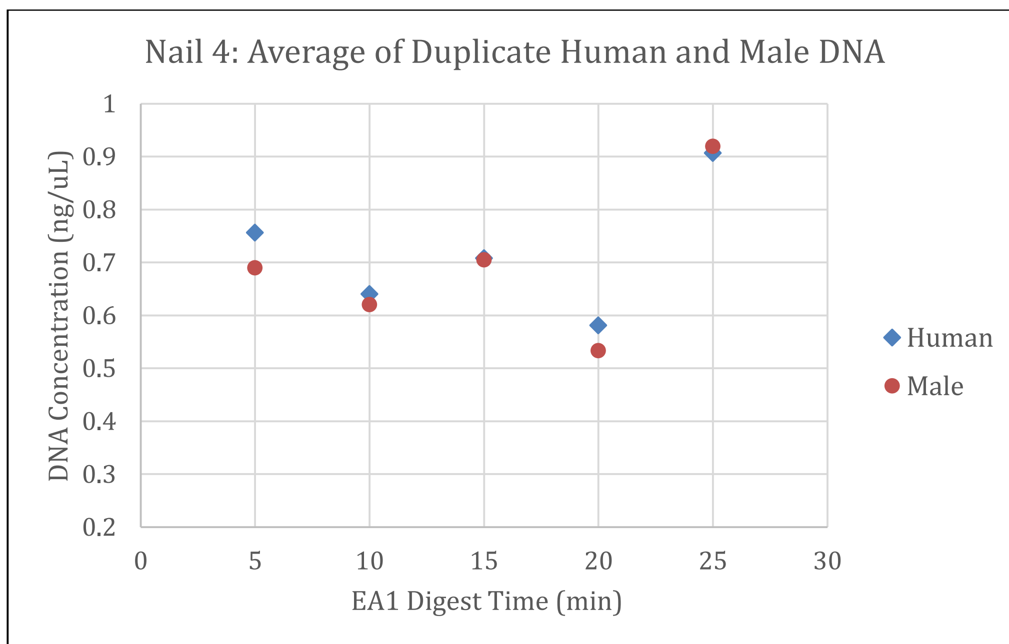

Figure 9. Nail 4: Time Course. Nail 4 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95℃. The values plotted are the average of the samples taken in duplicate for sample Nail 4.

The extraction results for Nail 7 show a greater concentration of total human DNA than male DNA throughout the entire time course. This likely means that female donor DNA was being extracted from the fingernail clipping along with the male saliva and will

produce a mixed, two-contributor profile. The initial total human DNA concentration was 1.05 ng/µL and the male DNA concentration was 0.71 ng/µL. The final concentrations were 1.01 ng/µL and 0.59 ng/µL respectively, as seen in Figure 10. There was a slight decrease from beginning to end for the male DNA, and a general increase throughout the time course for the total Human DNA. Based on these quantitation results, it is likely to see a greater presence of the female donor in the resulting profiles through the time course.

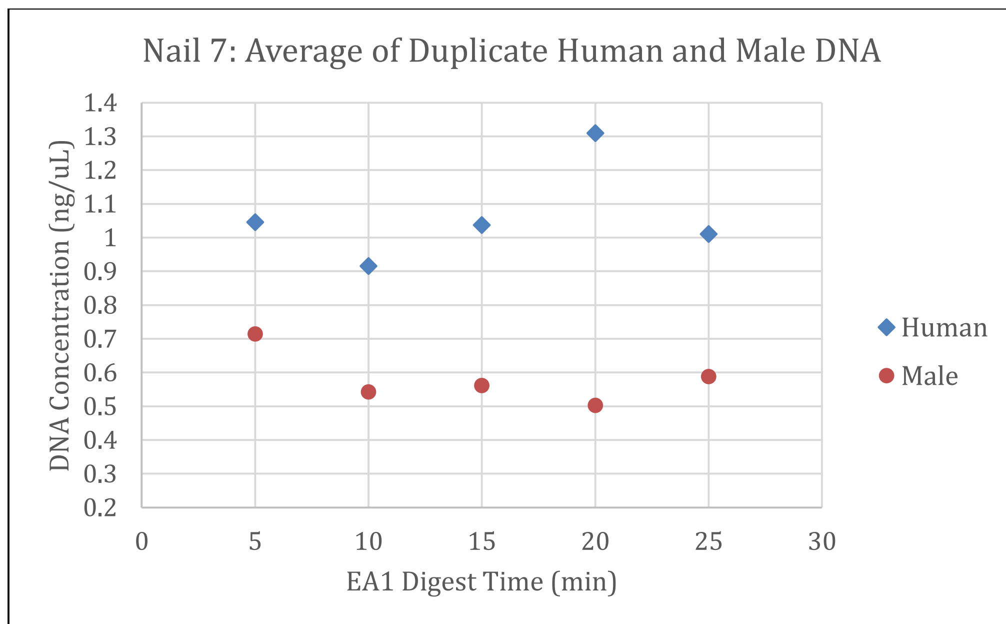

Figure 10. Nail 7: Time Course. Nail 7 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 7.

Extraction for Nail 18 produced a similar pattern of concentration values to Nail 7 but in lower amounts. Nail 18 also had a greater concentration of total human DNA than male DNA throughout the entire time course, which again likely means that female donor DNA was being extracted from the fingernail clipping along with the male saliva and will likely produce a mixed profile. The initial total human DNA concentration was 0.55 ng/µL

and the male DNA concentration was 0.41 ng/µL. The final concentrations were 0.64 ng/µL and 0.49 ng/µL respectively, as seen in Figure 11. A general decrease in concentration for male DNA from 5 to 20 minutes of the time course; then, after protease inactivation, there is a slight spike in concentration. The total human DNA concentration has slight, steady increase throughout the time course. Based on these quantitation results, it is likely to see a greater presence of the female donor in the resulting profiles through the time course, somewhat similar to Nail 7.

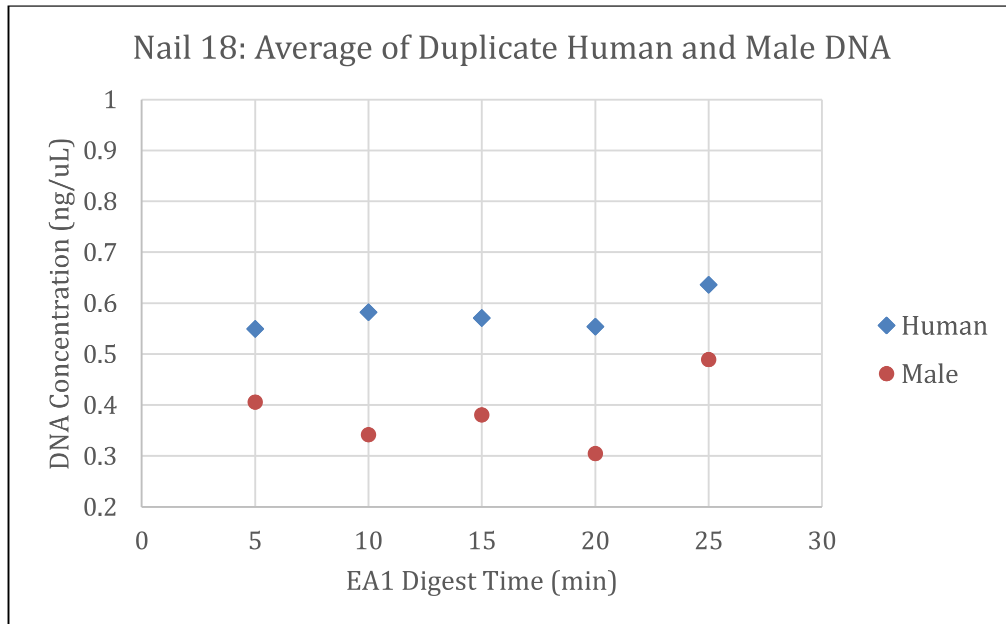

Figure 11. Nail 18: Time Course. Nail 18 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 18.

The extraction results for Nail 20 are interesting. The total human DNA is relatively consistent and, for the most part, greater than the male DNA. However, the male DNA concentrations are high for the initial and final readings, being greater than the total human DNA at these two points, but subsequently lower than total human DNA at the middle time

points. Based on this, it's likely to see the male profile as the major contributor with either a distinguishable minor contributor or only a handful of loci with additional alleles. The initial total human DNA concentration for Nail 20 was 0.78 ng/µL and the male DNA concentration was 0.85 ng/µL. The final concentrations were 0.96 ng/µL and 0.97 ng/µL respectively, as seen in Figure 12.

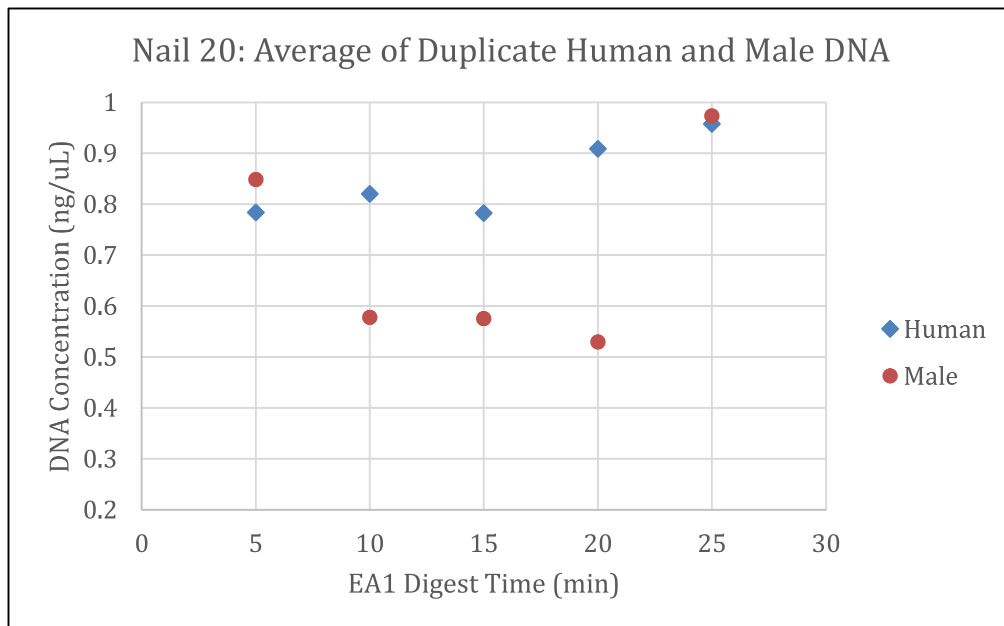

Figure 12. Nail 20: Time Course. Nail 20 DNA concentration recovery over time course of 20 minutes with 25-minute marker representing the protease inactivation at 95ºC. The values plotted are the average of the samples taken in duplicate for sample Nail 20.

The first e-cell control, B1, was extracted and quantified with Nails 5 and 14. The quantitation values did not vary significantly with each five-minute incubation, so the remainder of the e-cell controls followed the manufacturer's protocol for saliva samples. In three of the four e-cell controls that were run, male DNA was greater than the total human DNA. This is seen in Table 1.

Table 1. Male-to-Human Ratio of DNA in Male Saliva E-Cell Controls. Sample B1 underwent the same time course for extraction and quantified in the same run as Nails 5 and 14, so the results of each time point were averaged and the standard deviation was calculated. Samples E1, B2, and E2 did not undergo a time course. Sample E1 was run with Nails 3 and 16. Samples B2 and E2 were run with Nails 4, 7, 18, and 20.

	Concentration (ng/μL)		
E-Cell Controls	Human	Male	Male: Human Ratio
B1	0.30 ± 0.124	0.35 ± 0.13	1.16 ± 0.035
E1	0.64	0.77	1.21
B2	0.43	0.53	1.23
E2	0.57	0.51	0.89

3.2 Electropherogram Analysis

A preliminary look at the quantitation values gave an indication as to what was likely happening in the extractions when the concentrations of the total human and male DNA were compared. Analyzing the capillary electrophoresis results from nails 4, 7, 18, and 20 solidified what was initially thought. An electropherogram was produced for every 5-minute period of incubation for each of the four nails. Each electropherogram was compared to the nail donor profile, male donor profile, and each other to determine the number of contributors as well as major and minor profiles, if possible.

As predicted from the quantitation values, Nail 4 produced only reportable male profiles for all time periods of the time course. Figure 13 provides a comparison of the

yellow dye loci (seen as black for easy viewing—D2S441, D19S433, TH01, and FGA) from the *forensic*GEM extraction of saliva-spiked Nail 4 for each of the five-minute time points. The yellow dye was selected on the basis of having the fewest shared alleles between the nail donor and the foreign saliva donor. The profiles produced for all time points indicate the presence of a single, male contributor. It can be seen that only a few alleles at a couple of loci come from the female nail donor; all of the alleles present can be attributed to the foreign male donor, but there are not enough alleles present to determine a minor contributor.

The results from Nail 4 are the best-case scenario for the purpose of this study. The foreign male donor was detected for each STR profile produced from the time course samples; the additional alleles are not enough to be considered as a reportable minor donor.

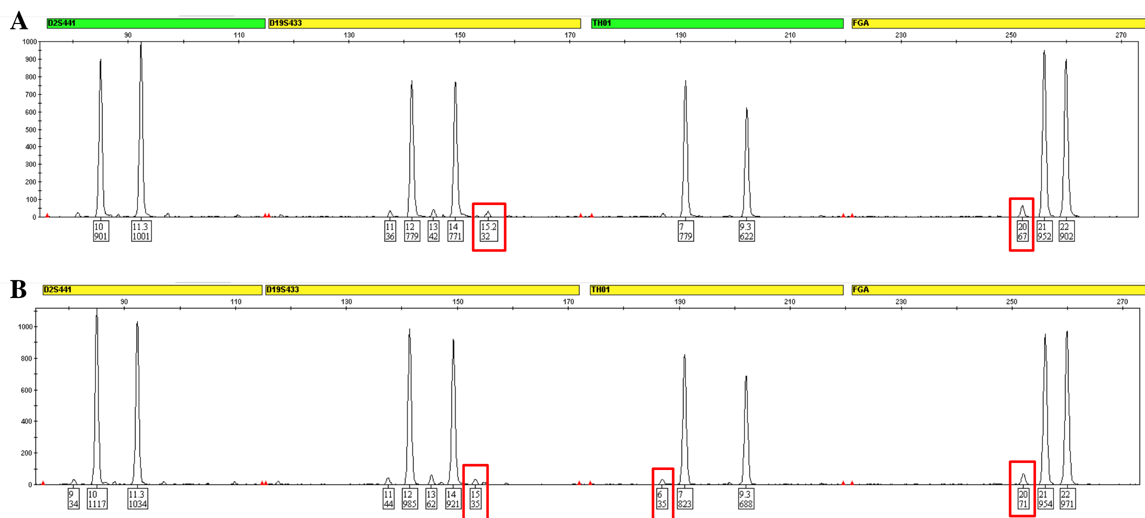

Figure 13. Profiles of Saliva-Spiked Nail 4 Time Course Extraction. Yellow (pictured as black for easy viewing) dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 4. A) 5-minute time point. B) 10-minute time point. Unique nail donor alleles are marked with red boxes. (Figure 13 Continues on next page.)

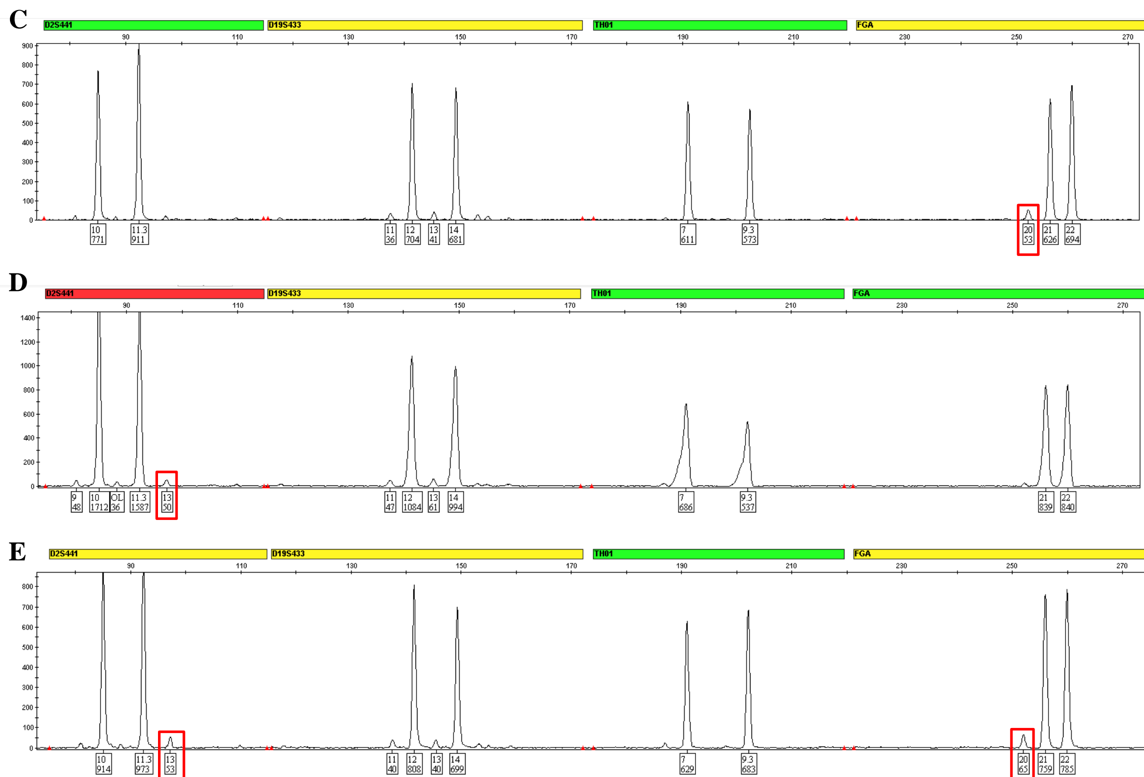

Figure 13. Profiles of Saliva-Spiked Nail 4 Time Course Extraction Continued. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 4. C) 15-minute time point. D) 20-minute time point. E) 25-minute time point, after protease inactivation. Unique nail donor alleles are marked with red boxes.

Based on the quantitation values, it was expected to see the extraction profiles of Nail 7 with two contributors for all time points and a possibility of the male profile being the major contributor for at least the 5- and 10-minute markers of the time course. Figure 14 provides a comparison of the yellow dye loci from the *forensic*GEM extraction of saliva-spiked Nail 7 for the 5-minute incubation periods of 5, 10, 15, 20, and 25 minutes. The yellow dye was selected on the basis of having the fewest shared alleles between the nail donor and the foreign saliva donor. From analyzing the electropherograms, extractions from 5 and 10 minutes produced a two-contributor profile and the majors and minors could be determined. Allele sharing is seen only at the first locus, D2S441. The alleles of the

major contributor could be attributed to the foreign male profile, and the alleles of the minor contributor could be attributed to the donor female profile. A major and minor contributor could not be easily determined from the profile produced for the 25-minute extraction period. The alleles present could be attributed to either the foreign male DNA or female donor DNA. The individual contributors at the 15- and 20-minute time points were not as easily discernible as at the 5- or 10-minute extractions, but not as difficult as at the 25-minute extraction. The red boxes represent unique nail donor alleles.

The results for Nail 7 are more realistic to what could be observed in a forensic case. However, the results could still be used to include the foreign male donor as a "suspect" because his alleles are present in the entire profile for each point of the time course and are sometimes discernible as the major contributor. This can also be used as a quality control check since the samples were not switched and both donors are present. In some forensic laboratories, the known victim's profile, in this case the fingernail donor, would be assumed to be present in the profiles and could be readily attributed to the victim. Then, the other contributor could be deduced more easily, and there's sufficient data from the male contributor to be eligible for a database search.

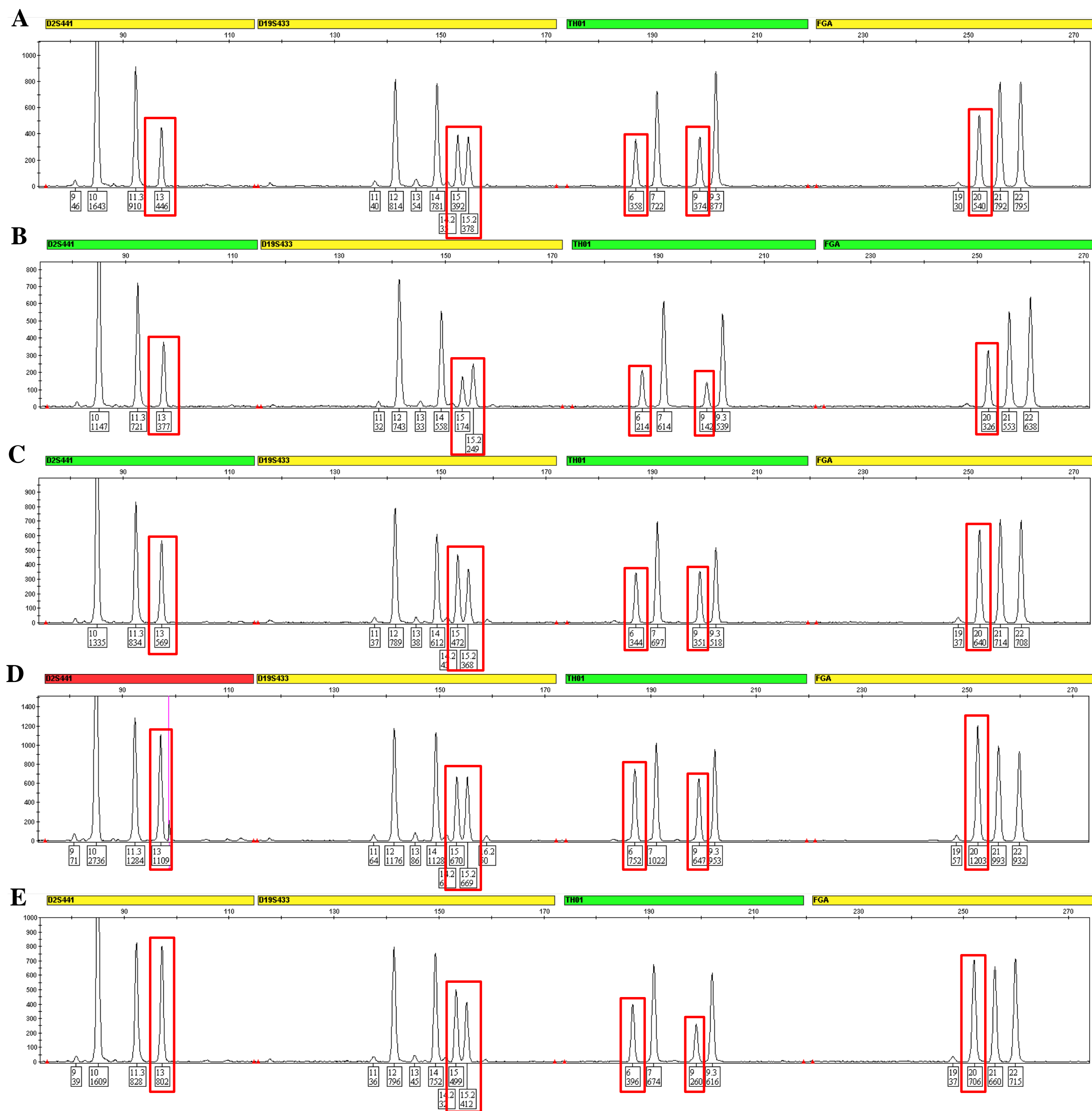

Figure 14. Profiles of Saliva-Spiked Nail 7 Time Course Extraction. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 7. A) 5-minute time point. B) 10-minute time point. C) 15-minute time point. D) 20-minute time point. E) 25-minute time point, after protease inactivation. Unique nail donor nail alleles are marked with red boxes.

Nails 4 and 7 had 12 loci sites where there was no allele sharing between the nail donor and saliva donor, making calculations simple for determining the percent of the donor nail present in the total sample. Nail 4 did not produce a significant amount of donor

nail DNA for calculations, but Nail 7 did. The average amount of nail donor DNA for the enzyme digest time course is depicted in Table 2. The loci that do not have shared alleles are CSF1PO, TPOX, D8S1179, D2S11, D19S433, TH01, FGA, D13S317, D7S820, SE33, D1S1656, and D12S391. The combined results from these loci are displayed in Table 2 and show a gradual increase in the percent of the contribution of endogenous DNA as the time of digest increases.

Table 2. Average Percent and Standard Deviation of Nail 7 Donor. The percent of nail donor DNA peak heights to the total DNA peak heights from each of the 12 loci that did not contain allele sharing was calculated. These values were averaged for each time point and the standard deviation from the individual loci to the average was found.

Nail 7		
Time Point (min)	Average % Nail Donor Present	% Standard Deviation
5	21%	4%
10	26%	7%
15	28%	8%
20	33%	8%
25	32%	8%

Based on the quantitation values, it was again likely to see the extraction profiles of Nail 18 with two contributors for all time points and the possibility of the male profile being the major contributor for all of the time points of the time course. Figure 15 provides a comparison of the yellow dye loci (D2S441, D19S433, TH01, and FGA) from the *forensic*GEM extraction of saliva-spiked Nail 18 for the 5-minute incubation periods of 5, 10, 15, 20, and 25 minutes. The yellow dye was selected on the basis of having fewer shared alleles between the nail donor and the foreign saliva donor. From analyzing the electropherograms, extractions from each time point produced a two-contributor profile

and the majors and minors could be determined relatively easily for the 5-, 10-, and 15-minute periods. The 20- and 25-minute extraction profile depicted two contributors but a major and minor contributor would not be deducible without prior knowledge of either donor's alleles. Allele sharing is seen at only one locus, D19S433, with both contributors having allele 14. If a major and minor were discernible, the alleles of the major contributor could be attributed to the foreign male profile, and the alleles of the minor contributor could be attributed to the donor female profile. The red boxes represent unique nail donor alleles.

The results for Nail 18 are also more realistic to what could be observed in a forensic case. However, the results could still be used to include the foreign male donor as a "suspect" because his alleles are present in the entire profile for each point of the time course and sometimes discernible as the major contributor.

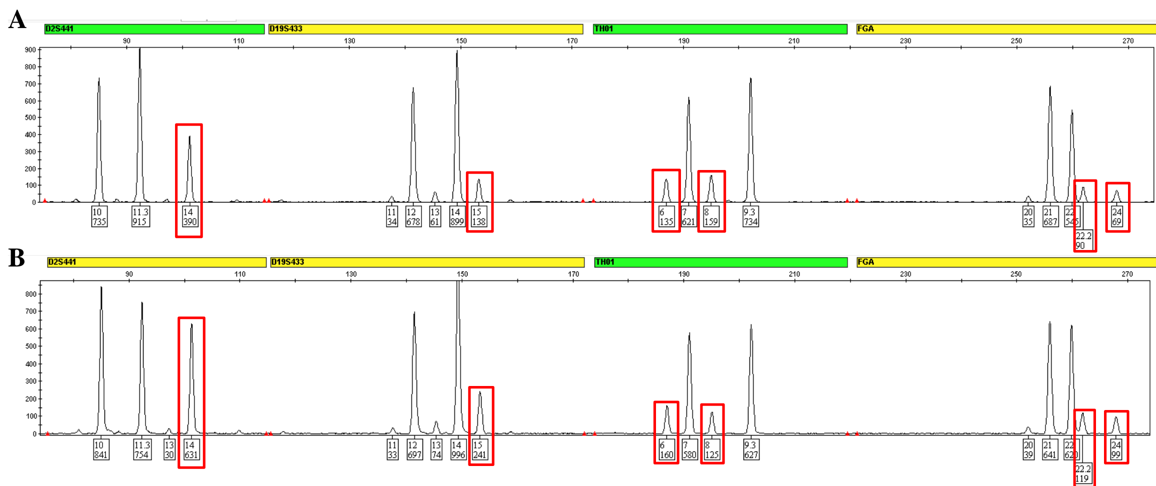

Figure 15. Profiles of Saliva-Spiked Nail 18 Time Course Extraction. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 18. A) 5-minute time point. B) 10-minute time point. (Figure 15 continues on next page.)

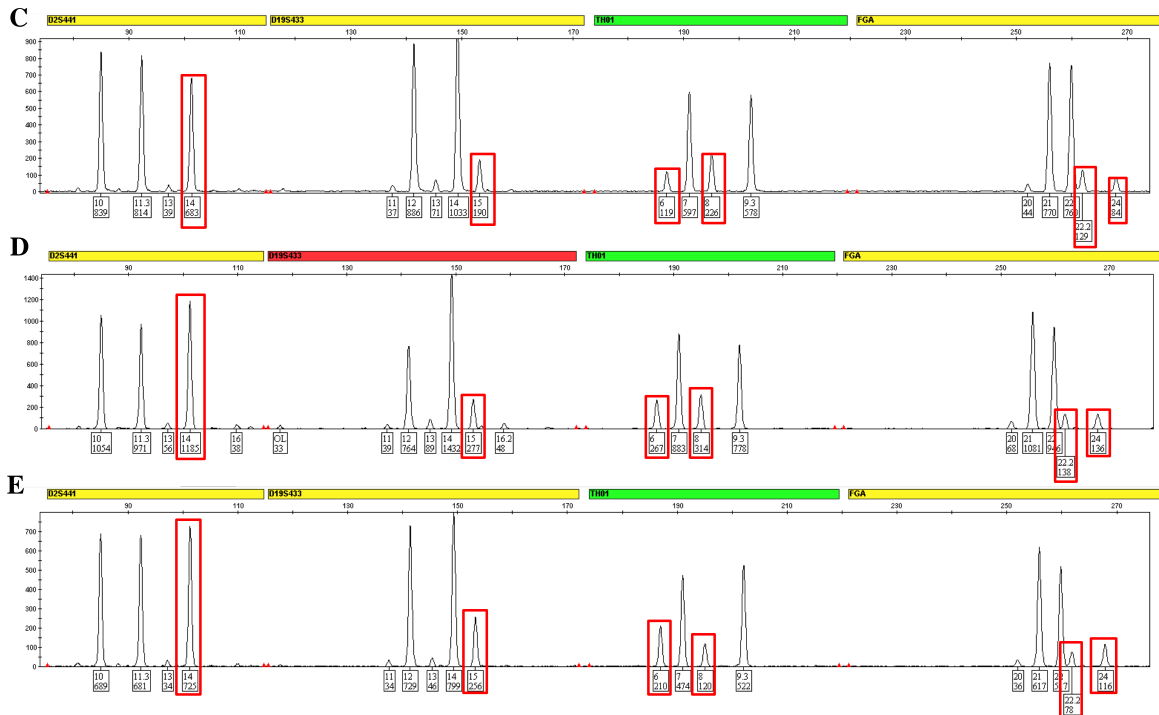

Figure 15. Profiles of Saliva-Spiked Nail 18 Time Course Extraction Continued. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 18. C) 15-minute time point. Unique nail donor alleles are marked with red boxes. D) 20-minute time point. E) 25-minute time point, after protease inactivation. Unique nail donor alleles are marked with red boxes.

As predicted from the quantitation values, Nail 20 produced two-contributor profiles with the major contributor being the foreign male donor and 3 to 6 loci that indicate the presence of a second minor contributor for all time periods of the time course. Based on the quantitation results, it was not expected to see a minor contributor at the 5- and 25-minute marks because the total human DNA and male DNA concentrations were relatively close in values. Figure 16 provides a comparison of the yellow dye loci (D2S441, D19S433, TH01, and FGA) from the *forensic*GEM extraction of saliva-spiked Nail 20 for each of the 5-minute incubation periods: 5, 10, 15, 20, and 25 minutes. The yellow dye was selected on the basis of having fewer shared alleles between the nail donor and the foreign saliva donor. All of the extraction profiles for Nail 20 resulted with a major male

contributor that came from the foreign male saliva. A minor contributor was detected for all of the extractions, but it was only discernible at 3 to 6 loci across all of the electropherograms. There are fewer loci in the resulting electropherograms than what was expected based on the quantitation results for the middle time points. There was more total human DNA than male DNA at the 10-, 15-, and 20-minute marks, so more female alleles were expected. There was not a great enough presence to determine if the minor contributor was male or female, but the female nail donor could not be excluded from the additional alleles present. The red boxes represent unique non-major contributor alleles.

The profiles of Nail 20 are another example of the types of mixture ratios that are frequently seen in forensic cases. An obvious major contributor is present, and there are detectable alleles of a minor contributor but not enough information produced from the minor to confidently include or exclude someone as the contributor.

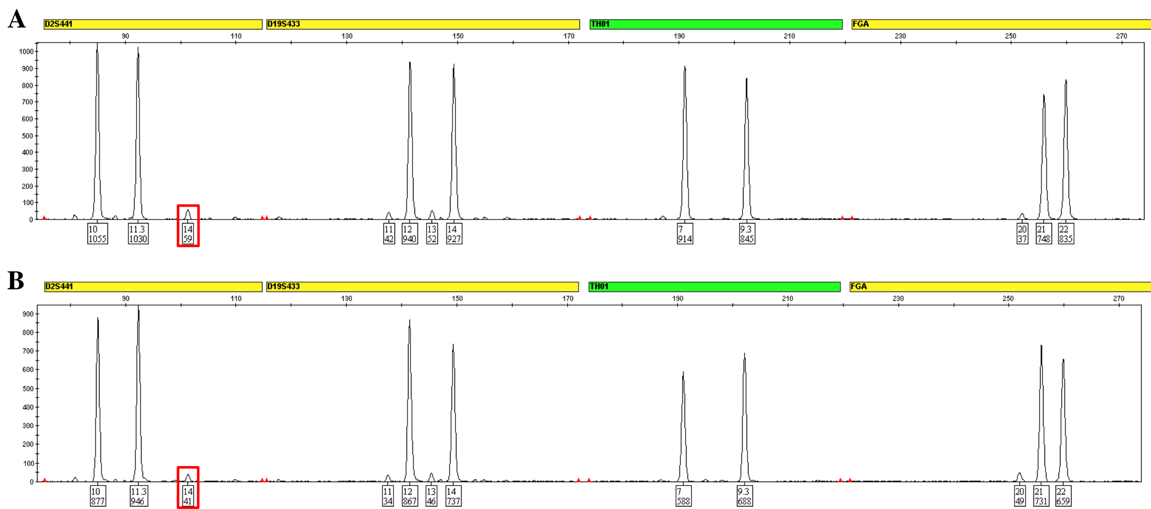

Figure 16. Profiles of Saliva-Spiked Nail 20 Time Course Extraction. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 20. A) 5-minute time point. B) 10-minute time point. The red boxes represent unique non-major contributor alleles. (Figure 16 continues on next page.)

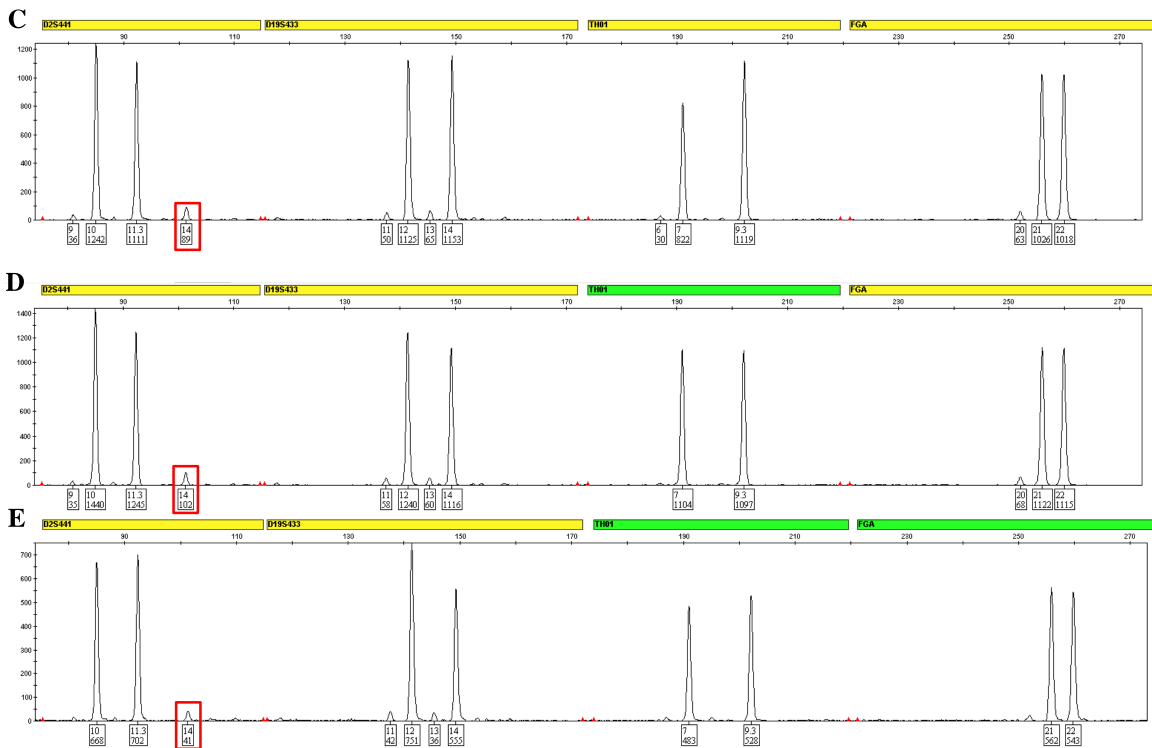

Figure 16. Profiles of Saliva-Spiked Nail 20 Time Course Extraction Continued. Yellow dye loci (D2S441, D19S433, TH01, and FGA) electropherograms for extraction of Nail 20. C) 15-minute time point. D) 20-minute time point. E) 25-minute time point, after protease inactivation. The red boxes represent unique non-major contributor alleles.

Nails 18 and 20 had 8 loci where there was no allele sharing between the nail donor and saliva donor, making calculations simple for determining the percent of the donor nail present in the total sample. Nail 20 did not produce a significant amount of donor nail DNA for calculations, but Nail 18 did. The average amount of nail donor DNA for the time course is depicted in Table 3. The loci that do not contain allele sharing are D3S1358, TPOX, D2S441, TH01, FGA, D7S820, SE33, and D1S1656. The combined results from these loci are displayed in Table 3 and show a gradual increase in the percent of the contribution of endogenous DNA as the time of digest increases.

Table 3. Average Percent and Standard Deviation of Nail 18 Donor. The percent of nail donor DNA peak heights to the total DNA peak heights from each of the eight loci that did not contain allele sharing was calculated. These values were averaged for each time point and the standard deviation from the individual loci to the average was found.

Nail 18		
Time Point (min)	Average % Nail Donor Present	% Standard Deviation
5	14%	4%
10	19%	7%
15	18%	8%
20	22%	10%
25	21%	9%

4. CONCLUSIONS

Extraction from fingernail clippings directly has its advantages and disadvantages. More exogenous DNA is likely to be recovered but so is endogenous DNA from the fingernail itself. The goal of this study was to maximize the amount of foreign DNA being recovered by extracting directly from nail clippings while trying to keep the recovery of endogenous DNA low by creating a time course. Instead of one extraction period of 15 minutes, there were 4 extraction periods with 5 minutes each. This method was put in place to try and identify at what point the extraction of mostly foreign DNA transitions to mostly endogenous DNA. A 20-minute window for incubation was decided on because it was similar in the amount of time as the original protocol created by the *forensic*GEM™ manufacturer for saliva samples, but it also allowed for a little more time for further extraction of exogenous DNA. With a small data set, it is not possible to draw firm conclusions. However, with a summation of the data that was collected, it is reasonable to state that from the reported results, the majority of the DNA extracted in the first 10 minutes can be attributed to exogenous DNA, but beyond 15 minutes, the endogenous DNA begins to equate or surpass the exogenous DNA. The use of *forensic*GEM for 5 to 10 minutes exhibited promising results suggesting a shorter extraction time period for fingernail clippings is a promising method of recovery for exogenous DNA.

4.1 Future Directions

The present research may be expanded upon with a greater sample size to more definitively assess whether a shorter extraction period results in a greater recovery of exogenous DNA and lower recovery of endogenous DNA. The nails were only spiked with

neat saliva, so this study would benefit from extracting samples with various dilutions of saliva. It would also benefit from a utilization of other body fluids, such as blood or semen, and dilutions of those as well. Another direction this study could take is testing the time course on other extraction methods, such as QIAGEN or Chelex, as well as other proteases.

APPENDIX

Table 4. Nail Donor and Weight. Nails were paired together for analysis based on similarity in weight and from different donors.

Nail	**Donor**	**Weight (mg)**
3	R-189	6.7
4	R-189	5.9
5	R-189	4.8
7	R-189	8.2
14	R-200	4.9
16	R-162	6.6
18	R-162	5.5
20	R-162	8.6

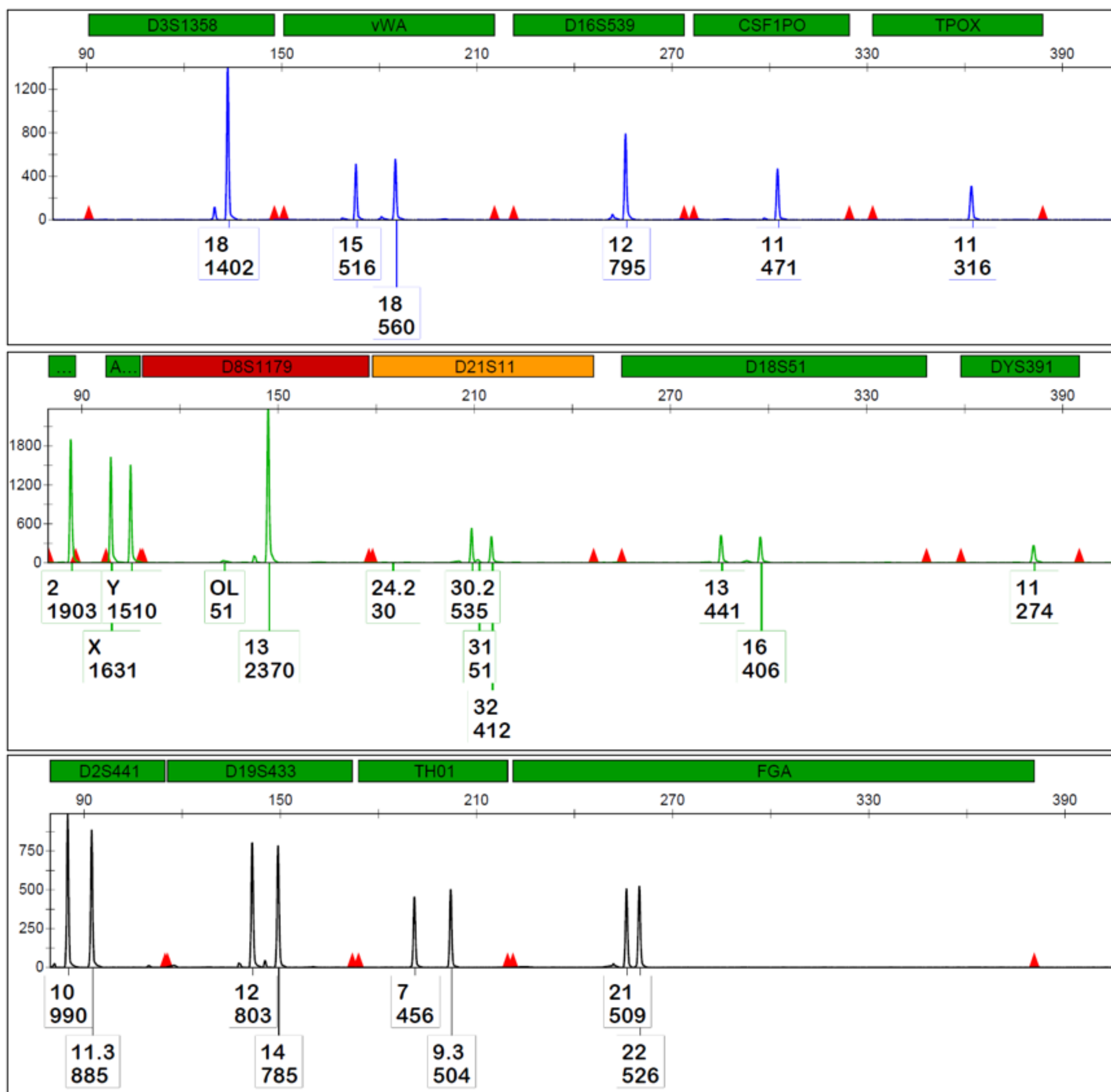

Figure 17. Male Donor Profile. Single donor profile from male saliva using GlobalFiler™. (Figure 17 continued on next page.)

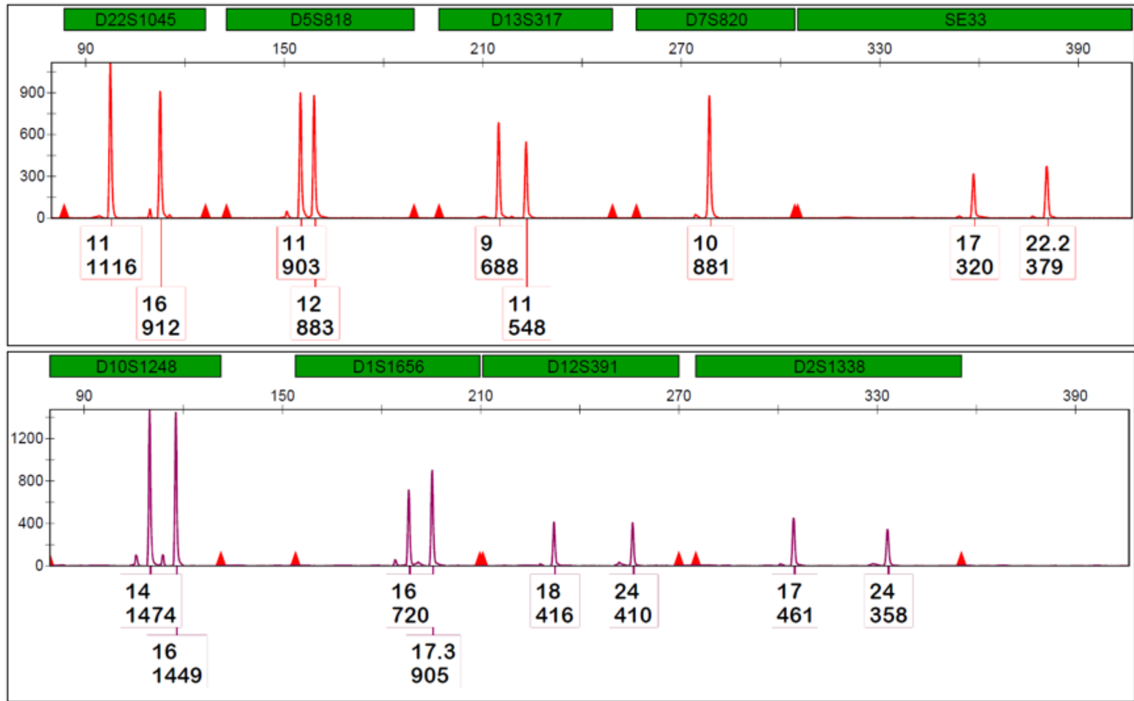

Figure 17. Male Donor Profile Continued.

Table 5. Genotypes of Female Donor for Nails 4 and 7. Genotypes are separated and color-coded by channel. The genotypes that were not identified in the IdentiFiler™ profile were deduced from the results of the time course using GlobalFiler™.

Nail 4 and 7	
Loci	**Genotypes**
D3S1358	16, 18
vWA	14, 18
D16S539	9, 12
CSF1PO	12, 14
TPOX	8, 8
Y	N/A
Amelogenin	X, X
D8S1179	14, 14
D21S11	29, 30
D18S51	16, 19
DYS391	N/A
D2S441	10, 13
D19S433	15, 15.2
THO1	6, 9
FGA	20, 20
D22S1045	11, 11 or 16
D5S818	10, 12
D13S317	12, 12
D7S820	9, 11
SE33	28.2, 28.2
D10S1248	13, 16
D1S1656	12, 14
D12S391	23, 23
D2S1338	17, 20

Table 6. Genotypes of Female Donor for Nails 18 and 20. Genotypes are separated and color-coded by channel. The genotypes that were not identified in the IdentiFiler™ profile were deduced from the results of the time course using GlobalFiler™.

Nail 18 and 20	
Loci	**Genotypes**
D3S1358	16, 16
vWA	16, 18
D16S539	10, 12
CSF1PO	11, 11
TPOX	8, 8
Y	N/A
Amelogenin	X, X
D8S1179	13, 14
D21S11	30.2, 32
D18S51	13, 14
DYS391	N/A
D2S441	14, 14
D19S433	14, 15
THO1	6, 8
FGA	22.2, 24
D22S1045	16, 17
D5S818	12, 14
D13S317	8, 11
D7S820	8, 8
SE33	28.2, 32.2
D10S1248	14, 14 or 16
D1S1656	11, 12
D12S391	18, 23
D2S1338	17, 20

LIST OF JOURNAL ABBREVIATIONS

J Forensic Sci	Journal of Forensic Sciences
PLoS One	Public Library of Science

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