THE COLLECTION, PRESERVATION, AND PROCESSING OF DNA SAMPLES FROM DECOMPOSING HUMAN REMAINS FOR MORE DIRECT DISASTER VICTIM IDENTIFICATION (DVI)

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A Dissertation

Presented to

The Faculty of the Department of Forensic Science

Sam Houston State University

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In Partial Fulfillment

of the Requirements for the Degree of

Doctor of Philosophy

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by

Amy Sorensen Holmes

May, 2018

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DEDICATION

To my advisor, Dr. Sheree Hughes-Stamm, through all the urgent e-mails, late nights of crunching data and generating power point slides. I truly could not have asked for a wiser, and more encouraging, patient, and caring mentor through the hardest of times. I've grown as a person and as a forensic science professional because of your endearing guidance and support.

To my parents and my sisters, for their endless love and encouragement. I am forever grateful.

To my husband, Steven, for all the times I was working late or on weekends instead of fishing with him. I love you more!

ABSTRACT

Holmes, Amy Sorensen, *The collection, preservation, and processing of DNA samples from decomposing human remains for more direct disaster victim identification (DVI)*. Doctor of Philosophy (Forensic Science), May 2018, Sam Houston State University, Huntsville, Texas.

Forensic DNA analysis plays a vital role in forensic casework and mass fatality incidents to identify victims. In these situations, human remains are often subjected to extreme heat, humidity, possible mutilation or fragmentation, microbial activity, decomposition and putrefaction, which can all complicate disaster victim identification (DVI). In many cases, the disaster event results in damage to the local infrastructure causing a loss of electricity and lack of facilities with refrigeration to house large numbers of human remains. Without refrigeration, the DNA in tissues become more degraded and damaged making traditional short tandem repeat (STR) typing more difficult. Therefore, immediate and simple in-field collection and stabilization of DNA samples from decomposing human remains without the need for refrigeration would be of great benefit to the forensic community.

In addition, quick victim identification is also a goal for DVI operational teams as they attempt to bring closure to the victim's families, and assist government agencies that must account for the deceased. As a result, the demand for a rapid turnaround time is often stressed; however, due to the overwhelmingly large number of samples requiring processing, this may not be feasible. Significant rate-limiting steps in the STR typing workflow are DNA extraction (approx. 3-4 hours), DNA quantification (1-2 hours), and standard PCR amplification (approx. 2-3 hours). Digestion of dense connective tissues such as skin and muscle prior to DNA purification may add another 8-12 hours, or hard

tissues such as bone or teeth another 24-48 hours to the overall time required for generating an STR profile. If some of these procedures could be reduced (or avoided) during a mass fatality incident involving thousands of victims, sample throughput could be substantially increased.

This doctoral research focuses on testing various in-field DNA collection and room temperature preservation methods for decomposing human remains as mock DVI samples. We compared rapid DNA purification protocols or direct amplification approaches that will eliminate unnecessary steps in the DNA analysis workflow, increasing the throughput and reducing the costs of analysis. Overall, results indicate that sufficient DNA can be collected and preserved at ambient temperature using some of these methods, provided that DNA is not already severely degraded before collection.

In-field sample collection from human remains using biopsy punches or simply making an incision in the skin and swabbing the underlying muscle with cotton or foam swabs proved to be the most successful and easiest methods of DNA collection. Biopsy punches should be immediately stored in a modified TENT preservative, and swabs allowed to dry for transport to the laboratory for refrigeration, or stored until DNA analysis is possible. TENT and swab samples were processed with both traditional DNA analysis workflows and a direct PCR approach. In all cases, complete profiles were obtained from fresh tissues using all methods tested in this study. However, severely decomposed tissues were more challenging, with most samples yielding partial (or no) STR profiles.

We proposed a method for triaging swab samples based on the quantification results of samples prepared for direct PCR in order to increase the first-pass success rate. Results indicate that foam swabs used to collect from muscle tissue may generate the most

complete STR profiles for the majority of decomposed tissues, with cotton swabs yielding similar results. In addition, aliquots of TENT containing DNA leached from tissues were successfully diluted and directly added to the PCR reaction, thereby skipping DNA extraction and quantification all together. This protocol is the quickest of all methods tested, generating STR profiles in a fraction of the time it takes for traditional DNA processing. If this first-pass approach fails due to insufficient amounts of DNA, then the tissue itself stored in the TENT buffer can be quickly extracted in under 20 minutes with the PDQeX DNA extraction system. We found that this method generated the most complete STR profiles from severely decomposed tissues.

Overall, we have demonstrated that tissues preserved in a modified TENT buffer or collected and stored using cotton and foam swabs show potential as alternate methods for the immediate in-field collection and preservation of DNA at room temperature for human identification purposes. However, these methods warrant further investigation to optimize protocols to achieve more efficient DNA preservation and higher STR success rates from severely decomposed human tissues.

KEYWORDS: Forensic science, STR typing, Mass disasters, Disaster victim identification, DNA collection, DNA preservation, Rapid purification, Direct amplification

ACKNOWLEDGEMENTS

This research was supported by Award No. 2013-DN-BX-K034 and Graduate Research Fellowship Award No. 2015-R2-CX-0029 (National Institute of Justice, Office of Justice Programs, U.S. Department of Justice). This funding source had no involvement in the study design, in the collection, analysis and interpretation of data, or in the writing of the thesis or publications. The opinions, findings, conclusions, or recommendations expressed in this dissertation are those of the authors and do not necessarily reflect those of the National Institute of Justice. I greatly appreciate the support from NIJ, which has provided me with an unparalleled opportunity to interact with various professionals in my field and allowed me to travel throughout the US and internationally to prestigious scientific meetings.

I must also acknowledge the immense support from faculty and staff at Sam Houston State University (SHSU), as well as Graduate Studies for additional financial support to travel to various conferences nation-wide. I would also like to thank the staff at the Applied Anatomical Research Center (AARC) at SHSU for their assistance, and the individuals and families of those who donated their bodies for scientific research.

I would like to sincerely thank my mentors, Dr. Sheree Hughes-Stamm and Dr. David Gangitano, for their guidance and support throughout my academic career as a Forensic Scientist. They have been instrumental in my development of the necessary skills to advance my career in forensic DNA research. Through their encouragement, I have gained confidence in my abilities and a strong desire to pursue academia and mentorship roles in the future.

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LIST OF TERMS AND ABBREVIATIONS

DNA	Deoxyribonucleic acid
DVI	Disaster victim identification
STR	Short tandem repeat
bp	Base pairs
ng	Nanograms
µL	Microliters
M	Molar
mM	Millimolar
epg	Electropherogram
IPC	Internal PCR control
SNP	Single nucleotide polymorphism
INDEL	Insertion/deletion polymorphisms
mtDNA	Mitochondrial DNA
PCR	Polymerase Chain Reaction
HID	Human identification
OSAC	Organization of Scientific Area Committees for Forensic Science
FBI	Federal Bureau of Investigations
LST	Lysis, storage, and transportation buffer
TENT	Tris, EDTA, NaCl, Tween 20
DESS	DMSO, EDTA, saturated salt
dsDNA	Double stranded DNA
mini-STR	Mini-short tandem repeats
H_2O	Water

GLOSSARY

Polymerase Chain Reaction	The method used to replicate a specific region of the genome over and over again to produce millions of copies of a particular sequence.
Degraded DNA	Double stranded DNA that has broken into small fragments.
Damaged DNA	Destruction of the DNA molecule resulting in base modifications and lesions such as nicks, double strand breaks, crosslinks, and base mismatches.
Decomposition	A process that occurs in human remains after death.
PCR Inhibition	Any factor that prevents the process of amplification of nucleic acids via the polymerase chain reaction.
Direct PCR	The process of adding DNA sample directly to the PCR reaction without prior DNA extraction or quantification.

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CHAPTER I

Introduction

Every year mass disasters claim the lives of hundreds to thousands of people around the world [1, 2]. These disasters are categorized as natural (hurricanes, tsunamis, earthquakes, floods, tornadoes, typhoons) or man-made events (terrorist attacks, airplane and train crashes, acts of genocide and wars) [2-7]. Each disaster event poses unique challenges and involves varying approaches for the recovery and identification of human remains [5]. Disaster victim identification (DVI) is a necessary action in mass fatality events providing legal and general closure to the victims' families [1, 3, 6]. Many factors complicate identification efforts such as the number of victims, severity of body destruction and fragmentation, exposure to harmful degradation processes, the speed and accessibility of body/sample collection, and availability of reference samples [3, 6]. In most cases, the aftermath of events such as Hurricane Katrina and the 2004 Indian Ocean tsunami, pose even more difficult challenges to identification methods due to extreme heat, humid conditions or bodies submerged in water, and microbial activity [8, 9]. The responsibility of forensic personnel is to quickly recover all human remains and process the disaster site according to the International Criminal Police Organization (INTERPOL) DVI Guidelines [10].

The process of identifying victims of mass disasters is multidisciplinary, which typically involves forensic pathologists, anthropologists, radiologists, odontologists, fingerprint examiners, and forensic biologists [3, 6, 10, 11]. Other methods and techniques can be employed for screening or triage of victims' remains including visual examination of the body, recognition of the face and markings on the body such as birthmarks, scars

and tattoos, any personal effects recovered with or on the body, and unique medical features such as numbered implants or prosthetics [4, 6]. These methods may not be entirely reliable, especially for severely decomposed or fragmented remains, and therefore are typically used as secondary identifiers. The primary methods of identification include dental and fingerprint comparisons, and DNA analysis [1, 4, 11, 12]. Only experts in these fields can establish and report an identification [11], and often the consensus of an expert panel or reconciliation team is required [13].

Forensic odontology is the science that deals with evidence surrounding dental structures and matching the post-mortem (after death) dental examination with ante-mortem (before death) records [2]. Likewise, fingerprint pattern analysis can generate many positive identifications, in many cases, using the Automated Fingerprint Identification System (AFIS) to search ante-mortem prints [13]. However, in many circumstances, bodies are severely fragmented, commingled, burned, or decomposed. These first two primary methods can only make identifications on largely intact remains, and rely on the availability of previously collected ante-mortem data [2, 4]. But DNA can theoretically be recovered from any biological material [1, 4, 7], and multiple DNA reference sources (personal items or family members) for comparison are often available [3]. Personal items such as a hairbrush, toothbrush, or unlaundered clothing may be used for direct comparison; however, if these items are not available, family members of the victim can provide DNA samples for identification via kinship analysis [14]. Therefore, forensic DNA typing is usually the last identification method to be employed, as it is the most timely and costly of the three primary methods. However, when other methods are exhausted, DNA analysis is always performed.

DNA Typing

The process of forensic DNA typing involves five steps consisting of DNA extraction, DNA quantification, short tandem repeat (STR) amplification, capillary electrophoresis that separates and detects the amplified products resulting in a STR profile, and data analysis. STR typing has been the gold standard in forensic DNA analysis for human identification since 1993 after the Waco siege, when the more than 70 people that died in a fire outbreak were identified by DNA analysis [3, 4]. STRs are small stretches of DNA units typically between two and seven nucleotides in length (eg. TCAT), which repeat a variable number of times within a particular region in the genome [15]. The number of times the repeat occurs at each site (locus) is variable among unrelated individuals, and the human genome contains thousands of these STR markers spread across all chromosomes [16]. With the advancement of the polymerase chain reaction (PCR), multiple STR loci can be simultaneously amplified (multiplexing) from a small quantity of DNA resulting in complete STR profiles in the vast majority of high-quality DNA samples [7, 15, 17]. In the forensic community, a core set of thirteen loci were originally selected for human identity testing and databasing in the United States via the Combined DNA Index System (CODIS) [3]. However, the CODIS core loci has more recently expanded to 20 loci making it more useful globally and increasing the data sharing efforts with international law enforcement [18-20]. The combination of these core STR markers enable a higher power of discrimination for profile "matching" and identification purposes to reduce the likelihood of adventitious matches [18, 19].

In addition, multiplexing of these 20 loci is achieved by utilizing different fluorescent dyes and varying the sizes of PCR products [15]. Current commercial STR kits

have expanded from 5-dye to 6-dye chemistries in order to incorporate more loci (20-24) under 450 base pairs (bp) in a single PCR reaction [18]. The 6-dye STR kits used within the USA include Investigator® 24plex QS Kit (QIAGEN, Hilden, Germany), GlobalFiler™ PCR Amplification Kit (Thermo Fisher Scientific, Waltham, MA, USA), and PowerPlex® Fusion 6C System (Promega, Madison, WI, USA). These newer commercial STR kits have more sensitive chemistries, are more tolerant to PCR inhibitors, and have shorter processing times (60-80 minutes) [18]. However, successful STR typing is still impacted by severely degraded template (<200 bp) and low amounts of DNA (<100 pg) [21].

DNA Damage and Degradation

In routine casework, a sample from the deceased would be collected in the morgue and stored at very low temperatures (<4°C) to inhibit DNA degradation by enzymatic and microbial activity [17]. As the level of sample degradation increases DNA breaks into smaller and smaller fragments, typically resulting in the longer loci (>250bp) failing to amplify during PCR producing partial STR profiles [17, 22, 23]. The reduced genetic information results in a lower power of discrimination for identification, and may also increase the chances of adventitious matches [17], especially between family members that are victims in the same mass disaster. The use of mini-STRs has become the most common approach for increasing the success rate from highly degraded DNA samples since the World Trade Center attacks in 2001, where severe disruption of remains led to unsuccessful STR typing [4]. With mini-STRs, the target amplicon size is reduced by moving the PCR primers closer to the repeat sequence [17, 21]. Commercial mini-STR kits such as AmpFℓSTR® MiniFiler™ PCR Amplification Kit (Thermo Fisher Scientific) and PowerPlex® S5 System (Promega) were developed specifically for the forensic market to

improve the profiling success from degraded DNA templates by complementing standard autosomal STR kits when the larger loci in these kits failed to amplify [15]. However, autosomal STR kits such as GlobalFiler™, PowerPlex® Fusion, and Investigator® 24plex QS have been designed to maximize the number of small loci (<220bp) in each kit to more successfully amplify degraded and problematic samples [18].

Other approaches for genotyping highly degraded samples include the analysis of single nucleotide polymorphisms (SNPs) [7, 14, 21, 23-25] and mitochondrial DNA (mtDNA) [7, 14, 26]. Analysis of SNPs may provide higher success with severely degraded DNA as the amplicons are very short (45-80bp) [23, 25], whereas mitochondria are organelles that contain maternally inherited DNA and are abundant in cells compared to a single copy of nuclear DNA [26, 27]. In addition, maternal relatives separated by several generations can be used for comparison due to lack of recombination [26, 28]. For this reason, mtDNA is commonly performed in mass disasters, missing persons cases, or war remains for HID of skeletal remains [26, 28, 29]. An analysis method relatively new to the field of forensic biology is massively parallel sequencing (MPS). This technology holds great promise for forensic analysis, as it enables deeper interrogation of the genome in multiple samples in parallel [7, 30]. MPS has been used to analyze samples for HID purposes using identity markers such as SNPs, microhaplotypes, STRs, and mitochondrial DNA [25-27, 31-34]. However, these alternate methods for genotyping highly degraded samples were not an area of focus for this project.

In order to maximize the STR typing success from biological material, analysts aim to amplify adequate amounts of DNA template (0.5ng-1ng) that is as pristine as possible (little or no DNA damage, degradation, or PCR inhibition). Although DNA can be

recovered from essentially all biological tissues (except red blood cells), the condition of the sample is an important factor in determining the quantity and quality of the DNA retrieved [1, 5]. This is a significant consideration when identifying human remains after a mass disaster as severe DNA damage and degradation can occur in tissues exposed to harsh environmental conditions [4, 22].

When a cell or an organism dies, the DNA in each cell is susceptible to damage and degradation by endogenous nucleases and exogenous insults such as microbial activity [12]. Cell death is a result of apoptosis (programmed cell death) or necrosis. Characteristic cell changes after apoptosis include condensation of cytoplasm, nuclear fragmentation, chromosomal DNA fragmentation, and an overall reduction in cell volume [35]. Necrosis of cells is a passive energy process and is accelerated by higher ambient temperatures. This pattern of cell death is associated with a temporary increase in cell volume, swelling of cytoplasmic organelles, and condensation of chromatin, which causes rupturing of the cell membranes, organelle breakdown, and leakage of lysosomal enzymes. As a consequence, the DNA is released into the surrounding environment and exposed to damaging enzymes and external insults resulting in a random pattern of degradation [35]. Other forms of DNA damage that may complicate DNA typing include radiation, oxidative and hydrolytic damage causing base modifications, strand breaks, crosslinks, and mismatches [22, 35].

Prolonged exposure to heat, ultraviolet (UV) radiation, humidity, and microorganisms further increases the rate of DNA damage and degradation [4, 22, 36, 37]. Microbial activity is common in humid environments and increases the vulnerability of the DNA to degradation processes like hydrolytic damage [35, 36]. The bond between the deoxyribose backbone and nucleic bases is most susceptible to cleavage and hydrolytic

attacks leading to loss of bases [22, 35, 38]. Single strand breaks or "nicks" in the DNA can lead to fragmentation (double strand breaks) if they are on opposite strands and in close proximity to one another. Single strand breaks can also result in fragmentation during the first step of PCR (denaturation of double stranded DNA into single strands) [22]. Because STR analysis is based on size differences in the DNA, these damaging processes that lead to fragmentation of the DNA template into progressively smaller pieces, may result in poor or complete failure of PCR amplification [35]. Therefore, successful DNA-based identification is reliant on timely sample collection after death to ensure the optimal tissues are sampled, and adequate preservation of the DNA sample prior to processing.

DNA Collection

The preferred sample type for DNA collection following mass fatality incidents are buccal swabs or blood from fresh remains [1]. When human remains are moderately decomposed or fragmented, human soft tissue is collected including skeletal muscle, organ tissue, and skin [3]. However, hard tissues like bone and teeth (or hair or nails) must be collected when the soft tissues have undergone severe decomposition and putrefaction, or extensive commingling of remains have occurred [1, 3, 8, 39]. DNA methods for bone and teeth are more complicated, costly, time-consuming, and requires specialized equipment and analyst training [29]. Therefore, these samples are typically used as a last resort [1]. Despite their resistance to degradation and damage, extreme environmental conditions can still degrade DNA in bones and teeth [39], but by necessity hard tissues are relied on as the last source of DNA for HID.

Swabs are another type of sample routinely processed by forensic crime laboratories. Various types of swabs are used to collect DNA from biological stains or

items that have been touched in order to link a suspect or victim to a crime scene. Humans shed several thousands of epithelial cells when they come into contact with items [40]. Therefore, these touched evidentiary items may contain tens to hundreds of shed epithelial cells depending on several factors including the amount of time the item was held, whether the person was a good shedder or a bad shedder, and the surface of the item (rough versus smooth surface) [41, 42]. One study by Albujja et al. tested the effectiveness of swabbing the skin surface of living volunteers for reference DNA samples compared to buccal swabs, and found skin surface cells to be a viable source for DNA typing [16]. This principle could be applied to disaster victim identification samples by swabbing the skin of human remains to recover epithelial cells more easily and immediately in the field.

Typically, tissues and swab samples would be housed in a refrigerator until DNA analysis is performed or archived in freezers. However, in the aftermath of a mass fatality incident, local mortuaries and DNA laboratories are not usually prepared to quickly store and identify thousands of victims. In fear that human remains will cause a disease epidemic, decomposing bodies are often buried in temporary mass graves without identification or sample collection [43]. The lapse of time between death and recovery of these remains will complicate DNA typing. Therefore, the condition and preservation of these biological samples collected from the deceased are also important considerations for DNA-based identification [5].

DNA Storage and Preservation

Although DNA is a relatively stable molecule, it can be subjected to degradation by endogenous and exogenous enzymes, microbial activity, hydrolysis, oxidation, and ultraviolet light [22, 35]. It is common practice to store DNA samples at low temperatures

to inactivate damaging nuclease enzymes and slow down other degradation processes [35]. However, in many cases refrigeration of samples may not be possible. Several methods have been developed to store DNA at room temperature for later analysis. Some studies have investigated room temperature storage of swabs prior to DNA analysis [16, 44-47]. Swabs treated with chemicals to lyse cells and inhibit microbial activity have also been studied [45-48]. Another alternate solution may be to collect a small tissue sample from each cadaver immediately in the field and store it on a Whatman® FTA® (fast technology for analysis) card or in a chemical preservative at room temperature until it can be processed weeks or months later. This process could potentially prevent DNA damage and degradation in the tissue sample without the requirement for cold or freezing temperatures.

Classic FTA® cards are treated papers with chemicals intended to capture and lyse cell membranes for stabilization of DNA molecules until later processing [12, 49-51]. On the contrary, FTA® Elute cards are specifically designed to release DNA into solution prior to STR typing [52]. These cards also contain proprietary chemicals to inhibit microbial activity and chelating agents to inactivate nucleases [12, 51]. FTA® cards have been shown to be effective for storing blood and saliva samples at room temperature for long periods of time [12, 51, 53, 54]. Studies have also looked at storage of other biological samples such as vitreous fluid and bone marrow [8], cerebrospinal fluid [55], vaginal fluid [56], organ tissues and tumors [57], and trace DNA samples on steering wheels [58]. However, no studies have looked at decomposing tissues for storage on FTA® cards.

Several studies have also investigated the potential for alternate methods and room temperature storage of biological tissue samples prior to genotyping. These treatments range from dehydration and desiccation to storage in chemical preservatives to inhibit the

destructive nuclease activity that leads to DNA degradation [5, 9]. The utility of commercial products such as RNAlater RNA Stabilization Reagent (QIAGEN) [9, 59, 60] and DNAgard® Tissues & Cells (Biomatrica, San Diego, CA, USA) [9, 59], as well as various in-house solutions have been reported [9, 59]. These in-house preservative solutions include a lysis, storage, and transportation (LST) buffer [1], DESS [9, 59], and TENT buffer [9]. These buffers are quick and easy to make, and contain chemicals that are commonly used in laboratories. This is a substantial benefit when responding to mass disasters, as they often occur unexpectedly, and personnel need to mobilize quickly. Commercial products may not be available for immediate purchase in the large amounts required, or cannot be shipped to isolated locations in a timely fashion. These products could be purchased and stored in preparation for a mass disaster, but they often have short shelf lives (6–12 months) and would require regular restocking. Therefore, the development of an in-house DNA preservative, which can preserve DNA in human tissue samples would be of great benefit for DVI operations.

The constituents of common tissue preservatives include salts (most commonly NaCl), detergents, chelating agents, and alcohols [9, 60]. Salts in solid form desiccate the tissues by removing moisture, which inactivates endogenous nucleases and inhibits microbial growth. In an aqueous form, salt also denatures proteins. Detergents, such as Tween 20, lyse cell membranes and aid in the release of genomic material. Chelating agents, such as ethylenediaminetetraacetic acid (EDTA), inhibit nuclease activity by binding to metal ions that are required for normal function of nucleases. Ethanol is the most commonly used alcohol for tissue preservation because it inhibits microbial activity, removes water from the sample, and denatures proteins [9]. Dimethyl sulphoxide (DMSO)

increases the permeability in tissues and promotes absorption of chemicals across cell membranes, although it is not thought to directly protect the DNA from degradation [9, 60].

Previous studies have shown that the liquid preservatives investigated in this project (LST, TENT, DESS, DNAgard®, and RNAlater) preserve the integrity of DNA within biological tissues to various degrees [1, 9, 60]. However, in order to make the process of DNA extraction faster, it would be ideal if DNA could be isolated directly from the storage solution surrounding the tissue sample. The ability of these tissue preservatives to promote the lysis of tissue and release "free" DNA into solution, while also protecting that DNA in solution from further degradation has not been widely investigated.

One study conducted by Graham et al., 2008 investigated the use of tissue preservatives that could potentially aid DNA-based DVI. Two preservative solutions, LST buffer and Oragene™ DNA self-collection kit (DNA Genotek, Ottawa, ON, Canada), were evaluated based on their ability to preserve fresh human muscle at room temperature over a 12-month period. The findings of this study concluded that both preservatives were effective over this time period. The Oragene™ solution yielded the highest DNA quantities. However, the LST buffer was superior in the quality of DNA recovered from the liquid preservative [1].

The utility of tissue preservatives to suspend DNA damage and degradation without the need for refrigeration was also investigated by Allen-Hall et al., 2012. The DNA quantity and quality was assessed from fresh human muscle in eight preservatives (salt, DESS, ethanol, ethanol with EDTA, TENT buffer, RNAlater, DNA Genotek Tissue Stabilizing Kit, and DNAgard®) stored at 35°C with high humidity (9-26%) to mimic

harsh, tropical climates. The results of this investigation showed that DNA was effectively preserved in the tissue after one month of storage in solid salt, DESS, ethanol-based preservatives, DNA Genotek, and DNAgard®. One interesting observation in that study was that various amounts of good quality DNA was leaching into the surrounding liquid preservative when muscle was stored in DESS, DNA Genotek, and DNAgard®. The ethanol-based preservatives failed to produce DNA in the liquid preservative, but effectively preserved DNA in the tissue. RNAlater was relatively poor at providing good quality DNA for genotyping in tissue, and also failed to leach DNA into the liquid preservative. Of all the preservatives tested in that study, the TENT buffer yielded the highest quantities of DNA in the surrounding solution, but the DNA degraded quickly, resulting in partial STR profiles [9]. In addition, several tissue preservative solutions have been shown to be effective for fresh human tissue [1, 9]. However, none have been tested on decomposed cadaveric tissue.

If DNA from decomposing human tissues can successfully be preserved from further DNA degradation processes either in chemical preservative or bound on swabs or FTA® cards, then the lengthy tissue digestion step can be avoided and DNA extraction and purification methods can be performed more rapidly.

DNA Extraction and Rapid Purification Methods

The traditional DNA analysis workflow includes DNA extraction as the first step to purify DNA from cellular debris and remove PCR inhibitors that interfere with downstream STR typing [61]. Several methods of DNA extraction such as organic extraction with phenol-chloroform, Chelex®, and silica based methods have been used by the forensic DNA community for several decades [7, 39, 61]. Organic extractions are

inexpensive but require toxic chemicals such as phenol-chloroform [39]. In addition, Centricon® spin columns are often used as part of this extraction to clean up and concentrate the DNA, but significant loss of DNA has been contributed to Centricons® [42]. The Chelex® method is based on an ion-exchange principal, where polar resin will bind to polar substances, and denatured non-polar DNA and RNA will remain in solution [7]. Chelex® is sometimes preferred as it is quick and does not require toxic chemicals, but this method does not efficiently remove PCR inhibitors in the sample [7, 61]. In fact, the Chelex® resin is itself a PCR inhibitor, capturing ions such as Magnesium (Mg2+) ions required for the Taq polymerase during PCR [61]. In addition, studies have shown that cell free DNA obtained in many biological samples is lost in the supernatant during Chelex® extractions, resulting in a reduction of starting DNA material [62].

Silica-based extractions like QIAGEN's QIAamp® DNA Investigator Kit follow four basic steps: 1) disruption of cell membranes with enzymes, heating and shaking, 2) binding of DNA to a silica column in the presence of chaotropic salts, 3) several washes with buffers to remove proteins and contaminates, and 4) elution of DNA off the silica column into a new tube [7, 61]. This extraction method is the most successful in removing PCR inhibitors and concentrating the DNA in a small volume (50-100µL). The numerous steps of chemical additions, incubations, and centrifugation make this extraction method laborious and time consuming, especially when hundreds of samples require processing [39]. In addition, it has been reported that irreversible binding of DNA to the silica columns can lead to significant loss of sample, especially for samples already in low template amounts [63, 64]. In a similar fashion, DNA purification using silica-coated magnetic beads has emerged as one of the most popular approaches for DNA extraction in forensic

laboratories [7]. The method is based on DNA binding to the silica-coated magnetic beads in a certain ionic charge, while unbound contaminates are removed [7]. These DNA purification systems also allow for automation via various DNA extraction platforms, thereby reducing human manipulation and the risk of personnel error [7, 39, 61].

A relatively new rapid DNA extraction platform, the PDQeX System (ZyGEM Hamilton, New Zealand) [65], with enzyme-based buffers is currently being explored by the forensic DNA community [66, 67]. This novel DNA extraction utilizes the enzymatic activity of Antarctic Bacillus sp. EA1, which is used to lyse cells and degrade nucleases and proteins in a sample in under 20 minutes [66]. A cocktail of other enzymes is customized for particular DNA samples, such as animal tissues vs plant tissues, and also used to help lyse cells to obtain the DNA [65]. This instrument utilizes several incubation steps, where the various enzymes function at certain temperatures and deactivate at higher temperatures (>95°C) during the final step [66]. The resulting lysate contains DNA, cellular debris, and denatured proteins that is then extracted through a proprietary column designed to remove PCR inhibitors and enzymes [65].

Although most current STR kits have been developed to be more sensitive to lower amounts of DNA template and more tolerant to common PCR inhibitors, a robust and valid DNA extraction or rapid purification method should ideally yield sufficient DNA quantities and remove PCR inhibitors from the sample in order to produce complete STR profiles.

PCR Inhibitors

PCR inhibitors are often co-extracted with DNA complicating downstream analysis. Common PCR inhibitors recovered from forensic casework samples include indigo dyes from clothing and denim, melanin from hair, bile salts in feces, hemoglobin

from blood, humic acid from soil, myoglobin from skeletal muscle [68], calcium and collagen from bone [39]. In addition, chemicals on swabs and FTA® cards used to lyse cells and stabilize DNA could also contribute to PCR inhibition, making direct PCR more challenging [49]. Similarly, many chemicals used during the DNA extraction process itself such as EDTA, phenol/chloroform, salts and detergents can also cause PCR inhibition [39].

PCR inhibitors vary in their activity by affecting the template DNA, nucleotides, primers, Mg2+ and/or the DNA polymerase [69]. Severe inhibition can cause complete amplification failure, and produce false negative profiles, or produce similar patterns as severely degraded DNA, where most commonly smaller loci are preferentially amplified [35, 70]. However, some PCR inhibitors also have a tendency to affect particular sequences of DNA and the dropout of alleles can be more random. Some of these PCR inhibitors (such as collagen and urea) have a mixed mode of inhibition, affecting both the DNA template and the DNA polymerase. However in general, most PCR inhibition will result in the drop-out of larger loci. This is most likely because longer sequences require more interaction with the DNA polymerase and there is more sequence variation [70].

One common technique to eliminate PCR inhibition prior to amplification is simple dilution of the sample to reduce the amount of inhibitor added to the reaction [69, 70]. However, this also dilutes the DNA in a sample. When a sample with high amounts of good quality DNA is diluted, no negative affect on the downstream STR profile is observed, but dilution may significantly affect the genotyping success of low template samples [70, 71]. Another common strategy to overcome PCR inhibition is the addition of Bovine Serum Albumin (BSA) to the PCR reaction [39, 69]. BSA has been found to significantly reduce PCR inhibition and increase alleles reported during STR typing [39]. Due to the

effectiveness of this PCR enhancer, most forensic STR amplification kits contain BSA in their PCR master mixes [39, 72, 73]. Other methods that remove PCR inhibitors include silica-based methods (QIAquick® Spin Columns and MinElute® Spin Columns, QIAGEN) including magnetic beads, synchronous coefficient of drag alteration (SCODA), and organic extraction with phenol-chloroform [71].

Although the DNA extraction methods commonly used within forensic laboratories are robust and have been shown to effectively remove many PCR inhibitors, some samples may still contain various levels of inhibitory agents [71]. Therefore, the assessment of sample quality is necessary before timely and costly STR typing is performed.

DNA Quantification and Assessment of DNA Quality

The second step in the traditional DNA analysis workflow is DNA quantification to determine the concentration of DNA (ng/μL) in an extracted sample. During the extraction process, other sources of DNA from bacteria, fungi, or other animals may be co-extracted with the human DNA of interest. Therefore, it is important to quantify the amount of human-specific DNA for downstream genotyping [74]. The amount of human DNA must be normalized prior to PCR because of the narrow concentration range (0.5-1ng) for optimal STR typing [74, 75]. Too much DNA will result in sample overload with artifacts such as split peaks, off-scale markers, exaggerated stutter peaks, and pull-up of peaks from other dye channels making interpretation of true alleles in the electropherogram difficult, while too little DNA input will most likely result in the significant loss of reportable alleles or false negative results [75].

A wide range of DNA quantification methods exist; however, methods such as UV spectrophotometry, PicoGreen™ assay, agarose gel electrophoresis, and slot blot

hybridization are unable to distinguish human DNA from non-human DNA and/or assess intact DNA versus degraded [74]. With the advancement of PCR, a more sensitive and human-specific quantification method called real-time or quantitative PCR (qPCR) was developed. During qPCR, the concentration of amplifiable DNA in a sample is determined rather than the total amount of DNA in a sample [74, 75]. The qPCR instrument consists of a thermal cycler, an integrated excitation light source, a fluorescence detection system, and typically connected to a computer with analysis software that displays the results [76]. The process works by incorporating fluorescent dyes into the reaction, either as a double strand DNA (dsDNA) intercalating dye or a fluorophore-labeled probe. SYBR® Green is an intercalating dye that binds to the minor groove of dsDNA, and as the target products are amplified cycle by cycle, more fluorescence is incorporated into the increasing copies of dsDNA and the fluorescence signal increases. Because SYBR® Green binds to all dsDNA, it will also bind to nonspecific products and primer-dimers, and as a result may overestimate the DNA in a sample. On the contrary, fluorophore-labeled probes are oligonucleotides that contain a fluorescent reporter dye on the 5'end suppressed by a quencher on the 3' end, which bind to a specific sequence of DNA between the forward and reverse primer. As the DNA polymerase extends the new sequence using a single strand of DNA as a template, the reporter dye is cleaved and emits fluorescence [75, 76]. The most common use of such primer and probe combinations is known as the TaqMan® assay, and is used by most of the commercial forensic DNA quantification systems [74, 75, 77-79].

The qPCR process occurs in four phases: baseline, exponential, linear, and plateau [75]. The baseline phase occurs at the beginning when the signal of fluorescence is similar

to normal background levels, and when the fluorescence increases beyond these levels, the process enters the exponential phase [75]. A cycle threshold (CT) is set at the beginning of this phase, and the starting DNA template is measured in an inversely proportional fashion to the number of amplification cycles at which this occurs for each sample [74, 75]. During the linear phase, the reagents required for PCR are in sufficient quantities for amplification of DNA to continue, but as the reagents are depleted then amplification of products begin to slow down and enter into the plateau phase. During the exponential phase, reagents are at their optimum concentrations and amplification of DNA product is theoretically performing at 100% efficiency. Therefore, the exponential phase is where fluorescence versus cycle number is measured. The CT value at which this occurs is then compared to a set of known concentrations of DNA (DNA standards) to determine the DNA concentration of the sample [75]. In forensic DNA analysis, qPCR amplification of a small DNA target (approximately 80-90bp) is used to determine how much DNA sample should be added to a downstream assay.

Multiplexing qPCR using multiple fluorescent dyes allows for more than one target to be amplified in order to assess more information about the quality of a given DNA sample, such as detecting any PCR inhibition [76]. The Quantifiler Human DNA Quantification Kit (Thermo Fisher Scientific) was one of the first multiplex qPCR kits specifically designed for forensic use, utilizing two TaqMan® probes labelled with two different reporter dyes. One dye is used to detect the amplified sequence of a human autosomal target (human telomerase reverse transcriptase gene, hTERT) while the other detects a synthetic piece of DNA not resembling any sequence in the human genome (serving as an internal PCR control, IPC) [80]. The IPC is contained in the master mix and

amplified simultaneously with the human DNA sample. Therefore, the IPC amplification should be constant for all samples if there is no PCR inhibition [70, 80]. Any delay in amplification as detected by an increased CT value for a sample compared to the known, pristine DNA samples (standards) indicates PCR inhibition (see Fig. 1.1.) [70].

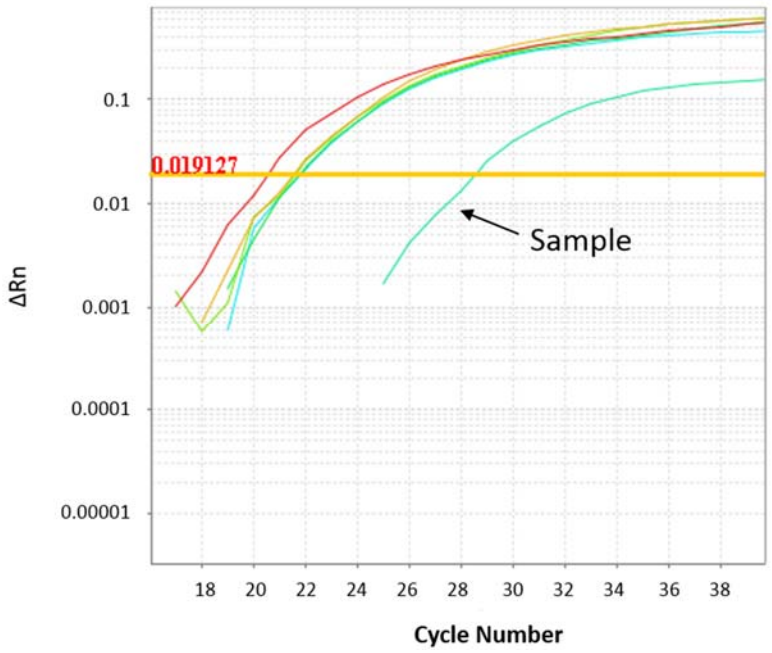

Fig. 1.1. An example amplification plot from HID Real-Time PCR Analysis Software, where fluorescence signal (ΔRn) is plotted against cycle number. The horizontal line is CT. The figure shows a delay in a sample's IPC target amplification in green compared to the five standards on the left, indicating a delay of approximately 6 cycles

Prior to the addition of an IPC target in qPCR, negative quantification results could not be distinguished between lack of DNA, or PCR inhibition [80]. In addition to detecting PCR inhibition, forensic DNA quantitation kits have been expanded to also detect other quality metrics such as the level of DNA degradation. Therefore, samples can be simultaneously assessed for sample quantity and quality, making it an important tool for triaging samples. Analysts may decide based on the quantification results to re-extract or

dilute a sample prior to STR typing or employ a totally different analysis method such as Y-STR typing, mitochondrial DNA, SNP, or INDEL typing.

More recently, commercial DNA quantification kits used in forensic laboratories include four targets: 1) a small human autosomal target, 2) a large human autosomal target, 3) a male (Y-chromosome) target, and 4) and an internal PCR control (IPC). Current kits on the market include the Quantifiler Trio DNA Quantification Kit (Thermo Fisher Scientific), Investigator® Quantiplex Pro Kit (QIAGEN), PowerQuant® System (Promega), and InnoQuant® HY (InnoGenomics Technologies). The small autosomal target is typically employed to normalize the DNA input for PCR, while the large target is used to determine the level of DNA degradation based on the ratio of the concentration of the small DNA target over the large DNA target (degradation index, DI). The male target is used to determine the amount of male DNA in a given sample or determine if a mixture of female and male DNA is likely present based on a ratio of the small human DNA target concentration to male DNA. Lastly, the IPC target in these kits is simultaneously amplified with the human targets to detect PCR inhibition.

The various DNA quantitation kits vary in their design. Differences in the human and IPC targets chosen, and buffer chemistry will affect the quantitation results, sensitivity, and tolerance to PCR inhibitors for each kit. For example, a recent study showed that an IPC target with a long sequence would provide an increased detection for PCR inhibitors [70]. However, the IPC sequence is often proprietary. Therefore, it would be beneficial for laboratories to test each qPCR kit and decide which kit is best suited for their application and sample types. For example, this research project would benefit from using a qPCR kit that has a small target tolerant to the chemicals and buffers used in this study in order to

provide the most accurate quantification result, or at the very least one that is a reliable predictor of inhibition in the sample. However, other applications may benefit more from a qPCR kit that better predicts the level of DNA degradation, or is able to detect the presence of minute amounts of male DNA in mixtures.

DNA quantification is required by the Federal Bureau of Investigation (FBI) for all samples other than reference samples [81]. Therefore, for DVI samples these informative DNA quantification assays could serve as a tool for triaging samples based on the quantity and quality of DNA for more direct amplification and maximum first pass success rates.

Standard PCR and Direct Amplification of DNA Samples

In order to simplify and standardize procedures and STR markers for CODIS and international databases, commercial STR kits include pre-mixed primers (including those for the core CODIS loci) and a cocktail of DNA polymerase, dNTPs, and other required constituents and buffers [15]. In previous years, amplification of samples would take three to four hours amplifying 15 STR loci simultaneously [82, 83]. However, current STR kits now only take one to two hours to perform amplification of more than 20 loci in a single reaction with the use of fast chemistries and more rapid cycling conditions. The new chemistries include polymerases with faster activation times and increased extension rates to shorten the overall time of amplification [83].

Recent studies comparing current commercial STR kits reported that the GlobalFiler™ Kit offers the highest sensitivity with low template DNA, the Investigator® 24plex QS Kit demonstrated the highest tolerance to common inhibitors [18, 84], and the PowerPlex® Fusion 6C Kit generated better heterozygote balance which would benefit mixture samples [18].

On the other hand, direct amplification (direct PCR) is the addition of DNA samples without prior DNA extraction and quantification directly to PCR reactions [85, 86]. Direct amplification is currently utilized in forensic DNA laboratories for single source reference and databasing samples. This method utilizes amplification of the same CODIS loci, but the analysis time is greatly shortened by skipping DNA extraction and quantification steps altogether [49, 85, 87]. For example, databasing laboratories can process samples in less than 2 hours [87]. In addition, labor and reagent costs can be significantly reduced for these types of samples.

As forensic databasing samples do not undergo a DNA extraction step to produce pure DNA extracts for amplification, improvements are made in the direct PCR kits' chemistry in order to address the challenges of "dirty" DNA samples and presence of inhibitors that would normally be extracted out of the sample [49, 51, 54]. In addition, these kits are more tolerant with large quantities of DNA input (up to 10 ng) [86]. Current direct PCR kits include PowerPlex 18D (Promega), IdentiFiler Direct and GlobalFiler Express (Thermo Fisher Scientific), Investigator 24plex GO! and ESSplex SE GO! (QIAGEN) to meet the demand of rapidly amplifying reference samples worldwide [82].

Unlike any of the other direct STR kits available, the Investigator® 24plex GO! Kit contains two novel quality sensors (QS1 and QS2) to monitor PCR success and detect PCR inhibition in a sample (See Fig. 1.2.) [18, 84, 88]. This kit provides an advantage for direct PCR analysis when DNA quantification and assessment of the sample quality is not performed. These quality sensors therefore serve in the place of an IPC target in quantification to determine PCR success or failure. For example, if drop-out of larger loci is observed in the profile but the quality sensors are both present (and balanced) then the

sample most likely contains degraded DNA, whereas if the sample shows a similar pattern but one or both sensors' peaks are missing from the profile then the sample most likely contains PCR inhibitors.

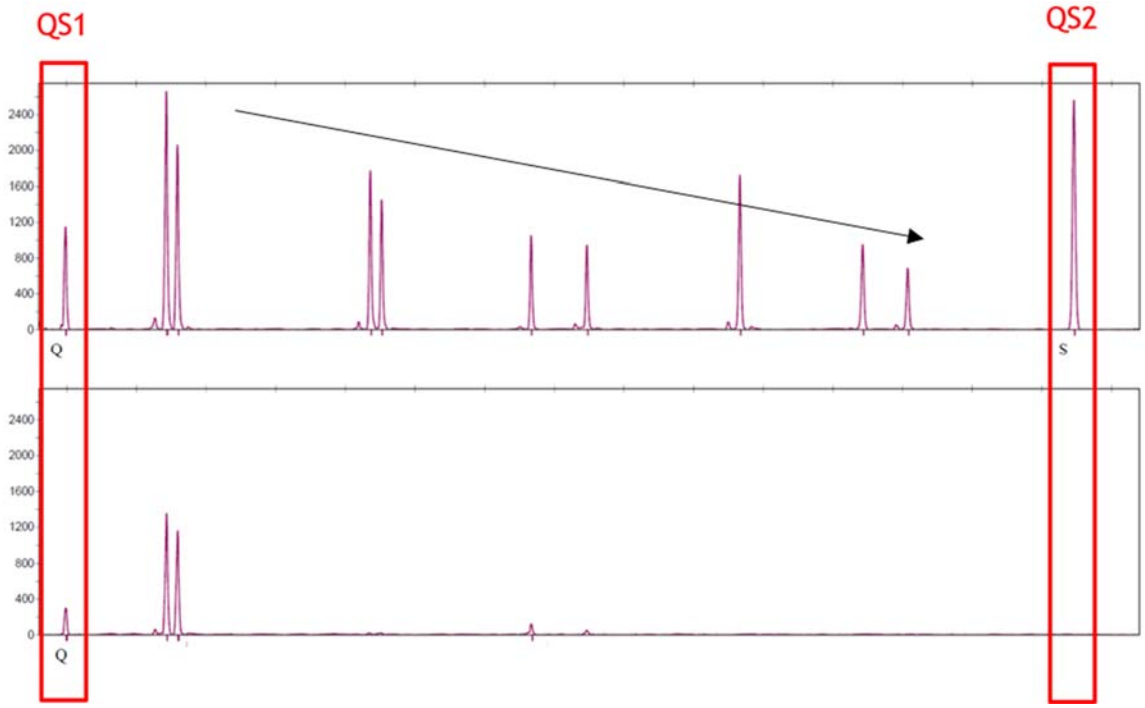

Fig. 1.2. A representative electropherogram (purple channel only) containing the quality sensors, which indicate PCR success and minor DNA degradation via ski-slope (top) versus PCR failure due to inhibition of the QS2 sensor (bottom)

Direct PCR kits are designed for use with FTA® cards and swabs [50, 54, 87, 88]. But direct PCR has also proven to be a successful approach for touch samples that may be degraded and low template, as well as difficult samples containing PCR inhibitors [63, 85, 86, 89, 90]. Significant amounts of DNA are lost during the extraction and quantification steps [86]. Furthermore, cell-free DNA that could contribute to more successful DNA typing for low template samples can also be lost during extraction [62, 86]. Therefore, the Organization of Scientific Area Committees for Forensic Science (OSAC) has recently

published a document requesting more research for optimization of forensic casework type samples and direct amplification, especially for low template samples [91]. This recommendation and shift in the forensic field may also be useful for other DNA-based human identification samples such as those encountered after mass disasters.

Statement of the Problem

One of the most important considerations following mass fatality incidents is the recovery and identification of human remains in a timely manner. Ideally, bodies (and DNA samples) are collected in a laboratory and stored at freezing or refrigerated temperatures prior to DNA typing. However, human remains may be subjected to harsh environmental conditions following mass disaster situations. In addition, DNA laboratories may take several months to set up before they can begin to process or transport samples, and the DNA in those tissues will begin to degrade and fragment into smaller pieces making successful DNA typing more difficult. Often by the time the bodies are retrieved, the soft tissues are too decomposed to be used for STR typing, and therefore DNA laboratories must rely on sampling the skeletal remains (bone and teeth) in order to obtain enough DNA for STR or mitochondrial DNA typing. However, these methods are more laborious, time consuming, expensive and require specialized equipment and analyst training. Therefore, methods need to be optimized for faster and easier collection of DNA samples in-field that can also be preserved without refrigeration. Because DNA analysis is the most costly and time-consuming DVI method, this process would also benefit from faster and more cost-efficient methods.

The aims for this project focused on providing solutions to the aforementioned problems of sub-optimal DNA based methods for DVI are:

1. Evaluation of several commercial and home-made DNA preservatives that facilitate lysis of tissue from fresh and decomposing human remains, leaching of DNA into solution, and preservation of that DNA from further degradation for successful STR typing.
2. Comparison of DNA preservatives based on their ability to facilitate lysis of tissue and leaching of DNA, thereby skipping DNA extraction completely, allowing direct PCR methods for successful STR typing.
3. Identify the concentration of salt in the TENT buffer that facilitates better DNA preservation but also allows successful direct PCR amplification.
4. Evaluation of several forensic DNA quantification kits to compare their ability to detect DNA degradation and inhibition in challenging samples.
5. Assessment of in-field collection and preservation methods for tissues in liquid preservative and on FTA® cards that produces comparable STR typing results using both traditional DNA analysis and direct PCR methods.
6. Investigation of methods to directly collect DNA from decomposing human tissues using various types of swabs and processed using both traditional and direct DNA analysis workflows for successful STR typing.

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CHAPTER II

Preservation and Rapid Purification of DNA from Decomposing Human Tissue Samples[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1]Sorensen A, Rahman E, Canela C, Gangitano D, Hughes-Stamm S (2016) Preservation and rapid purification of DNA from decomposing human tissue samples. Forensic Sci Int Genet 25:182-190.

Abstract

One of the key features to be considered in a mass disaster is victim identification. However, the recovery and identification of human remains are sometimes complicated by harsh environmental conditions, limited facilities, loss of electricity and lack of refrigeration. If human remains cannot be collected, stored, or identified immediately, bodies decompose and DNA degrades making genotyping more difficult and ultimately decreasing DNA profiling success. In order to prevent further DNA damage and degradation after collection, tissue preservatives may be used. The goal of this study was to evaluate three customized (modified TENT, DESS, LST) and two commercial DNA preservatives (RNA*later* and DNAgard®) on fresh and decomposed human skin and muscle samples stored in hot (35°C) and humid (60–70% relative humidity) conditions for up to three months. Skin and muscle samples were harvested from the thigh of three human cadavers placed outdoors for up to two weeks. In addition, the possibility of purifying DNA directly from the preservative solutions ("free DNA") was investigated in order to eliminate lengthy tissue digestion processes and increase throughput. The efficiency of each preservative was evaluated based on the quantity of DNA recovered from both the "free DNA" in solution and the tissue sample itself in conjunction with the quality and completeness of downstream STR profiles. As expected, DNA quantity and STR success decreased with time of decomposition. However, a marked decrease in DNA quantity and STR quality was observed in all samples after the bodies entered the bloat stage (approximately six days of decomposition in this study). Similar amounts of DNA were retrieved from skin and muscle samples over time, but slightly more complete STR profiles were obtained from muscle tissue. Although higher amounts of DNA were recovered from

tissue samples than from the surrounding preservative, the average number of reportable alleles from the "free DNA" was comparable. Overall, DNAgard® and the modified TENT buffer were the most successful tissue preservatives tested in this study based on STR profile success from "free DNA" in solution when decomposing tissues were stored for up to three months in hot, humid conditions.

Keywords: Forensic science, STR typing, Tissue preservation, Disaster victim identification (DVI)

Introduction

Mass disasters may occur due to natural (hurricanes, tsunamis, and earthquakes), accidental (airplane and train crashes), or man-made events (terrorist attacks and war) and may result in the loss of hundreds or thousands of lives [1,2]. In hot and humid climates, rapid decomposition and putrefaction of bodies create a public health risk, and also makes human identification more difficult [3]. As bodies decompose, DNA starts its degradation process. When a cell or an organism dies, the DNA is susceptible to damage and degradation by endogenous cellular nucleases and exogenous insults. Oxidative and hydrolytic damage may cause base modifications, strand breaks, crosslinks, and mismatches [4,5]. Prolonged exposure to heat, humidity, ultraviolet (UV) radiation, and microorganisms further increases the rate of DNA damage and degradation resulting in fragmentation of the DNA molecule into smaller lengths [4,6,7]. Although DNA typing using short tandem repeats (STRs) is currently the gold standard for human identification purposes [3], highly degraded samples often result in partial STR profiles because the larger loci (>250bp) commonly fail to amplify due to fragmentation of the DNA structure [4–8]. Successful DNA typing of mass disaster victims may therefore be reliant on the speed of sample collection and the immediate preservation of the sample [1,2]. This is particularly true in tropical climates, as warm temperatures and high humidity accelerate the entire decomposition process [9], and therefore storage of samples at 4°C or -20°C conditions are recommended to prevent further DNA damage and degradation [1,10,11]. However, after a mass fatality event, the loss of electricity and lack of adequate storage facilities often prevent this action [2,3,9]. Recommendations published by the DNA Commission of the International Society for Forensic Genetics state that tissue samples

may be stored in a chemical preservative at room temperature (with the exception of formalin) [1]. Several studies have investigated the potential for room temperature storage of biological samples for DNA analysis [2,3,9,11–14]. These treatments range from dehydration and desiccation [3,9,12] to storage in various chemical preservatives in order to inhibit the nuclease activity that leads to DNA degradation [2,9,11–14]. Several preservative solutions have been shown to effectively preserve fresh human tissue [2,3,9,11]. However, none have been tested on decomposed human tissues. Therefore, three customized (modified TENT, DESS, LST) and two commercial DNA preservatives (RNA*later* and DNAgard®) were evaluated in this study to determine which solutions most effectively preserve DNA in skin and muscle tissues from decomposing human cadavers. The quantity and quality of DNA released from these tissues into the preservative solution ("free DNA") were also investigated. If DNA of high quantity and quality can be extracted directly from the preservative solution, this will reduce sample-processing time by avoiding the long tissue digestion process prior to DNA purification. Previous studies have shown that DNA can be released from tissues into chemical preservatives ("free DNA") [2,9,14], but the quantity and quality of that DNA may not be adequate for successful genotyping, or stable over time. A study by Allen-Hall [9] reported that high amounts of DNA leached into solution when tissues were stored in TENT buffer, but downstream STR profiling often failed suggesting that TENT did not preserve the "free DNA" during storage. Therefore, in this study we modified the TENT buffer by increasing the salt concentration in an effort to retain the leaching effectiveness of the buffer, but improve preservation of the "free DNA" over time. This study assessed the quantity and quality of DNA released from decomposing human skin and muscle samples into the preservative

solution and compared to the DNA preserved in the tissue itself when stored at 35°C in humid conditions (60–70%) over a three-month period.

Materials and Methods

Sample Collection

Skin and muscle samples were collected from three human cadavers provided by the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, Texas, USA. The cadavers were caged to prevent animal scavenging and left outdoors for two weeks in October 2013. The weather during those two weeks was moderate (high: 24°C, low: 12°C) with high humidity (average 76%). It also rained four days out of the two weeks (Days 0, 1, 3, and 12). Skin samples were taken from the left thigh and muscle tissue was removed from the left quadriceps muscle group (directly under the skin sample) of each cadaver at day 0, 2, 4, 6, 8, 10, and 12.

Chemical Preservation

Skin or muscle tissue (30mg) was added to each of the five preservative solutions (300µL) tested in this study (Table 2.1.). Control samples (no preservative) consisted of tissue (30mg) with 20µL of distilled water to prevent desiccation. Samples were stored at 35°C with relative humidity of 60–70% in a Forced Air Lab oven (SHEL LAB, Cornelius, OR, USA) for one, two, and three months. Control tissue samples (no storage or preservation) were also collected from each cadaver every second day and processed immediately.

Table 2.1. Chemical Preservatives

Preservatives	*Constituents*
DNAgard® Tissues & Cells	Proprietary (Biomatrica)
RNAlater RNA Stabilization Reagent	Proprietary (QIAGEN)
LST Buffer [a]	0.1M Tris-HCL, 0.5M KCl, 4.5% IGEPAL CA-630, 4.5% Tween 20, 1% NaN_3 (100mL, pH 7.5)
Modified TENT	10mM Tris, 10mM EDTA, 2M NaCl, 2% Tween 20 (120mL, pH 8.0)
DESS Buffer [b]	20% DMSO, 0.25M EDTA, saturated NaCl (100mL, pH 8.0)

[a] As described in Graham et al. [2]
[b] As described in Allen-Hall et al. [9]

DNA Extraction

DNA extraction was performed after one, two, and three months of storage. Skin and muscle samples were removed from the preservative solution for separate DNA extraction as described in the next section (*Tissue*). The QIAamp® DNA Investigator kit was used for all DNA extractions. This kit uses MinElute® silica columns to purify double stranded DNA (70bp–4kb) [15].

Tissue

The DNAgard® tissues were washed with ultra-pure sterile water as per manufacturer's instructions before being placed into a new tube [16]. Tissue samples were digested with 30µL of Proteinase K (20mg/mL) and 270µL of ATL Buffer (QIAGEN) and incubated at 56°C overnight as per the QIAamp® DNA Investigator kit recommended protocol for tissues [17] with one minor change. The volume of Proteinase K and ATL Buffer were increased in proportion to a total volume of 300µL, which was required prior to loading onto the QIAcube robotic station (QIAGEN) using the forensic casework

samples protocol B (purification step only). If the tissue was not completely digested, another 10µL of Proteinase K was added and incubated for an average of 4–6h until complete digestion was achieved.

Liquid Preservative

An aliquot (100µL) of the preservative solution was removed from each storage tube and placed into a new tube with 200µL of PB buffer (QIAGEN) prior to loading on the QIAcube (QIAGEN) using the forensic casework samples protocol B (purification step only).

DNA Quantification

DNA quantification was performed on a StepOne™ Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA USA). Each reaction contained 2µL of DNA, 10µL 2X SYBR® Green PCR Master Mix (Thermo Fisher Scientific), 2µL 10µM hTERT primers (63bp amplicon) [18], and 6µL diH_2O. To generate a standard curve, a 1:2 dilution series (9 standards—50ng/µL to 0.0977ng/µL) was prepared using K526 control DNA (Promega, Madison, WI, USA). The cycling conditions were as follows: 10min at 95°C, and 40 cycles of 15s at 95°C then 1min at 60°C. Data were considered reliable if the R^2 value of the standard curve was 0.99 or greater.

STR Genotyping

Genotyping was performed using the AmpFlSTR® Identifiler® Plus PCR Amplification Kit (Thermo Fisher Scientific) according to manufacturer's instructions [19] with the modification of using a half reaction volume (12.5µL). The target amount of DNA template was 0.8ng. For low quantity DNA samples (less than 0.16ng/µL), 5µL of neat DNA extract was added to the PCR reaction. K526 control DNA was used as the positive

control, and sterile water was used as the no template control. PCR was performed on a GeneAmp® PCR System 9700 (Thermo Fisher Scientific) using the recommended cycling parameters [19]. Separation and detection of PCR products was performed using a 3500 Genetic Analyzer (Thermo Fisher Scientific) with a 50cm capillary array and POP7 polymer with an injection time of 8s at 1.6kV. The reaction was prepared by adding 1μL of the amplified product or allelic ladder to a mix of 9μL Hi-Di™ Formamide and 1μL of LIZ 500 (Thermo Fisher Scientific). The samples were denatured using the GeneAmp® PCR system 9700 at 95°C for 3min. Data were analyzed with GeneMapper™ software v4.1 (Thermo Fisher Scientific). An analytical threshold of 100 relative fluorescence units (RFUs) was applied.

Statistical Analysis

DNA quantity was expressed as the concentration of DNA recovered from 30mg of tissue (skin or muscle) and 100μL of neat preservative solution. STR typing success was measured as the percentage of alleles that were called and were concordant with the reference profiles. To assess the degree of DNA degradation a Degradation Ratio (DR) was calculated as the average ratio of the peak heights (RFUs) of the shortest to longest markers in three channels on each STR profile (6-FAM, VIC and NED). The average size of the amplicons in each marker across the three individuals used in this study were 144bp (D8S1179) and 325.9bp (CSF1PO) in the 6-FAM channel, 130.1bp (D3S1358) and 330.1bp (D2S1338) in the VIC channel, and 120.9bp (D19S433) and 298.7bp (D18S51) in the NED channel. A DR calculation was not possible for samples when locus drop out occurred at all longest markers (assigned as UND). Data were tested for statistical significance by Factorial ANOVA analysis with Fisher LSD post-hoc comparisons, using

the software Statistica 12.5 (StatSoft Inc., Tulsa, OK). $P < 0.05$ was accepted as the level of significance.

Results and Discussion

DNA Quantity

Controls

DNA from skin and muscle tissue was extracted immediately after collection on days 0, 4, 6, 8, 10 and 12 to determine the quantity of DNA before storage or chemical preservation (no storage controls). As expected, the amount of DNA recovered from the decomposing tissue declined with time (Fig. 2.1.). Interestingly, a rapid decrease in the amount of DNA recovered was observed in each cadaver after the bodies had initiated the "bloat" stage of decomposition at day 6, although DNA was still recovered until the cadavers reached advanced decomposition at day 12 (Fig. 2.1.). Similar amounts of DNA were recovered from skin and muscle samples (Fig. 2.1.). However, amplifiable DNA was detected for longer time during the decomposition process with skin compared to muscle (days 10 and 8 respectively) (Fig. 2.1.). These data suggest that skin may be a more resilient tissue to decomposition than muscle tissue. This result is consistent with other studies by Michaud et al. [13] and Clare et al. [20]. Tissue samples were also stored under hot and humid conditions for up to three months without chemical preservative (no preservative controls). As expected, the DNA in skin and muscle decomposed more rapidly in the absence of a chemical preservative (Fig. 2.1.). Only fresh tissue (day 0) for all three months of storage yielded amplifiable DNA (45, 10, and 28ng/μL for one, two, and three months storage, respectively for skin, and 27, 13, and 18ng/μL for one, two, and three months storage, respectively for muscle) (Fig. 2.1.). Tissue samples that were already decomposing

when collected (day 4 to day 12) did not yield amplifiable DNA when stored without any chemical preservative. Not surprisingly, these data indicate that decomposing tissues stored in hot and humid conditions without any chemical preservative degrade rapidly over time.

Tissue

The results of average DNA concentrations extracted from skin and muscle samples from three cadavers stored for up to three months in various preservatives are shown in Fig. 2.1. In general, common trends were observed in DNA quantity from skin and muscle samples for each preservative. DNA yields from skin and muscle samples over time of decomposition and storage were similar, and a decrease in DNA quantity was observed with each month of storage in all preservatives (Fig. 2.1.). All preservatives (except for LST) effectively preserved adequate amounts of DNA for STR typing (>0.2 ng/μL) in fresh and decomposed skin (up to day 10) for up to three months of storage (Fig. 2.1.). The LST buffer failed to preserve the DNA in skin after Day 6 and in muscle after day 4 for all three months of storage (Fig. 2.1.). Compared to the other four preservatives tested, RNA*later* yielded the highest DNA concentrations in skin and muscle for up to three months of storage (Fig. 2.1.). However, these results differ from those previously reported by Allen-Hall et al. [9], which found RNAlater to be relatively poor at preserving DNA for STR typing in fresh muscle tissue. These opposing results may be attributed to different DNA extraction methods, tissue amounts, and preservative volumes used in both studies. Higher amounts of DNA were recovered from skin tissue than from muscle in RNA*later*, and for longer in the control samples, also confirms that skin is a more resilient tissue (Fig. 2.1.). Factorial ANOVA showed that tissue type ($F_{6,67}$ = 7.8, p = 0.000002), choice of preservative ($F_{30,270}$ = 5.2, p = 0.0000001), and time of storage ($F_{12,1347}$ = 4.2, p = 000016)

all had statistically significant effects on the amount of DNA recovered from preserved tissues.

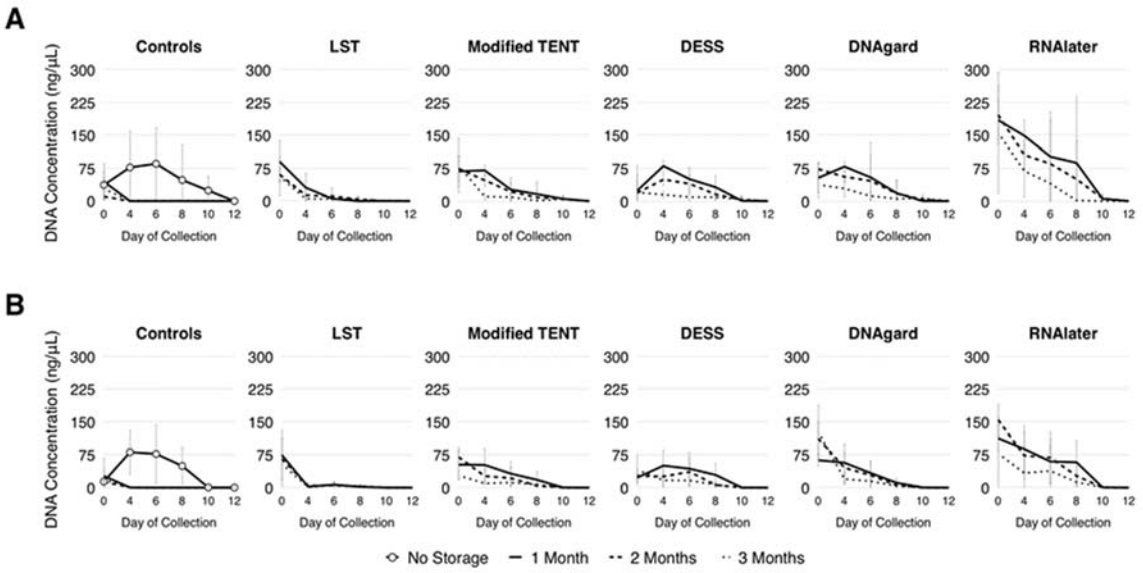

Fig. 2.1. Average DNA concentration (ng/µL) (± SD) of the DNA from (A) skin and (B) muscle samples stored for up to three months at 35°C and 60-70% humidity without preservation (controls) or in the five preservatives tested in this study (LST, modified TENT, DESS, DNAgard®, and RNA*later*)

Liquid Preservative

DNA was extracted directly from each preservative solution for skin and muscle samples stored for up to three months (Fig. 2.2.). As previously shown in Fig. 2.1., higher amounts of DNA were obtained from the tissues preserved in RNA*later* compared to the other preservatives tested. However, no "free DNA" was detected in the RNA*later* solution (Fig. 2.2.). This observation was also previously reported by Allen-Hall et al. [9]. Adequate amounts of DNA for STR typing (greater than 0.16ng/µL), were detected in all preservatives (except for DESS skin samples) for up to 8 days of decomposition stored for up to three months (Fig. 2.2.). The amount of purified DNA from preservative solution was lower than that extracted from tissue. The amount of "free DNA" recovered from DESS

was much higher for muscle when compared to skin (Fig. 2.2.). These data suggest that DESS may better promote lysis and release of DNA into solution from softer tissue than from more resistant tissues such as skin. Higher amounts of "free DNA" were retrieved from modified TENT and DNAgard® across time (day 0 to day 10) and storage (one, two, and three months) compared to the other preservatives tested (Fig. 2.2.). DNA yields from skin and muscle stored in these preservative solutions were relatively stable for up to three months, with little decrease in the amount of DNA recovered with longer storage (in fact, slight increases were observed in some cases; Fig. 2.2.). In addition, the "free DNA" recovered directly from DNAgard® skin samples did not notably decrease in quantity over the three month storage time (Fig. 2.2.). High amounts of DNA were recovered from the LST buffer after one month of storage. However, the DNA concentration in the LST buffer substantially decreased with two and three months of storage. This observation suggests that although LST promotes efficient release of DNA into solution, the buffer is unable to prevent further DNA damage and degradation over time. Factorial ANOVA analysis showed that tissue type ($F_{6,55} = 4.0$, $p = 0.002$) and choice of preservative ($F_{24,1937} = 3.4$, $p = 000001$) had significant effects on the amount of DNA recovered directly from liquid preservatives. In addition, the interaction between tissue and preservative was found to be significant ($F_{24,1937} = 1.9$, $p = 0.001$). Overall, the amount of DNA retrieved from all samples (tissue and "free DNA") decreased over time (days of decomposition) regardless of tissue type (Fig. 2.1. and Fig. 2.2.). Although higher amounts of DNA were recovered from tissue samples when compared to "free DNA", the amount of DNA recovered from skin and muscle samples over time was similar in both cases (tissue and "free DNA").

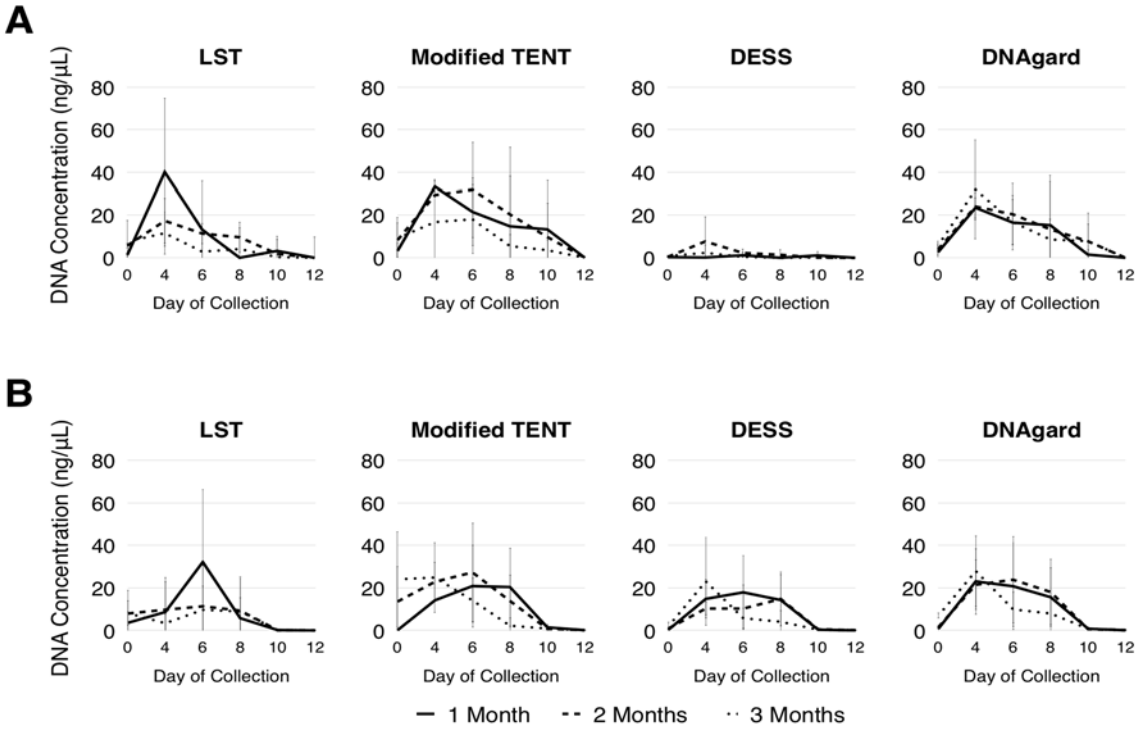

Fig. 2.2. Average DNA concentration (ng/µL) (± SD) of the "free DNA" in the preservative solutions surrounding (A) skin and (B) muscle samples stored for up to three months at 35°C and 60-70% humidity. Data for RNA*later* is not shown in this figure as no DNA was detected in solution during DNA quantification

STR Typing

Controls

When processed immediately after collection from decomposing cadavers, complete Identifiler® Plus profiles were generated from muscle tissue for up to 6 days of decomposition, and for skin up to 8 days (Fig. 2.3. – No Storage). However, when tissues were stored in hot and humid conditions for up to three months without a preservative, STR profile quality markedly decreased (Fig. 2.3. & Fig. 2.4. – No Preservative). In most cases, complete (or near complete) STR profiles were obtained for fresh tissues (day 0) stored for up to three months without a preservative, but the DNA in all decomposed tissues (day 4

to day 12) degraded quickly, resulting in partial or no STR profiles being obtained (Fig. 2.3. – No Preservative). DNA degradation was evident in all decomposing tissue samples and increased over time as evidenced by drop-out of the higher molecular weight loci, increasing DR (Fig. 2.3. and Fig. 2.4.), and the "ski-slope effect" observed in STR profiles (Fig. 2.5.).

Tissue

The skin and muscle samples stored in all preservatives, except for LST produced complete Identifiler® Plus profiles for up to 4 days of decomposition over the span of three months storage at 35°C and 60–70% humidity (Fig. 2.3.). In general, the completeness and quality of STR profiles declined as decomposition progressed and time of storage lengthened (Fig. 2.3. and Fig. 2.4.). By day 6 of decomposition (time of bloat), partial profiles (<98% alleles) were generated in 50% of the samples. Overall, the completeness and quality of STR profiles from skin samples declined more rapidly than profiles from muscle samples (Fig. 2.4. and Fig. 2.6. A and B). STR success did not necessarily correlate with DNA quantity. Comparable (or slightly higher) amounts of DNA were observed for skin compared to muscle samples, but on average muscle samples generated more complete and balanced STR profiles (Fig. 2.3. and Fig. 2.4.). The higher DR of STR profiles in skin samples suggest that DNA may be degrading more rapidly, and that although more DNA was detected from skin samples over time, the DNA may be better preserved in the muscle tissue. This finding differs from the results of Michaud et al. [13] and Clare et al. [20] that compared DNA preservation in skin and muscle from porcine and equine tissues respectively. Both studies found that DNA from skin was better preserved than DNA from muscle [13,20]. It has been suggested that the cellular structure of skin remains intact while

resisting degradative processes for longer periods of time compared to muscle, especially when the skin desiccates [13]. However, deep muscle tissue may also be protected from harsh external conditions by the desiccated skin. Interestingly, the Federal Bureau of Investigation recommends red skeletal muscle as the tissue of choice if submitting soft tissue for forensic DNA testing of unidentified remains [10]. Our results support that muscle tissue may indeed provide more complete STR profiles from decomposing human remains than other soft tissues such as skin. As previously mentioned, the quantity of DNA was not always a reliable indicator of STR success. Similar observations have been reported in the literature [7,8,21]. Our quantification method (63bp target) likely overestimated the amount of amplifiable DNA in these degraded samples. We would expect that using a system such as Quantifiler® Trio (Thermo Fisher Scientific) or PowerQuant® (Promega) with longer targets (>200 bases) would better correlate the amount of DNA detected with downstream STR results [7,20]. The number of reportable alleles and quality of STR profiles from samples stored in LST buffer rapidly decreased over time (Fig. 2.3., Fig. 2.4., and Fig. 2.7). Substantially fewer LST samples generated full profiles compared to the other preservatives (18% versus 43–56% respectively). The higher DR of STR profiles from LST samples compared to the other preservatives suggest that DNA was degrading most rapidly in LST (Fig. 2.4.). The preservation efficiencies of modified TENT, DESS, DNAgard®, and RNA*later* solutions were comparable based on allele recovery and degradation ratios of STR profiles. Although RNA*later* recovered the highest DNA concentrations from fresh and decomposing tissues for up to three months of storage (Fig. 2.1), STR success was comparable to the other preservatives (44% samples with partial STR profiles compared to 58%, 56% and 44% of modified TENT, DNAgard®,

and DESS samples respectively) (Fig. 2.3.). Factorial ANOVA analysis showed that tissue type ($F_{5,31} = 4.8$, $p = 0.0023$) and choice of preservative ($F_{25,116} = 3.3$, $p = 0.000009$) had significant effects on the completeness of STR profiles generated from preserved tissues.

Liquid Preservative

Similar trends in STR success rates were observed with "free DNA" in preservatives as with DNA from tissues with the exception of RNA*later*, which did not facilitate the release of DNA into solution (Fig. 2.3.). In general, the quality of STR profiles from "free DNA" in the preservatives of skin samples declined more rapidly than profiles generated from preservatives surrounding muscle samples (Fig. 2.3., Fig. 2.4., and Fig. 2.6. C and D). The STR degradation ratios also increased suggesting DNA was degrading more rapidly in skin samples (Fig. 2.4.) As with the tissues themselves, "free DNA" purified directly from the LST buffer also produced the least successful STR results (Fig. 2.7.). Complete profiles were only generated from LST surrounding fresh tissue (day 0) (Fig. 2.3. and Fig. 2.7.). The LST buffer generated the most samples with incomplete profiles (81% compared to 56% for both modified TENT and DESS buffers, and 50% with DNAgard®) and STR profiles with the highest DRs (Fig. 2.4.). Modified TENT, DESS, and DNAgard® produced comparable STR results when amplifying the "free DNA" in solution with decomposing tissues (Fig. 2.3., Fig. 2.4., and Fig. 2.7.). Similar results were also observed when comparing the DR of STR profiles produced from the "free DNA" in these three preservatives suggesting comparable levels of DNA preservation with these buffers (Fig. 2.4.). Factorial ANOVA analysis showed that tissue type ($F_{6,19} = 6.5$, $p = 0.00075$) and choice of preservative ($F_{18,54} = 2.8$, $p = 0.002$) had significant effects on the quality of STR profiles generated from DNA recovered directly from liquid preservatives.

In addition, the interaction between tissue and preservative was found to be significant ($F_{18,54} = 1.9$, $p = 0.031$). However, with the exception of cadaver 2 being too decomposed to collect tissue at Day 12, little difference in STR success was observed across the three cadavers for each day (Fig. 2.8.). Overall, STR success rates obtained from all samples (tissue and "free DNA" in solution) decreased over days of decomposition regardless of tissue type (Fig. 2.6.). The completeness and quality of STR profiles generated from "free DNA" in solution were similar to that obtained from the tissue itself for each sample (Fig. 2.3., Fig. 2.4., and Fig. 2.6.). It is also important to note that similar STR results were obtained from the "free DNA" surrounding decomposed tissue when stored in all preservatives (except for LST) for up to three months (Fig. 2.9.). These results demonstrate that DNA can be preserved for up to three months and extracted directly from the preservative solution eliminating the tissue digestion step and, in this way decreasing the extraction process time and increasing throughput, especially when many samples need to be processed in short periods of time such as the case of DVI. In summary, muscle samples yielded slightly more complete and balanced STR profiles from both tissue and "free DNA" for longer days of decomposition than skin samples (Fig. 2.3., Fig. 2.4., and Fig. 2.6.).

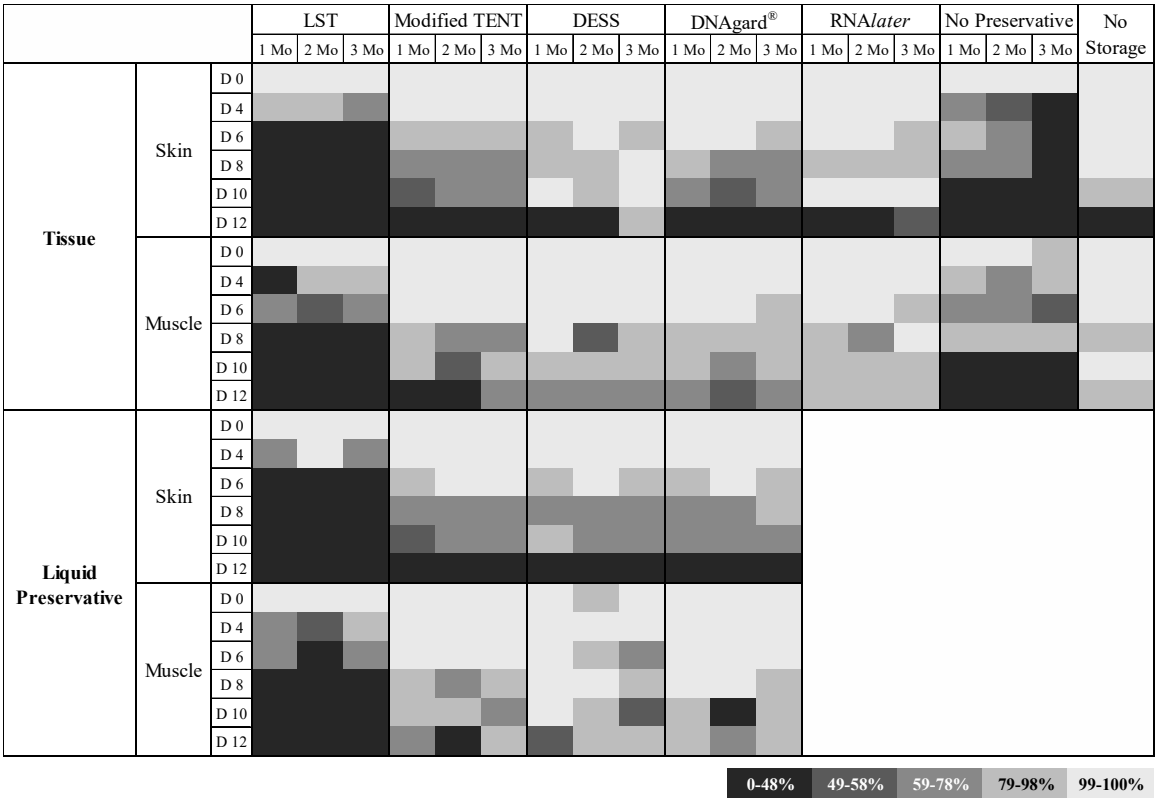

Fig. 2.3. Average STR results of all controls and experimental samples. Percentage of alleles correct (average of three cadavers) presented as a heat map. RNA*later* results for the "free DNA" in preservative solution are not shown due to the lack of DNA released into solution. (D0 = 0 days of decomposition, 1Mo = 1 month of storage)

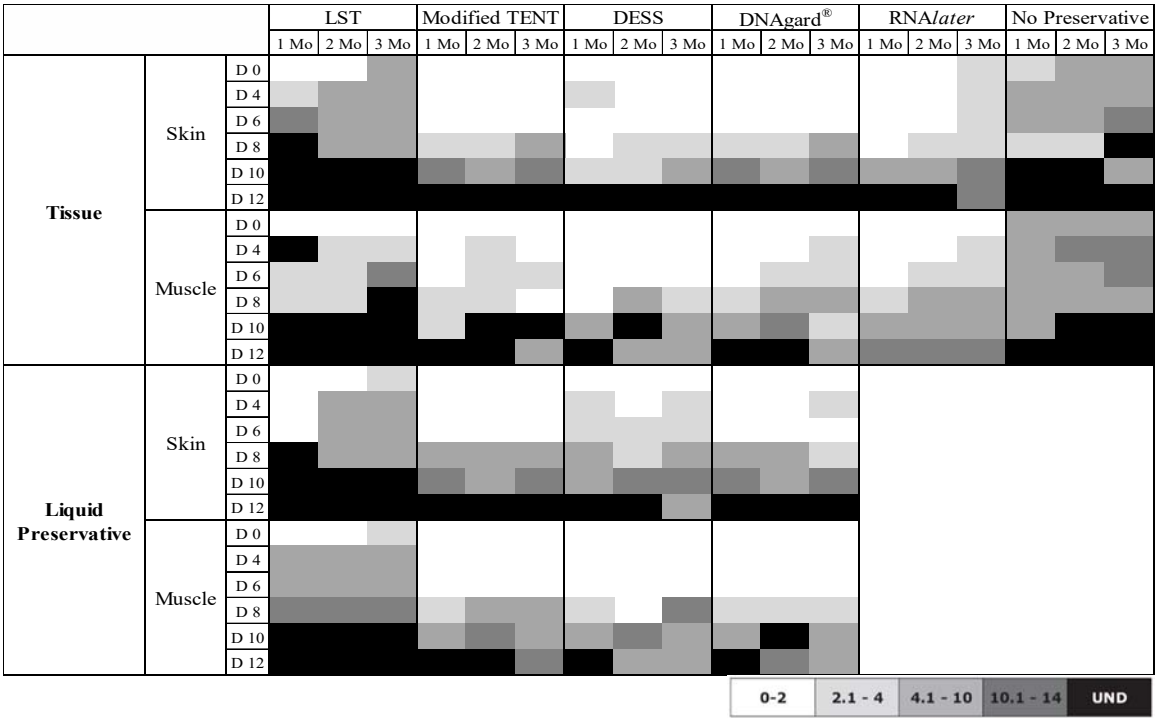

Fig. 2.4. Average degradation ratio (DR) based on STR results for all controls and experimental samples. The DR was calculated as the ratio between the peak heights of the shortest and longest markers in each channel. Average size of markers used were 144bp (D8S1179) and 325.9bp (CSF1PO) in the 6-FAM channel, 130.1bp (D3S1358) and 330.1bp (D2S1338) in the VIC channel, and 120.9bp (D19S433) and 298.7bp (D18S51) in the NED channel. A DR calculation was not possible for samples when locus drop out occurred at all longest makers (assigned as UND). RNA*later* results for the "free DNA" in preservative solution are not shown due to the lack of DNA released into solution. (D0 = 0 days of decomposition, 1Mo = 1 month of storage)

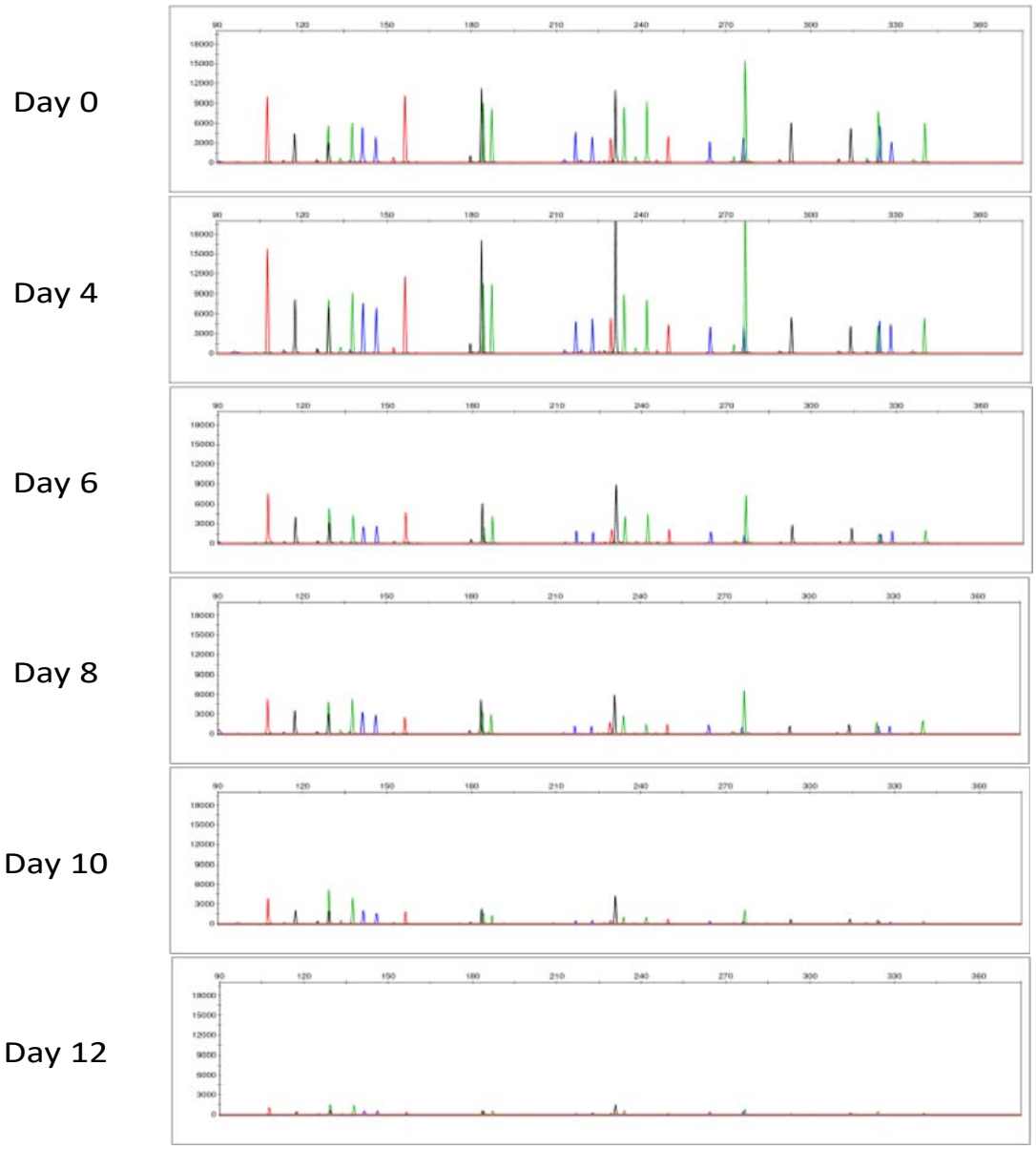

Fig. 2.5. Representative STR electropherograms produced from muscle tissue over 12 days of decomposition using the Identifiler® Plus kit

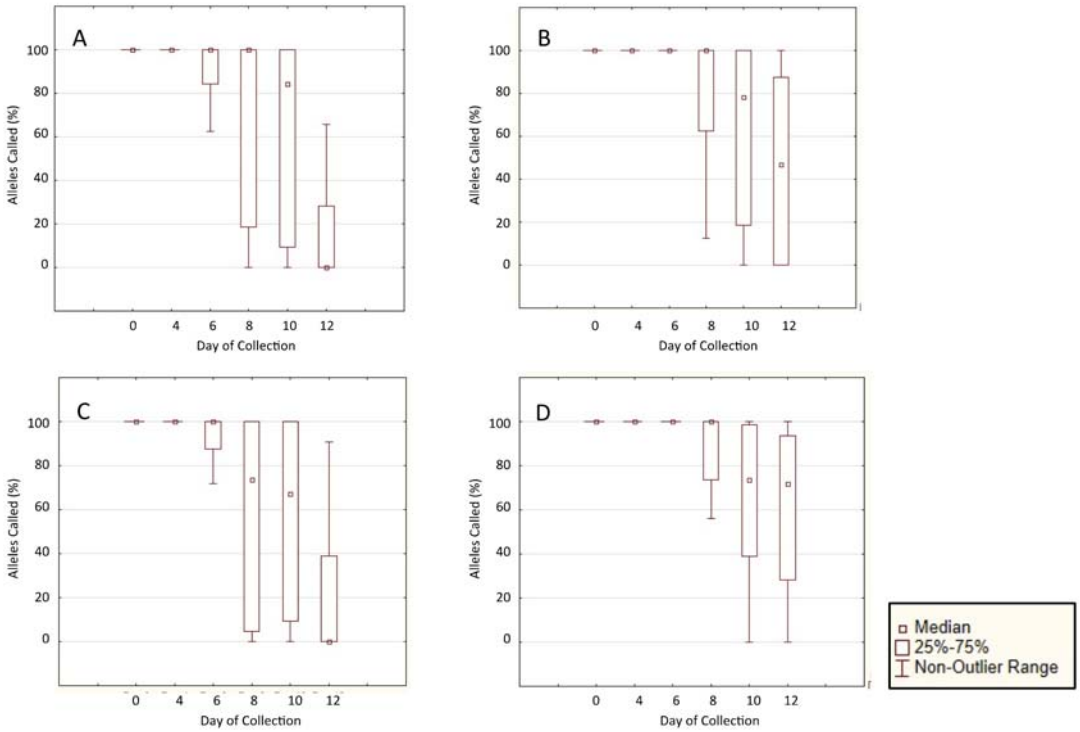

Fig. 2.6. STR success of the three cadavers as measured by the percentage of alleles correct from (A) skin and (B) muscle tissue stored in preservatives for up to three months at 35°C and 60-70% humidity. Average STR success of "free DNA" in the preservative solutions surrounding (C) skin and (D) muscle samples stored for up to three months at 35°C and 60-70% humidity

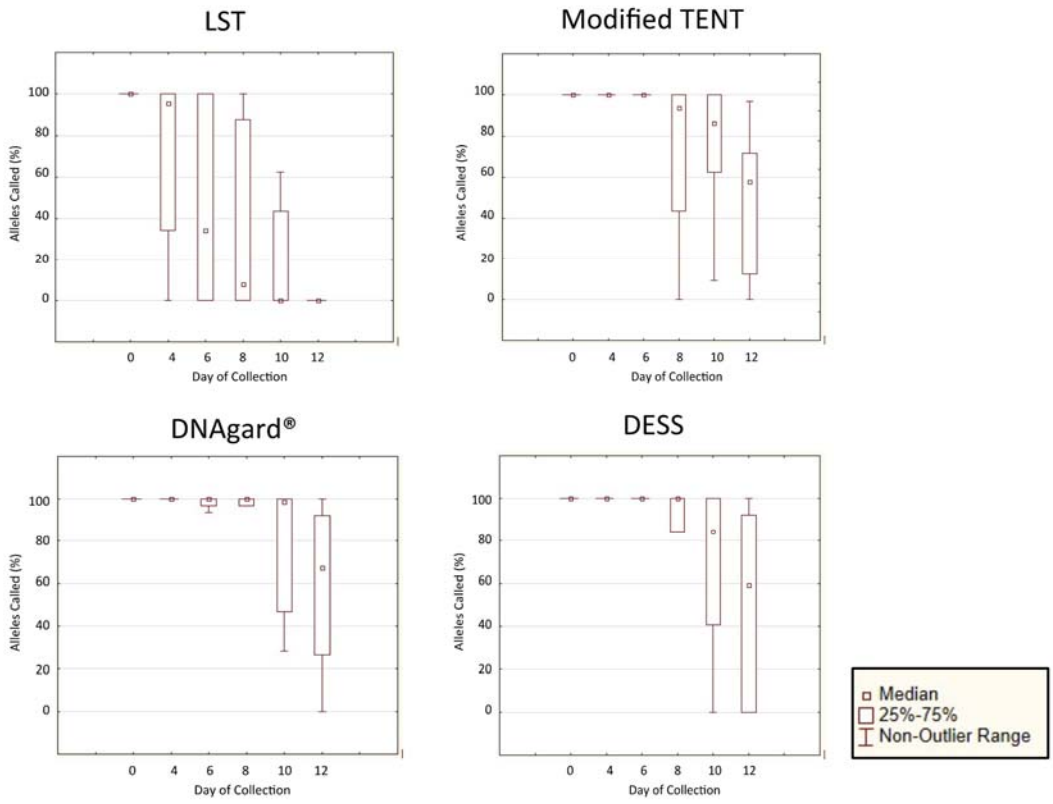

Fig. 2.7. STR success from the three cadavers as measured by the percentage of alleles correct from the "free DNA" in LST, modified TENT, DNAgard1 and DESS samples stored for up to three months at 35°C and 60–70% humidity. STR results generated from "free DNA" surrounding skin and muscle samples have been combined for each preservative

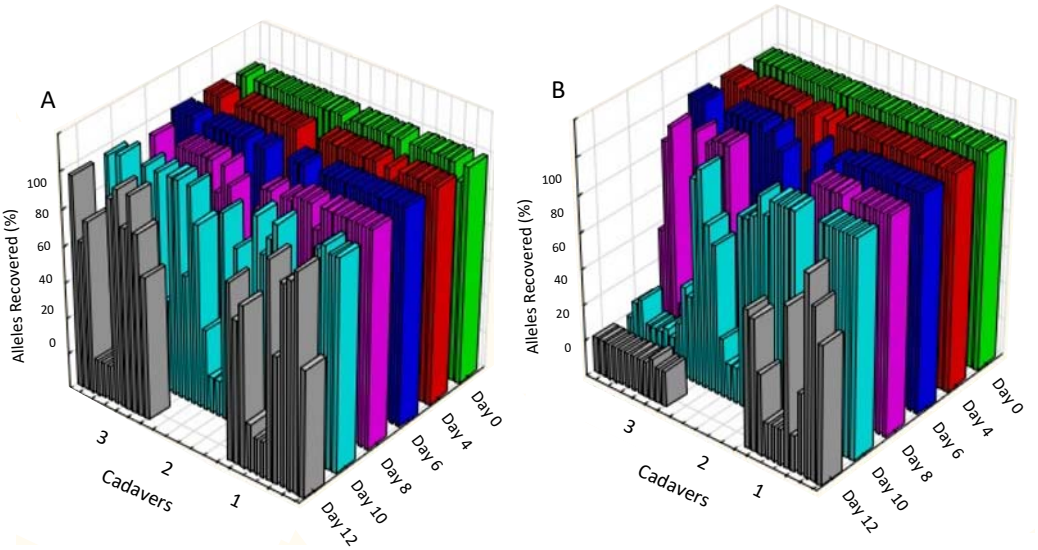

Fig. 2.8. STR success from the "free DNA" of decomposing A) muscle and B) skin showing the variation between the three cadavers. Data for all three months of storage and four preservatives have been combined. Due to advanced decomposition, no tissue samples were collected for cadaver 2 on Day 12

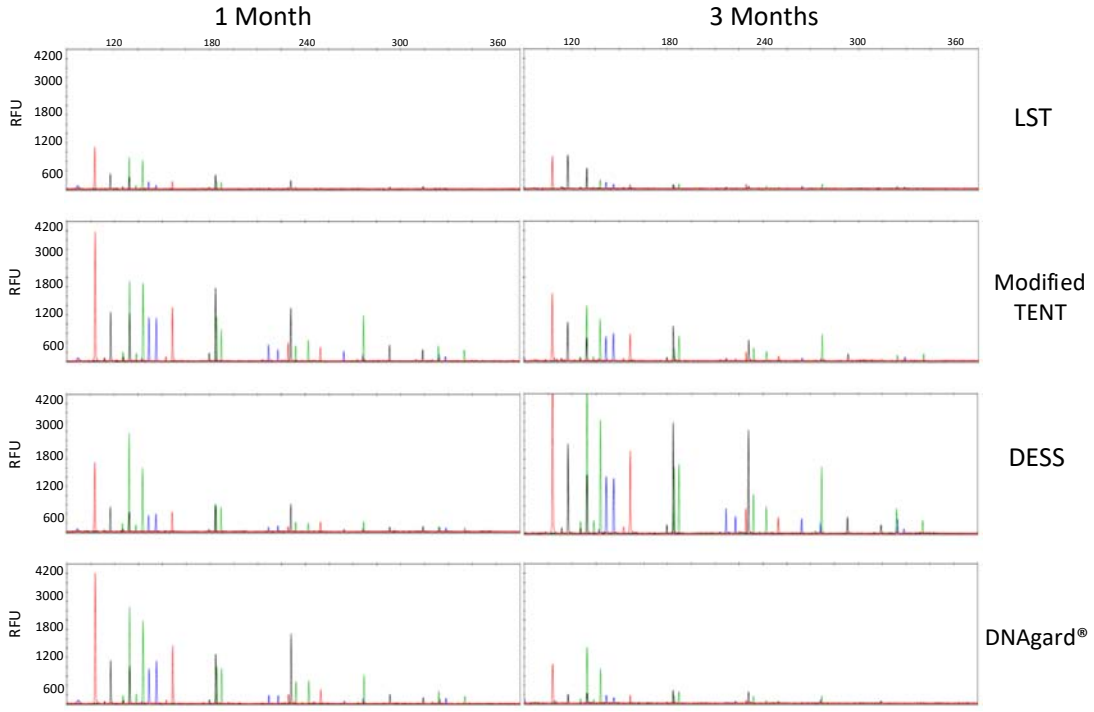

Fig. 2.9. Representative STR electropherograms produced from the "free DNA" surrounding decomposed muscle (Day 10) in LST, modified TENT, DESS, and DNAgard® stored for one and three months at 35°C and 60-70% humidity

Conclusion

Our conclusions support those of previous authors [2,9,11,13], suggesting that the use of preservative solutions can be beneficial for crime scene evidence collection and preservation in addition to DNA-based DVI operations. We have demonstrated that all solutions preserved DNA in fresh (day 0) and decomposed (days 4–12) skin and muscle for successful STR typing after storage for up to three months. As expected, there was a general decrease in the amount of amplifiable DNA as decomposition progressed. However, we observed that DNA quantity and STR quality markedly decreased after the body entered the bloat stage (Day 6 in this study), demonstrating the importance of sample collection as early as possible, and preferably prior to bloat to maximize downstream STR success. We also observed that similar quantities of DNA were extracted from skin and muscle for both tissue and "free DNA" with increasing decomposition time, but the average percentage of reportable alleles was higher for muscle samples. RNA*later* was found to preserve solution of DNA in tissues well, but it prevented the release of DNA into solution. On the other hand, LST, modified TENT, DESS, and DNAgard® seemed to favor the release of DNA into solution facilitating DNA purification without the lengthy digestion step. However, the LST buffer failed to preserve the "free DNA" from decomposing tissues and for extended periods of storage. While DESS, DNAgard®, and the modified TENT buffers all adequately preserved the "free DNA" in solution over time, modified TENT and DNAgard® may yield DNA of slightly higher quantity and generate more balanced and complete STR profiles from both the tissues and the liquid preservatives stored for up to three months at 35°C with 60–70% humidity. Although commercial products (such as DNAgard®) may be available for a mass disaster operation, they often have short shelf lives

(6–12 months) and would require regular restocking. Therefore, the development of a simple in-house solution that can preserve DNA in tissue samples and the surrounding liquid for direct purification (such as modified TENT) could be of benefit to DVI operations and crime scene evidence preservation in harsh environmental conditions alike.

Role of Funding

This study was supported by Award No. 2013-DN-BX-K034, awarded by the National Institute of Justice, Office of Justice Programs, U.S. Department of Justice. This funding source had no involvement in the study design, in the collection, analysis and interpretation of data, or in the writing of the report or the decision to submit this paper for publication. The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect those of NIJ.

Acknowledgements

The authors would like to thank the staff at the Applied Anatomical Research Center (AARC) at Sam Houston State University for their assistance, and the individuals and families of those who donated their bodies for scientific research. The authors would also like to thank James White for his assistance in the laboratory.

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CHAPTER III

Direct-to-PCR Tissue Preservation for DNA Profiling[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1]Sorensen A, Berry C, Bruce D, Gahan ME, Hughes-Stamm S, McNevin D (2016) Direct-to-PCR tissue preservation for DNA profiling. Int J Legal Med 130:607-613.

Contributions to Chapter III:

Work was divided into two sections for this project; 1) fresh tissues, and 2) decomposed tissues. All experimentation and data derived from the fresh tissues was performed by Clare Berry and Michelle Gahan under the direction of Dennis McNevin from the University of Canberra (Australia) and David Bruce from NSW Forensic and Analytical Science Service (Australia). All experimentation on the decomposed tissues was performed by Amy Sorensen under the direction of Sheree Hughes-Stamm at Sam Houston State University (SHSU).

Amy Sorensen:

- All bench work and data interpretation for decomposed tissue samples
- Writing of methods and results for decomposed samples

Clare Berry and Michelle Gahan

- All bench work and data interpretation for fresh tissue samples
- Writing of manuscript for all data regarding fresh samples

Sheree Hughes-Stamm

- Co-PI - (SHSU)

David Bruce

- Co-PI – (NSW Forensic and Analytical Science Service)

Dennis McNevin

- Co-PI - (University of Canberra) and corresponding author
- Writing of manuscript for all data regarding fresh samples

Abstract

Disaster victim identification (DVI) often occurs in remote locations with extremes of temperatures and humidities. Access to mortuary facilities and refrigeration are not always available. An effective and robust DNA sampling and preservation procedure would increase the probability of successful DNA profiling and allow faster repatriation of bodies and body parts. If the act of tissue preservation also released DNA into solution, ready for polymerase chain reaction (PCR), the DVI process could be further streamlined. In this study, we explored the possibility of obtaining DNA profiles without DNA extraction, by adding aliquots of preservative solutions surrounding fresh human muscle and decomposing human muscle and skin tissue samples directly to PCR. The preservatives consisted of two custom preparations and two proprietary solutions. The custom preparations were a salt saturated solution of dimethyl sulfoxide (DMSO) with ethylenediaminetetraacetic (EDTA) and TENT buffer (Tris, EDTA, NaCl, Tween 20). The proprietary preservatives were DNAgard (Biomatrica®) and Tissue Stabilising Kit (DNA Genotek). We obtained full PowerPlex® 21 (Promega) and GlobalFiler® (Life Technologies) DNA profiles from fresh and decomposed tissue preserved at 35°C for up to 28 days for all four preservatives. The preservative aliquots removed from the fresh muscle tissue samples had been stored at −80°C for 4 years, indicating that long-term archival does not diminish the probability of successful DNA typing. Rather, storage at −80°C seems to reduce PCR inhibition.

Keywords: Disaster victim identification (DVI), Mass disaster, Tissue preservation, DNA profile, Direct PCR, PCR inhibition

Introduction

During a mass fatality event, large numbers of bodies may be decomposing rapidly in harsh environmental conditions. The DNA in those tissues is also degrading which makes genotyping more difficult with the passage of time. The use of a simple field preservative to quickly halt DNA degradation and store large numbers of tissue samples at ambient temperature prior to genotyping would be a valuable tool.

As well as preserving tissue morphology, some tissue preservatives also preserve DNA for downstream DNA profiling. The notable exception to this is formalin (aqueous formaldehyde), historically used to preserve medical and museum specimens, which chemically modifies DNA by crosslinking proteins and nucleic acids, making polymerase chain reaction (PCR) difficult [1-3]. Both INTERPOL [4] and the International Society for Forensic Genetics (ISFG) [5] recommend against its use for DVI, although it is possible to obtain at least partial profiles from formalin-fixed and paraffin-embedded tissue (FFPET) [6,7].

However, there are a range of other preservatives which have been proven to be effective for DNA profiling: for a review, see Allen-Hall & McNevin [8]. INTERPOL and ISFG guidelines state that (non-formalin) preservatives can be used to conserve soft tissue at room temperature and we have previously shown that some are also effective at elevated temperatures likely to be encountered in tropical environments [9]. We demonstrated the ability to obtain full AmpFℓSTR® Identifiler® (Life Technologies) STR profiles from DNA extracted from fresh muscle tissue preserved in salt, ethanol, TENT buffer (10mM Tris, 10mM EDTA, 100mM NaCl, 2% Tween 20, pH 8.0), salt-saturated dimethyl sulfoxide solution (20% DMSO, 0.25M EDTA, saturated with NaCl, pH 8.0) and two proprietary

preservatives: DNAgard® (Biomatrica) and one from DNA Genotek, Inc. Three of the preservatives (salt-saturated DMSO-EDTA, DNAgard and DNA Genotek) also yielded full profiles from DNA extracted from aliquots of the preservative solution surrounding the muscle tissues.

The advantages of eliminating DNA extraction are many. Firstly, the path from tissue sampling to profile reporting is quicker and less expensive, with one less step involved. Secondly, there is less opportunity for loss of DNA as a result of DNA extraction. Thirdly, there is less risk for contamination of samples with a number of tube-to-tube and/or well-to-well transfers removed. Finally, the DNA is effectively archived in a small volume which can be re-accessed if required. The advantages of a direct-to-PCR approach have already been demonstrated for blood [10,11] hairs [12,13], fingernails [14], fabrics [15] and so-called "touch DNA" [16,17]. This may be a result of "cell free" DNA which has been demonstrated in a number of forensically relevant samples [18,19]. Proprietary DNA profiling assays have been validated for blood and buccal samples on FTA® card to be added directly to PCR and these are regularly used for reference samples [20-24].

However, the direct addition of preservatives to PCR does pose some unique challenges. Most of the preservatives contain high concentrations of salt and EDTA, as well as detergents, which are known PCR inhibitors [25,26]. Components of human tissue as well as the by-products of decomposition will also inhibit PCR. These include haemoglobin [26,27] and hematin [28]. There may be other inhibitors in contaminating material from soils including humic and fulvic acids [28-30].

In this study, we explored the possibility of obtaining DNA profiles without DNA extraction, by adding aliquots of preservative solutions surrounding fresh and decomposing human tissue samples directly to PCR. This approach has potential application to disaster victim identification (DVI) as well as to any form of field based biological evidence or intelligence collection [31].

Materials and Methods

Preparation of Preservative Solutions

Only those solutions for which we have previously found DNA in the solution surrounding preserved tissue were considered for this study [9]. These were salt-saturated DMSO/EDTA (DESS), TENT buffer, DNAgard® and DNA Genotek Tissue Stabilising Kit (described earlier). Solutions were prepared using purified water with a resistivity of at least 18MΩ·cm at 25ºC. DESS and TENT were subsequently autoclaved at 121ºC for 20 minutes.

Preservation of Tissue Samples

Fresh skeletal muscle tissue samples were obtained from three volunteers undergoing orthopaedic surgical procedures and approximately 300mg sections were preserved in 1 or 2 mL of each of the four preservatives at 35ºC as previously described [9]. After 3, 7, 14 and 28 days, a 20 or 50µL aliquot of each preservative surrounding each tissue was transferred to individual sterile 1.5mL microcentrifuge tubes and archived at -80ºC for four years.

Tissue samples for the decomposition study were sourced from two human cadavers provided by the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, TX, USA. The cadavers were placed at -20ºC upon receipt

by AARC, and stored for up to 45 days prior to use. Both cadavers were allowed to equilibrate to room temperature, and then placed on the same day (in October, 2013) in an open field (caged to prevent predation), and left to decompose. The AARC facility is adjacent to the Sam Houston State Forest with a humid, subtropical climate. The average temperature for the duration of this study was 18ºC (average high of 24ºC and low of 12ºC). Skin and muscle samples were collected from the thigh of each cadaver on day 0, 6, 8 and 10. Tissue samples (30mg) were preserved in 300µL of each preservative and stored at 35ºC for one month. Preservation in water was included as a "no preservative" control for tissue samples from each cadaver in order to demonstrate any preservation effect over and above not preserving. The water controls also served to mimic a high humidity environment, often encountered in DVI operations. Buccal (inside cheek epithelial cell) swabs were obtained from the donors of all tissue samples to provide reference DNA profiles.

Quantification of DNA in Preservatives

The Quantifiler® Human DNA Quantification Kit (Life Technologies) was used to quantify the DNA in the preservative aliquots of all samples according to the manufacturer's recommended protocol [32,33]. When required because of inhibition (as indicated by non-detection of the internal PCR control: IPC), a 1:10 or 1:20 dilution was made prior to quantification. This was only required for DESS, DNAgard® and DNA Genotek samples from decomposed cadavers. The Quantifiler® Human DNA standard was used to prepare a dilution series to establish a standard curve. A quantitation negative control was prepared with 2µL of TE buffer (10mM Tris, 0.1mM EDTA, pH 8.0) in place

of the DNA extracts. Thermal cycling and fluorescence detection were performed in a 7500 Real-Time PCR System (Life Technologies).

STR Genotyping

Preservative aliquots from fresh tissue were diluted where required to 0.05ng/μL and then 0.5ng of DNA from each aliquot was added to PowerPlex® 21 System (Promega) PCRs in a 25μL final volume according to the manufacturer's recommended protocol [33,34]. For the decomposition study, 0.5ng of DNA from each aliquot was added to a GlobalFiler® PCR (Life Technologies) in a 25μL final volume according to the manufacturer's recommended protocol [35]. Thermal cycling was performed according to the recommended protocols in a GeneAmp® PCR System 9700. Capillary electrophoresis was performed on an Applied Biosystems™ 3500 Series Genetic Analyzer (Life Technologies) fitted with 36cm capillaries and POP-4™ polymer according to the recommended protocol with the following settings: HID application type, HID36_POP4 run module, G5 (PowerPlex 21) or J6 (GlobalFiler) dye set, 1.2 kV injection voltage, 24 second (PowerPlex 21) or 15 second (GlobalFiler) injection time and 1,500 second (PowerPlex 21) or 1,550 second (GlobalFiler) run time.

For the fresh tissue samples, a volume of 1μl of each PowerPlex® 21 PCR product (or 1μl of PowerPlex® 21 Allelic Ladder Mix) was added individually to 2μL of CC5 Internal Lane Standard 500 (Promega) and 10μL of Hi-Di™ formamide (Life Technologies) [33]. For the decomposition study, a volume of 1μl of each PCR product (or 1μl of GlobalFiler® Allelic Ladder Mix) was added individually to 0.4μL of 600 LIZ® dye Size Standard (Life Technologies) and 9.6μL of Hi-Di™ formamide (Life Technologies). Immediately prior to loading, all samples were then denatured at 95°C for

3 minutes, and then chilled at -20ºC for 3 minutes. Electropherograms were analyzed using GeneMapper ID-X software (Life Technologies) with PowerPlex® 21 or GlobalFiler® STR panels, bins and stutter files, as appropriate. Peak amplitude thresholds (PATs) of 175 and 700 (PowerPlex® 21) and 175 and 600 (GlobalFiler®) relative fluorescence units (RFU) were used to designate reportable heterozygous and homozygous alleles, respectively. Where a single peak was concordant with a homozygous allele in the relevant reference profile for that donor and above the (higher) homozygote RFU threshold, it was counted as two (homozygous) alleles.

Statistical Analysis

Statistical analysis was performed using SPSS (IBM). A Shapiro Wilks test for normality was applied before either an ANOVA (normally distributed data) or Kruskall Wallis test (non-normal data) for significant difference. A p value of 0.05 was considered significant (with 95% confidence) [33].

Results

DNA Concentrations in Preservatives

DNA concentrations in each of the four preservative solutions surrounding fresh muscle samples at 3, 7, 14 and 28 days after storage at -80ºC for four years are shown in Fig. 3.1. [33]. No PCR inhibition (as measured by a delay in C_T for the IPC) was observed for any of the samples. The DNA yield was highly dependent on the donor tissue (Kruskall Wallis p value = 0.003) with the highest concentrations derived from Donor 1 across all preservatives. There was a significant difference between preservatives (p = 0.005) with TENT buffer consistently yielding the highest concentrations and DNAgard® the least.

DNA concentration appears to increase with initial preservation time from 3 to 28 days but this result was not statistically significant.

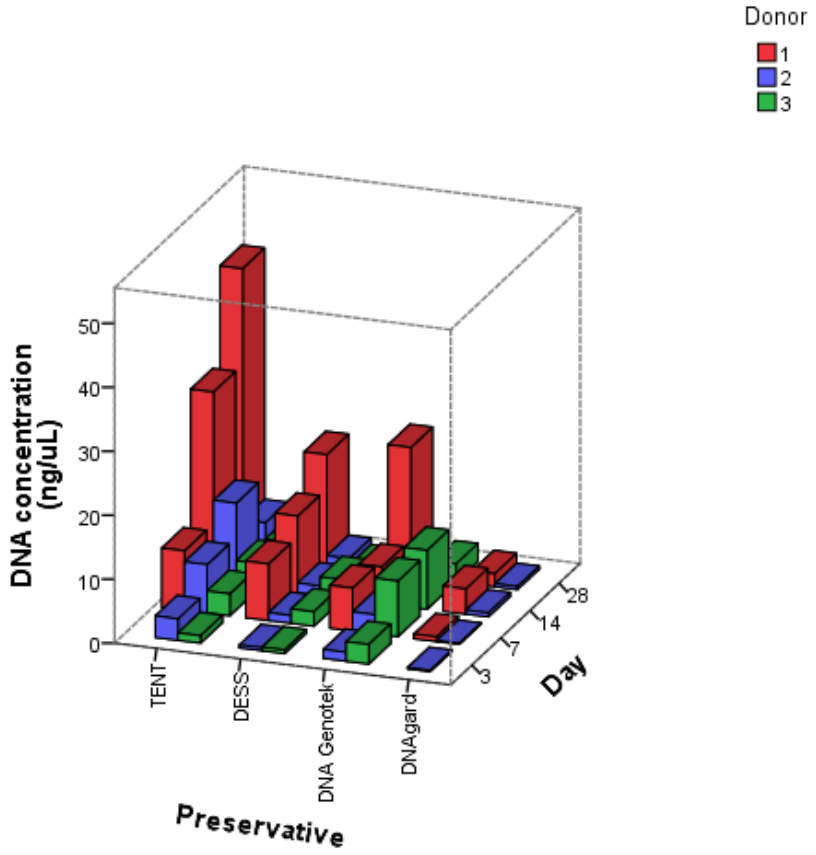

Fig. 3.1. DNA concentrations in aliquots of each of four preservative solutions surrounding fresh muscle tissue samples from each of three donors after 3, 7, 14 and 28 days followed by storage at -80°C for four years. TENT and DESS are custom preparations as defined in the text. DNA Genotek and DNAgard are proprietary preservatives. The preservative solutions were added directly to Quantifiler real time PCR assays (without DNA extraction)

In contrast to the preservatives surrounding fresh tissues (archived at -80°C), those surrounding decomposed cadavers were all inhibited (the IPC was only detected in the TENT buffer). However, a 1:10 dilution for DESS and DNAgard® and a 1:20 dilution for DNA Genotek were sufficient to relieve inhibition. DNA concentrations (adjusted for dilution) for both cadavers increased from day 0 to day 6 in both muscle and skin tissues (Fig. 3.2.). Concentrations decreased to near zero at day 8 for cadaver B (skin and muscle)

and at day 10 for cadaver A, corresponding with the appearance of bloat in both cases. All preservatives yielded more DNA than the water control. DESS and DNAgard® yielded disproportionately more DNA after 6 days for muscle (both cadavers) and after 8 days for skin (cadaver A) suggestive of a leaching effect.

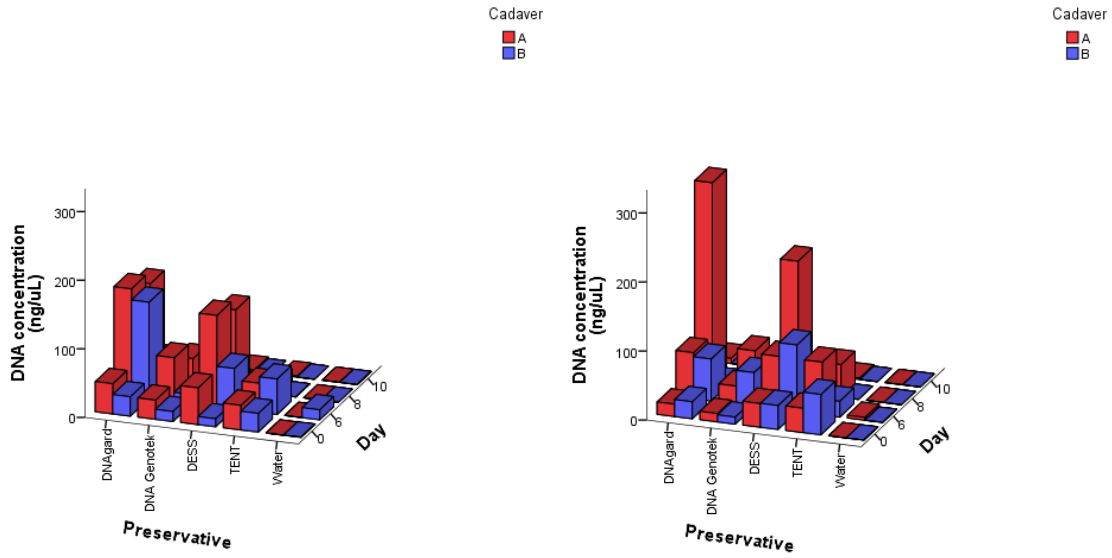

Fig. 3.2. DNA concentrations (adjusted for dilution) in aliquots of each of four preservative solutions and water surrounding muscle (left) and skin (right) tissue samples from each of two cadavers after 0, 6, 8 and 10 days of decomposition and subsequent storage at 35°C for one month. TENT and DESS are custom preparations as defined in the text. DNA Genotek and DNAgard are proprietary preservatives. The preservative solutions were added directly to Quantifiler real time PCR assays without DNA extraction after no dilution (water control and TENT buffer), 1:10 dilution (DESS and DNAgard) or 1:20 dilution (DNA Genotek)

STR Genotyping

The numbers of reportable and concordant alleles (of a total possible 42 in PowerPlex 21) generated from aliquots of each of the preservative solutions surrounding each of the fresh muscle tissue samples (subsequently stored at -80ºC) are shown in Fig. 3.3. [33]. DESS and DNA Genotek yielded full profiles of 42 reportable alleles for each donor at each time point. DNAgard generated full profiles for all aliquots except at day 7.

TENT buffer produced profiles with 26 to 42 reportable alleles. As expected, allele peak heights were generally higher for lower molecular weight markers (data not shown). Interestingly, except for a few samples, Donor 2 exhibited the highest peaks for all loci and all preservatives, even though Donor 1 had significantly greater DNA concentrations (Fig. 3.1.). In addition, the peak heights derived from TENT buffer diminished significantly (relevant to other preservatives) at higher molecular weight loci (Penta D and Penta E).

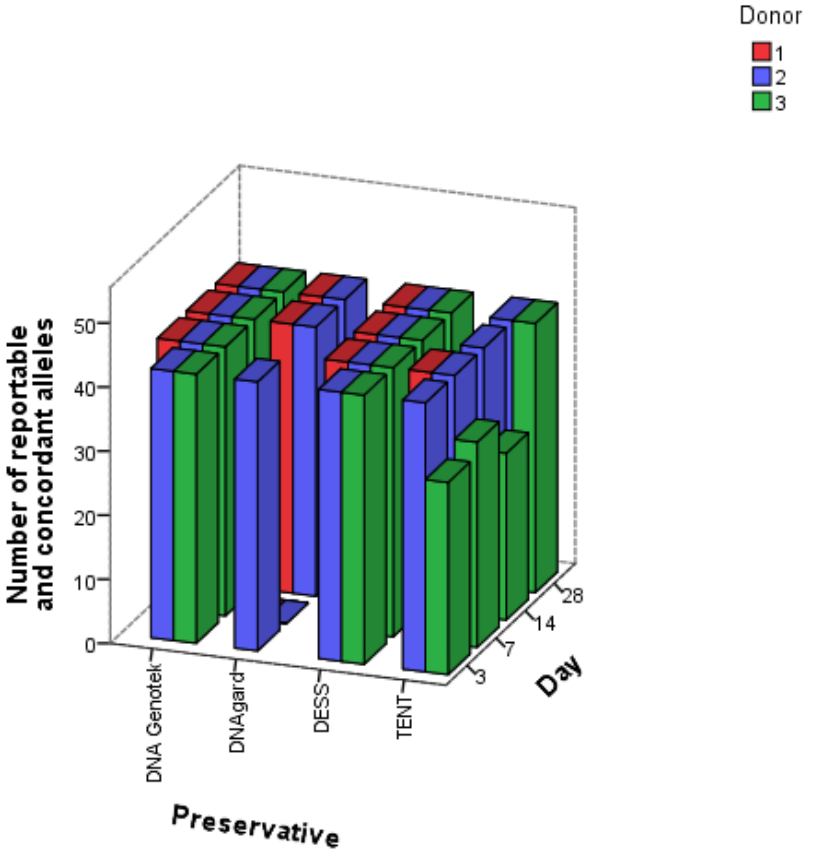

Fig. 3.3. Number of reportable alleles (of a total possible 42) in STR genotypes from aliquots of each of four preservative solutions surrounding fresh muscle tissue samples from each of three donors after 3, 7, 14 and 28 days followed by storage at -80°C for four years. TENT and DESS are custom preparations as defined in the text. DNA Genotek and DNAgard are proprietary preservatives. The preservative solutions were added directly to PowerPlex 21 PCRs (without DNA extraction)

Figure 3.4. shows STR typing success for aliquots of each of the preservative solutions and water surrounding skin and muscle tissue samples from cadaver A and B. Again, a 1:10 dilution for DESS and DNAgard and a 1:20 dilution for DNA Genotek were required to relieve inhibition. Full GlobalFiler profiles (all 44 possible alleles reportable and concordant) were obtained for DESS and TENT up to 8 days from the skin of cadaver A. Other preservatives were less successful but all yielded full profiles up to 6 days from the skin of cadaver A and all yielded full profiles at day 6 from the muscle tissue of cadaver B.

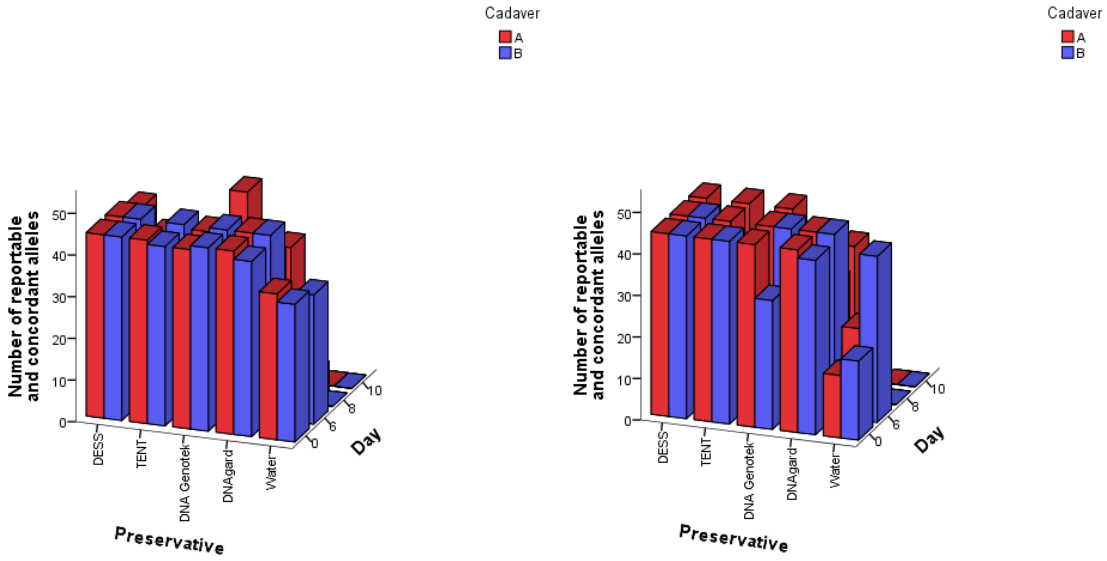

Fig. 3.4. Number of reportable alleles (of a total possible 44) in STR genotypes from aliquots of each of four preservative solutions and water (control) surrounding muscle (left) and skin (right) tissue samples from each of two cadavers after 0, 6, 8 and 10 days of decomposition and subsequent storage at 35°C for one month. TENT and DESS are custom preparations as defined in the text. DNA Genotek and DNAgard are proprietary preservatives. The preservative solutions were added directly to GlobalFiler PCRs without DNA extraction after no dilution (TENT buffer), 1:10 dilution (DESS and DNAgard) or 1:20 dilution (DNA Genotek)

Discussion

The advantages of eliminating DNA extraction in favour of adding DNA directly to PCR have been demonstrated by Swaran and Welch [36] who showed that samples subjected to direct PCR generated DNA profiles with higher peak heights and lower allele dropout when compared to samples subjected to extraction. In this study, we have shown that a similar approach is suitable for DVI. Provided that tissue samples (skin or muscle) are taken from decomposing corpses before they have reached full bloat or entered active decay and that DNA is of sufficient quantity and quality, there is good evidence to suggest that a full or partial DNA profile will be obtained directly from the preservative if the samples are submerged for up to one month in any of TENT buffer, DESS, DNAgard® or DNA Genotek® solutions, after appropriate dilution. Although we did not preserve beyond this time, it is likely that DNA profiles could be obtained after longer periods as no decrease in efficacy was observed over this time (Fig 3.1. and Fig 3.3.). Further, if these solutions are archived at -80ºC, all preservatives except TENT are likely to produce full profiles at least four years later. While DNA extraction (from either unpreserved tissue, preserved tissue or preservative solution) will also yield DNA [9], with potentially less inhibitors, this requires more time and resources. By eliminating DNA extraction, a faster DVI process is possible and any increase in inhibition (derived from the preservatives themselves) can be easily diluted out.

When samples were processed without storage at -80°C, both fresh and decomposed tissue samples exhibited substantial (or complete) inhibition for all preservatives except TENT. This observation would suggest that the process of archiving at this temperature may relieve inhibition in these preservatives. Although the mechanism

is not understood, a substantial decrease (or elimination) in PCR inhibition has been reported when freeze-thawing serum [37] and genital and urine samples [38,39]. Compared with DESS, DNAgard® and DNA Genotek solutions, TENT buffer produced less full profiles from fresh tissues after freezing (Fig 3.3.). This seems to be largely a result of drop out of larger molecular weight loci (*eg*. Penta D and Penta E).

In our hands, the amount of DNA retrieved from individual fresh skeletal muscle tissue samples and decomposed cadavers varied widely and bore no relation to the subsequent success of DNA profiling and the resultant allele peak heights. The same result would be expected in a mass disaster where the ability to take representative tissue samples would be even more difficult than in our controlled laboratory and field conditions. We stored our tissue samples at 35ºC to simulate worst-case temperatures that may be encountered in a disaster but this is another variable that would be expected to influence DNA retrieval and genotyping success.

Conclusions

Our study has shown that a direct PCR approach for identifying fresh and decomposing tissue samples preserved at room temperature is possible. By directly amplifying DNA in solution (with dilution in some cases), DNA extraction from the dense tissues can be avoided, and successful STR profiles can be obtained in a timelier manner. As a result, a significant impact can be made to address the demands for DNA preservation in rapidly decomposing remains and provide faster DNA identification during a mass disaster. Our results are limited to decomposition of only two cadavers in one set of environmental conditions and further research investigating the ability to obtain

forensically useful DNA under varying environmental conditions could establish more widely applicable time frames for sampling.

Acknowledgments

Some equipment and consumables were provided by the NSW Forensic and Analytical Science Service (Sydney, Australia). The authors thank Dr. Heloise Breton for providing valuable feedback on the manuscript; the staff at AARC (Sam Houston State University, Huntsville, TX, USA) for their assistance; and the individuals and families of those who donated their bodies to AARC for scientific research.

Compliance with ethical standards

Approval for collection and use of human tissue samples was granted by the University of Canberra Human Research Ethics Committee (Project number 09-01with extension 14-71). All samples were collected with the informed consent of the donors. For the decomposed samples used in this study, Code 45 of US Federal Regulations part 46102(f) exempts the requirement for Institutional Review Board (IRB) approval regarding the use of human cadaveric samples. All procedures were in accordance with the 1964 Helsinki declaration and its later amendments.

Conflict of interest

The authors declare that they have no conflict of interest.

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CHAPTER IV

Evaluation of Four Commercial Quantitative Real-time PCR Kits with Inhibited and Degraded Samples[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1]Holmes AS, Houston R, Elwick K, Gangitano D, Hughes-Stamm S (2017) Evaluation of four commercial quantitative real-time PCR kits with inhibited and degraded samples. Int J Legal Med

Abstract

DNA quantification is a vital step in forensic DNA analysis to determine the optimal input amount for DNA typing. A quantitative real-time polymerase chain reaction (qPCR) assay that can predict DNA degradation or inhibitors present in the sample prior to DNA amplification could aid forensic laboratories in creating a more streamlined and efficient workflow. This study compares the results from four commercial qPCR kits: 1) Investigator® Quantiplex® Pro Kit, 2) Quantifiler® Trio DNA Quantification Kit, 3) PowerQuant® System, and 4) InnoQuant® HY with high molecular weight DNA, low template samples, degraded samples, and DNA spiked with various inhibitors. The results of this study indicate that all kits were comparable in accurately predicting quantities of high quality DNA down to the sub-picogram level. However, the InnoQuant® HY kit showed the highest precision across the DNA concentration range tested in this study. In addition, all kits performed similarly with low concentrations of forensically relevant PCR inhibitors. In general, the Investigator® Quantiplex® Pro Kit was the most tolerant kit to inhibitors and provided the most accurate quantification results with higher concentrations of inhibitors (except with salt). PowerQuant® and InnoQuant® HY were the most sensitive to inhibitors, but they did indicate significant levels of PCR inhibition. When quantifying degraded samples, each kit provided different degradation indices (DI), with Investigator® Quantiplex® Pro indicating the largest DI and Quantifiler® Trio indicating the smallest DI. When the qPCR kits were paired with their respective STR kit to genotype highly degraded samples, the Investigator® 24plex QS and GlobalFiler® kits generated more complete profiles when the small target concentrations were used for calculating input amount.

Keywords: Forensic science, DNA quantification, Quantitative real-time PCR, Degradation index, PCR inhibition

Introduction

Short tandem repeat (STR) typing via polymerase chain reaction (PCR) is considered the gold standard in the forensic DNA community for human identification. As per the *FBI's Quality Assurance Standards for Forensic DNA Testing Laboratories*, an optimal input amount of DNA for STR typing should be determined prior to PCR amplification for samples other than reference DNA [1]. In a forensic setting, quantification of DNA is commonly performed *via* quantitative real-time PCR (qPCR) [2-4]. Forensic casework samples are often degraded, in low amounts, inhibited, or a combination thereof [5], which may complicate forensic STR analysis. Therefore, if analysts can better predict any DNA degradation or inhibition in the sample prior to STR amplification, costly resources and time for analysis could be reduced [2,3,6-8].

Current commercial qPCR kits are comprised of a four-target system that enable an analyst to predict downstream STR typing success. These targets include small and large human autosomal targets, male targets, and an internal PCR control. The small and large targets are used to generate a degradation index (DI) which is calculated by dividing the small target DNA concentration by the large target DNA concentration [3,4,6-10]. The inclusion of the large target is beneficial as it more closely reflects the amplicon sizes in current STR amplification kits with large STRs up to 475 base pairs. When DNA becomes fragmented during degradation processes, smaller STRs may be preferentially amplified, and the large STRs may drop out resulting in a partial profile [8,9,11]. The same principle applies to quantification where the small autosomal target will be preferentially quantified in degraded samples [3,7]. While the small target concentration is most commonly used for determining DNA input for STR amplification and the large target concentration is used

for calculating the DI [7,8], some have suggested the use of the large target DNA concentration to assess the input DNA volume for autosomal STR amplification with samples indicating possible degradation [3,4]. Alternatively, a different genotyping method may be chosen if the concentration of DNA is below the detection limit for STR analysis such as mitochondrial testing [10,11], SNP-typing [11,12], insertion and null alleles (INNULS) [10], insertion/deletion polymorphisms (INDELS) [12], or massively parallel sequencing [12].

Forensic casework samples may also include co-extracted PCR inhibitors, such as hematin from blood, humic acid from soil, calcium from bone, melanin from hair, or salt in aqueous solutions [13-15]. Current commercial qPCR kits include an internal PCR control (IPC) to detect any inhibition. This target is a synthetic fragment of DNA that is amplified simultaneously with the other targets [3,4,7,10,14]. PCR inhibition is determined for each sample by a shift in the cycle threshold of the IPC target when compared to the IPC cycle threshold of the DNA standards [3,6,7,10,13,14]. PCR inhibition can also reduce the efficiency of the PCR reaction resulting in a lower quantification value [13]. Inhibited samples can also generate STR results that mimic those obtained from low template or degraded DNA, such as drop out of larger loci [13,14]. However, the approach one would take to improve STR results from samples that are inhibited differs from degraded and low template samples. Instead of simply increasing the template amount into PCR and thereby increasing the amount of inhibitor, one might dilute the sample if the DNA concentration permits [4,13-15], add more BSA to the reaction [13,14], or perform additional purification methods [4,13,14]. Extremely high concentrations of inhibitors may affect the concentration of the small target DNA concentration, which may be problematic for

downstream STR success. Therefore, it is crucial to determine whether a sample is in trace amounts, degraded, or inhibited; it is even more important to be able to do so prior to STR amplification to increase first pass success rates [2-5].

Four commercial qPCR kits were compared in this study: 1) Investigator® Quantiplex® Pro Kit (QIAGEN, Hilden, Germany), 2) Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, South San Francisco, CA, USA), 3) PowerQuant® System (Promega Corporation, Madison, Wisconsin, USA), and 4) InnoQuant® HY (InnoGenomics Technologies, New Orleans, LA, USA). These qPCR kits utilize four-target systems that amplify multicopy loci and an IPC; however, the base pair (bp) size and location of these targets vary, as well as the size of the synthetic PCR controls and the calculation for the level of inhibition (Table 4.1.) [3,6,7]. The Investigator® Quantiplex® Pro Kit contains the longest amplicon for the large target (353bp) [16], most closely reflecting the higher range of loci in STR amplification kits. The large targets in the other kits fall between 207 and 294bp [3,6,7]. The differences in the lengths of these large targets may lead to varying degradation ratios [9]. The small and male targets are similar in size for all kits [6], but the PowerQuant® System and InnoQuant® HY contain two targets for the male quantification in order to increase detection sensitivity and minimize any effect that copy number variation may have on the quantification results [3,6]. The IPC targets vary in size with Investigator® Quantiplex® Pro Kit and the PowerQuant® System having the largest control amplicons (~435bp) [6]. In addition, the IPC target in Investigator® Quantiplex® Pro reflects the quality sensors in its respective STR kits (Investigator® 24plex QS & Investigator® 24plex GO! Kits) [5]. InnoQuant® HY also differs from the other kits as it targets two *Alu* retrotransposable elements on

autosomal chromosomes for the small and large target [3,4]. Loftus and Pineda found that these markers were better candidates for more accurate, sensitive, and reproducible DNA results due to their high copy number (approximately 10% of the human genome) [3,4]. In addition, the qPCR kits also have recommended values for when samples are flagged for possible degradation (Table 4.1.) [6]. Investigator® Quantiplex® Pro and the PowerQuant® System have default flags in their respective analysis tools [6], whereas Quantifiler® Trio and InnoQuant® HY have recommended ranges for samples with moderate to severe degradation [8,10,17]. Likewise, these four qPCR kits have different flags and varying calculations for inhibition, but the principle for determining inhibition is the same (Table 4.1.) [3,6,7]. Each manufacturer of these kits strongly suggests confirming or defining these flags during laboratory's internal validation [16-19].

Table 4.1. Design metrics and targets of the four qPCR kits used in this study

	Large Target	*Small Target*	*Male Target*	*IPC*	*DI*	*Degra-dation flags*	*IPC Δ CT*	*Inhib-ition flags*
Investigator® Quantiplex® Pro Kit	353bp*[a]	91bp[a]	81bp[a]	434bp[a]	DNA conc. of small target divided by DNA conc. of large target[bcdef]	>10[ab]	Average of standards' IPC CT minus sample IPC CT[b]	>1[ab]
Quantifiler® Trio DNA Quantification Kit	214bp[cd]	80bp[cd]	75bp[cd]	130bp[cd]		1-10, >10[d]	Sample IPC CT minus average of standards' (1-5) IPC CT[cd]	>2[cd]
PowerQuant® System	294bp*[e]	84bp[e]	81 & 136bp[e]	435bp[e]		>2[e]	Sample's IPC is compared to a standards' CT (based on nearest quant)[e]	>0.3[e]
InnoQuant® HY	207bp[f]	80bp[f]	79 & 79bp[f]	72bp[f]		2.5-20, >20[g]	Sample IPC CT minus average of standards' (1-5) IPC CT[f]	>2[f]

[a] As described in Investigator Quantiplex Pro Handbook [16] and [b] QIAGEN Quantification Assay Data Handling and STR Setup Tool v2.01
[c] As described in Holt et al. [7] and [d] Quantifiler HP and Trio DNA Quantification Kits User Guide, 2017 [17]
[e] As described in Ewing et al. and PowerQuant™ System Technical Manual [6,19]

[f] As described in Loftus et al. and InnoQuant HY User Guide [3,18] and [g] van den Berge et al. [10]
* Different region from the same locus as the small target

All of the aforementioned design differences in these qPCR kits may affect the human quantification, DI, and inhibition results when used to quantify the same sample prior to STR typing [9]. This study aims to evaluate the performance of four qPCR kits with high quality, low template, degraded, and inhibited DNA samples commonly seen in forensic casework and discuss the importance of the quality indicators for assessment prior to STR amplification.

Materials and Methods

DNA Standards and Samples

The accuracy and sensitivity of each kit to human DNA was tested using National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM) 2372 Human DNA Quantitation Standard (Gaithersburg, MD, USA). Serial dilutions were prepared from Components A and B, which were derived from a single male donor with a neat concentration of 57ng/µL and multiple female donors with a neat concentration of 61ng/µL, respectively [20]. The following DNA concentrations were included in the dilution series: 20, 2.5, 0.313, 0.039, 0.005, 0.000625ng/µL. The neat samples and dilution series were quantified in triplicate.

The following forensically relevant inhibitors were tested in this study: hematin (ICN Biomedicals, Aurora, OH, USA), humic acid (Alfa Aesar, Ward Hill, MA, USA), calcium hydrogen phosphate (Sigma-Aldrich, Milwaukee, WI, USA), melanin (Sigma-Aldrich), and sodium chloride in solution (VWR, Radnor, PA, USA). Hematin, humic acid, calcium, and melanin were prepared according to Thompson et al. [13]. Sodium chloride

in solution was prepared by dissolving 292.2g sodium chloride in 700mL deionized water and adjusted to a final volume of 1L. Two human male DNA standards, NIST SRM 2372 Component A and TaqMan® Control Genomic DNA (Thermo Fisher Scientific), were used to spike with aliquots of these inhibitors. Each DNA standard was diluted to 1ng/µL followed by a 1 in 2 dilution with various concentrations of inhibitors for a final DNA concentration of 0.5ng/µL. The final inhibitor concentrations are listed as the concentration in the DNA sample (Table 4.2.). Non-inhibited samples (0.5ng/µL of DNA) were also quantified as controls. All samples were quantified in duplicate.

Table 4.2. Inhibitor sample concentrations

Hematin (µM)	*Humic Acid (ng/µL)*	*Calcium (mM)*	*Melanin (ng/µL)*	*Salt (mM)*
30	10	1	10	100
50	20	1.5	25	200
70	30	2	50	300
90	40	2.5	75	400
110	50	3	100	500
500	100	15	175	600
600	200	20	200	700
700	250	25	225	800
800	300	30	250	900
900	400	35	275	1000

The degraded samples included DNA from bones (N=5), decomposed tissues (N=5), and formalin-damaged samples from embalmed tissues (N=5). Human remains were provided by the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, Texas, USA. Bone samples were extracted with either QIAamp®

DNA Investigator Kit (QIAGEN) or PrepFiler® BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific) following manufacturer's instructions [21,22]. Decomposed tissues were extracted with the QIAamp® DNA Investigator Kit on the QIAcube® (QIAGEN) using the forensic casework samples protocol [23]. Embalmed samples were extracted with QIAamp® DNA FFPE Tissue Kit (QIAGEN) following manufacturer's instructions [24].

DNA Quantification and Assessment

All samples were quantified with Investigator® Quantiplex® Pro Kit, Quantifiler® Trio DNA Quantification Kit, PowerQuant® System, and InnoQuant® HY following manufacturer's instructions [16-19]. DNA quantification was performed on a 7500 Real-Time PCR System (Thermo Fisher Scientific) using each kit's respective template. Data were analyzed using the HID Real-Time PCR Analysis Software v1.2, where a R^2 value $\geq$0.99 on the standard curve was accepted.

Additional analysis was required for each kit in order to calculate the DI and inhibition index. The DI is generated automatically for Quantifiler® Trio, whereas the InnoQuant® HY kit has a macro based analysis tool (InnoQuant Degradation Index Macro) [18]. The IPC ΔCT values (inhibition indices) were calculated manually for Quantifiler® Trio and InnoQuant® HY using manufacturer's recommendations [17,18]. The Investigator® Quantiplex® Pro Kit and PowerQuant® System include macro based analysis tools (QIAGEN Quantification Assay Data Handling and STR Setup Tool v2.01 and PowerQuant® Analysis Tool v1, respectively), which were used to produce the degradation and inhibition indices for the samples tested in this study [16,19]. Absolute values for the

inhibition indices from Investigator® Quantiplex® Pro were used for comparison with the other kits in this study.

STR Analysis of Degraded Samples

STR amplification and analysis was performed on all degraded samples (N=15) to assess any possible relationship of the DI with downstream STR success. Each commercial qPCR kit was paired with its corresponding STR amplification kit: 1) Investigator® 24plex QS Kit (QIAGEN), 2) GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific), and 3) PowerPlex® Fusion 6C System (Promega Corporation) using each manufacturer's instructions [25-27]. Although the InnoQuant® HY kit can be paired with any STR kit, STR amplification was not performed due to the limited DNA extract available. The DNA concentration assessed by the small target was used from each qPCR kit to target 0.8ng for each STR reaction, when possible. For samples with <0.053 ng/µL the maximum of 15µL was added to the PCR reaction. Amplification was performed on a ProFlex™ PCR System (Thermo Fisher Scientific) using each STR kit's recommended cycling parameters [25-27].

Separation and detection of PCR products was performed on a 3500 Genetic Analyzer (Thermo Fisher Scientific) using a 36cm capillary array and POP4 polymer platform. Data were analyzed with GeneMapper ID-X Software v1.4 (Thermo Fisher Scientific). The following thresholds were used for data interpretation: an analytical threshold (AT) of 100 relative fluorescent units (RFUs) and stochastic threshold (ST) of 200 RFUs for Investigator® 24plex QS, AT of 150 RFUs and ST of 600 RFUs for GlobalFiler®, and AT of 175 RFUs and ST of 400 RFUs for PowerPlex® Fusion 6C.

Statistical Analysis

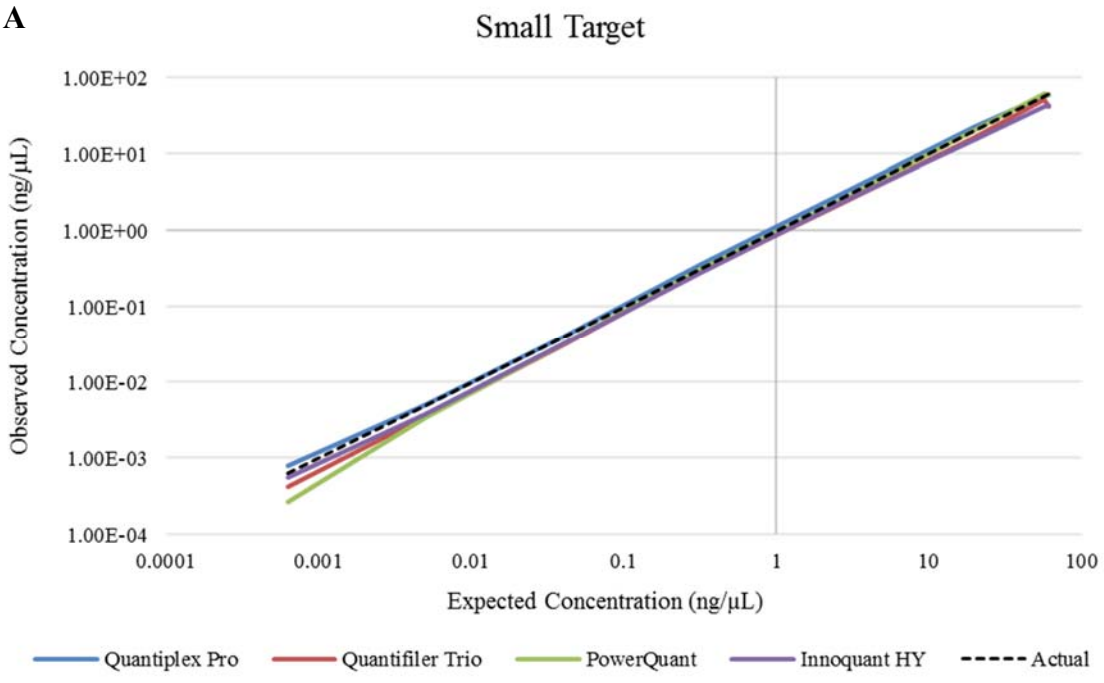
Data were tested for statistical significance by one-way ANOVA analysis in Microsoft Excel. $P<0.05$ was accepted as the level of significance.

Results and Discussion

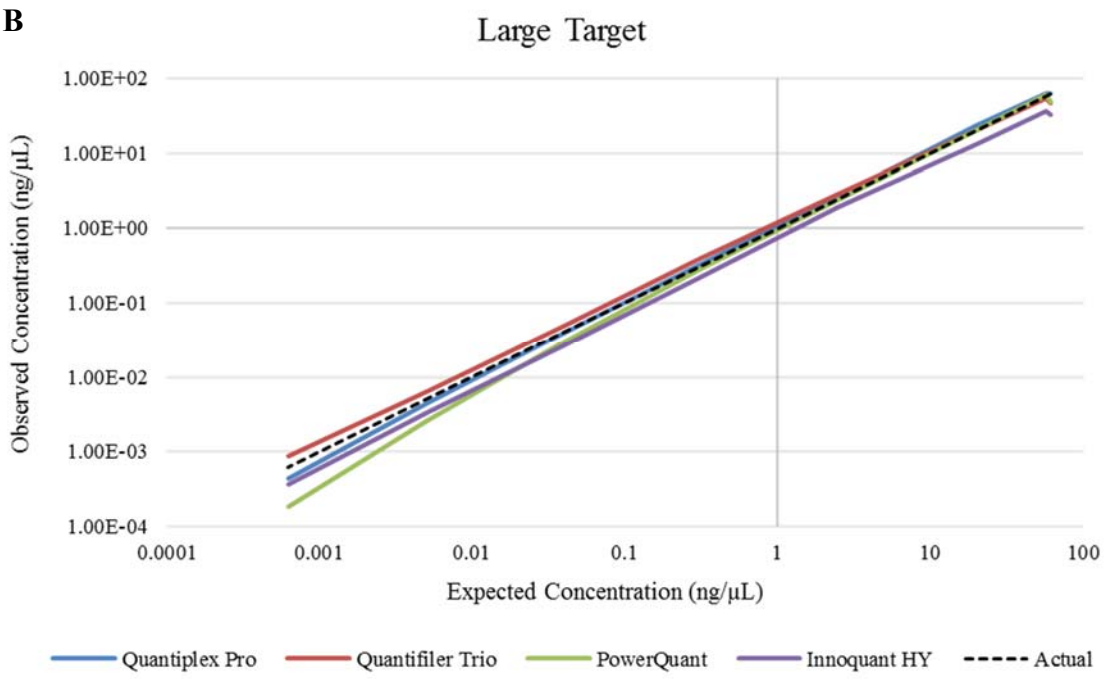
Sensitivity, Precision, and Accuracy

Forensic casework samples range in DNA concentration from nanograms down to picograms. In order to test each kits sensitivity, precision, and accuracy, we assessed the small and large target concentrations for two sources of human DNA and the male target concentration for the male DNA source. Data from two DNA sources (SRM A and SRM B) were combined in the logarithmic observed vs. expected graphs (Fig. 4.1. A and B). In general, all kits produced reliable autosomal and male DNA quantification results along the sensitivity range. However, more disparity was observed in DNA concentrations that fell outside the concentration range for each kit's respective standard curve (50 – 0.0025ng/µL for Investigator® Quantiplex® Pro; 50 – 0.005ng/µL for Quantifiler® Trio; 50 – 0.0032ng/µL for PowerQuant®; 20 – 0.005ng/µL for Innoquant® HY). These findings are consistent with Loftus et al. for the InnoQuant® HY kit [3]. At the lower DNA concentrations, the PowerQuant® System and Investigator® Quantiplex® Pro Kit were the least accurate for the human targets and the male target, respectively (Fig. 4.1. A-C). Accuracy for each of the kits is represented by the fold change and the precision along the dilution series can be observed with the %CV (Tables 4.3. – 4.6.). InnoQuant® HY had the overall lowest %CV compared to the other kits suggesting that this kit may have better precision across the range tested in this study (Table 4.6.).

A


Small Target
Observed Concentration (ng/µL)
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
0.0001
0.001
0.01
0.1
1
10
100
Expected Concentration (ng/µL)
Quantiplex Pro
Quantifiler Trio
PowerQuant
Innoquant HY
Actual

B


Large Target
Observed Concentration (ng/µL)
1.00E+02
1.00E+01
1.00E+00
1.00E-01
1.00E-02
1.00E-03
1.00E-04
0.0001
0.001
0.01
0.1
1
10
100
Expected Concentration (ng/µL)
Quantiplex Pro
Quantifiler Trio
PowerQuant
Innoquant HY
Actual

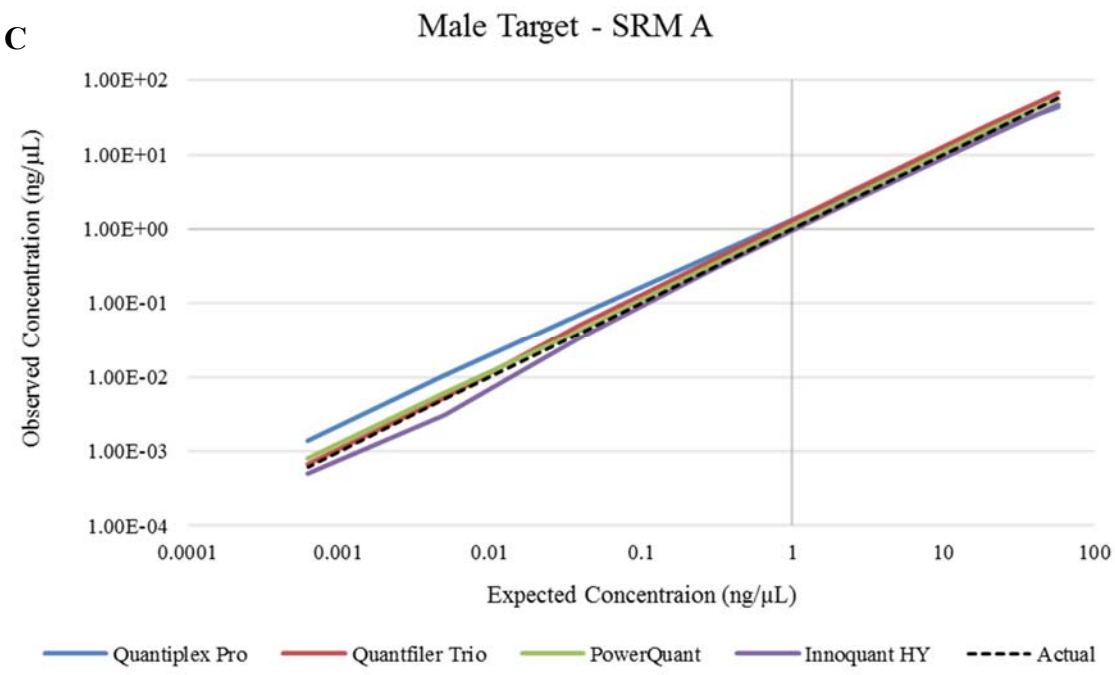


Fig. 4.1. Average observed vs. expected concentrations for each commercial qPCR kit presented as (A) the short target, (B) the large target for SRM A & B combined, and (C) the male target for SRM A. Data represents mean presented in a logarithmic scale

Table 4.3. Sensitivity, precision and accuracy results of NIST SRM 2372 Component A and Component B dilutions for Investigator® Quantiplex® Pro

Sample Source	Expected Conc. (ng/µL)	Observed Small				Observed Large				Observed Y			
		Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV
NIST A SRM	57.0	57.5	7.2	1.01	12.4%	62.1	7.7	1.09	12.5%	44.3	4.8	1.29	10.8%
	20.0	23.7	3.2	1.18	13.5%	25.4	4.4	1.27	17.5%	20.4	2.8	1.02	13.5%
	2.50	2.84	0.04	1.14	1.6%	2.81	0.04	1.12	1.4%	3.13	0.03	1.25	1.0%
	0.313	0.341	0.019	1.09	5.5%	0.327	0.013	1.04	4.1%	0.468	0.011	1.50	2.4%
	0.0390	0.0390	0.0034	1.00	8.8%	0.0390	0.0038	1.00	9.6%	0.0697	0.0029	1.79	4.1%
	5.00E-03	5.00E-03	6.93E-04	1.00	13.9%	4.40E-03	7.21E-04	1.14	16.4%	1.05E-02	5.77E-05	2.09	0.6%
	6.25E-04	8.00E-04	1.00E-04	1.28	12.5%	5.00E-04	1.00E-04	1.25	20.0%	1.40E-03	1.23E-03	2.24	87.8%
NIST B SRM	61.0	59.6	2.0	1.02	3.3%	62.1	2.7	1.02	4.3%	0	-	-	-
	20.0	22.0	0.8	1.10	3.7%	22.4	1.0	1.12	4.4%	0	-	-	-
	2.50	2.78	0.16	1.11	5.8%	2.66	0.17	1.06	6.3%	0	-	-	-
	0.313	0.350	0.013	1.12	3.7%	0.325	0.021	1.04	6.4%	0	-	-	-
	0.0390	0.0396	0.0043	1.02	10.9%	0.0391	0.0041	1.00	10.5%	0	-	-	-
	5.00E-03	5.00E-03	9.17E-04	1.00	18.3%	4.60E-03	1.32E-03	1.09	28.8%	0	-	-	-
	6.25E-04	7.67E-04	1.15E-04	1.23	15.1%	3.67E-04	2.08E-04	1.70	56.8%	0	-	-	-

Table 4.4. Sensitivity and Accuracy results of NIST SRM 2372 Component A and Component B dilutions for Quantifiler® Trio

Sample Source	Expected Conc. (ng/µL)	Observed Small				Observed Large				Observed Y			
		Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV
NIST A SRM	57.0	51.3	1.0	1.11	2.0%	55.0	3.2	1.04	5.9%	68.2	0.6	1.20	0.9%
	20.0	18.5	0.4	1.08	2.3%	21.9	1.1	1.10	5.1%	25.4	0.5	1.27	2.0%
	2.50	2.48	0.17	1.01	6.9%	3.11	0.11	1.25	3.4%	3.37	0.14	1.35	4.3%
	0.313	0.278	0.013	1.13	4.6%	0.382	0.014	1.22	3.6%	0.397	0.017	1.27	4.3%
	0.0390	0.0358	0.0042	1.09	11.6%	0.0523	0.0015	1.34	3.0%	0.0501	0.0049	1.29	9.7%
	5.00E-03	3.55E-03	1.58E-04	1.41	4.5%	5.85E-03	1.20E-03	1.17	20.5%	5.25E-03	1.48E-03	1.05	28.2%
	6.25E-04	5.42E-04	1.62E-04	1.15	29.9%	8.65E-04	6.24E-04	1.38	72.1%	6.74E-04	3.10E-04	1.08	46.1%
NIST B SRM	61.0	41.6	9.4	1.47	22.5%	46.2	8.0	1.32	17.2%	0	-	-	-
	20.0	15.3	2.2	1.30	14.2%	18.8	2.1	1.07	11.4%	0	-	-	-
	2.50	1.86	0.50	1.34	26.8%	2.66	0.57	1.06	21.3%	0	-	-	-
	0.313	0.288	0.058	1.09	20.1%	0.394	0.075	1.26	19.0%	0	-	-	-
	0.0390	0.0262	0.0067	1.49	25.6%	0.0449	0.0095	1.15	21.1%	0	-	-	-
	5.00E-03	3.55E-03	7.16E-04	1.41	20.2%	6.95E-03	8.67E-04	1.39	12.5%	0	-	-	-
	6.25E-04	3.01E-04	2.16E-04	2.08	71.6%	8.66E-04	6.00E-04	1.39	69.3%	0	-	-	-

Table 4.5. Sensitivity and Accuracy results of NIST SRM 2372 Component A and Component B dilutions for PowerQuant® System

Sample Source	Expected Conc. (ng/µL)	Observed Small				Observed Large				Observed Y			
		Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV
NIST A SRM	57.0	62.2	6.9	1.09	11.0%	61.0	1.1	1.07	1.8%	56.0	0.8	1.11	1.5%
	20.0	21.3	1.6	1.07	7.7%	23.5	1.2	1.17	5.0%	22.0	1.6	1.10	7.2%
	2.50	2.40	0.42	1.04	17.4%	2.67	0.25	1.07	9.3%	2.79	0.21	1.12	7.6%
	0.313	0.295	0.009	1.06	3.0%	0.302	0.015	1.04	4.9%	0.351	0.010	1.12	2.8%
	0.0390	0.0292	0.0017	1.33	5.9%	0.0289	0.0011	1.35	3.8%	0.0422	0.0089	1.08	21.2%
	5.00E-03	4.04E-03	6.27E-04	1.24	15.5%	3.37E-03	4.57E-04	1.48	13.5%	6.16E-03	1.16E-03	1.23	18.8%
	6.25E-04	3.00E-04	1.00E-04	2.08	33.3%	2.00E-04	0.00E+00	3.13	0.0%	8.00E-04	1.00E-03	1.28	125.0%
NIST B SRM	61.0	60.2	3.0	1.01	5.0%	49.0	1.8	1.24	3.6%	0	-	-	-
	20.0	20.1	2.3	1.00	11.4%	17.0	0.5	1.18	2.8%	0	-	-	-
	2.50	2.37	0.37	1.05	15.6%	2.07	0.34	1.21	16.4%	0	-	-	-
	0.313	0.324	0.017	1.03	5.3%	0.263	0.016	1.19	6.1%	0	-	-	-
	0.0390	0.0345	0.0042	1.13	12.3%	0.0287	0.0042	1.36	14.8%	0	-	-	-
	5.00E-03	2.84E-03	6.33E-04	1.76	22.3%	1.87E-03	3.00E-04	2.68	16.1%	0	-	-	-
	6.25E-04	2.29E-04	2.62E-04	2.73	114.4%	1.65E-04	1.70E-04	3.79	103.0%	0	-	-	-

Table 4.6. Sensitivity and Accuracy results of NIST SRM 2372 Component A and Component B dilutions for Innoquant® HY

Sample Source	Expected Conc. (ng/µL)	Observed Small				Observed Large				Observed Y			
		Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV	Avg. Quantity	Std. Dev.	Fold Change	%CV
NIST A SRM	57.0	42.0	3.6	1.36	8.6%	36.0	2.8	1.58	7.9%	46.6	2.5	1.22	5.3%
	20.0	16.2	0.3	1.23	1.5%	14.2	0.7	1.41	4.8%	17.5	0.6	1.14	3.6%
	2.50	2.33	0.07	1.07	2.9%	2.12	0.13	1.18	6.0%	2.40	0.09	1.04	3.6%
	0.313	0.278	0.016	1.13	5.9%	0.240	0.021	1.30	8.8%	0.297	0.023	1.05	7.7%
	0.0390	0.0306	0.0018	1.28	6.0%	0.0273	0.0058	1.43	21.1%	0.0334	0.0036	1.17	10.9%
	5.00E-03	3.96E-03	1.59E-04	1.26	4.0%	3.60E-03	2.81E-04	1.39	7.8%	3.10E-03	1.37E-03	1.61	44.0%
	6.25E-04	5.46E-04	1.83E-05	1.14	3.4%	3.66E-04	1.18E-05	1.71	3.2%	5.06E-04	-	1.23	-
NIST B SRM	61.0	43.3	3.6	1.41	8.4%	33.1	1.8	1.84	5.5%	0	-	-	-
	20.0	14.9	0.4	1.34	2.6%	12.0	0.5	1.66	4.4%	0	-	-	-
	2.50	2.18	0.03	1.15	1.6%	1.73	0.10	1.45	5.9%	0	-	-	-
	0.313	0.263	0.024	1.19	9.3%	0.209	0.010	1.50	4.9%	0	-	-	-
	0.0390	0.0329	0.0023	1.19	7.1%	0.0250	0.0015	1.56	6.1%	0	-	-	-
	5.00E-03	3.55E-03	2.11E-04	1.41	5.9%	3.05E-03	1.48E-04	1.64	4.9%	0	-	-	-
	6.25E-04	5.77E-04	4.82E-05	1.08	8.4%	3.55E-04	5.96E-05	1.76	16.8%	0	-	-	-

Inhibition

Control DNA sources (0.5ng/µL with no inhibitor added) were also quantified with each of the four commercial qPCR kits. Investigator® Quantiplex® Pro generated a small target DNA concentration of 0.52±0.12ng/µL, 0.44±0.18ng/µL using Quantifiler® Trio, 0.44±0.05ng/µL with PowerQuant®, and 0.33±0.05ng/µL with InnoQuant® HY. As expected no PCR inhibition was detected in any control samples.

In general, all kits provided similar inhibition indices with the lower inhibitor concentrations tested, but the DNA quantification results varied between kits. The inhibition indices for hematin were similar for all kits until the PowerQuant® System started flagging inhibition at 500µM, Quantifiler® Trio at 600µM, and InnoQuant® HY at 900µM (Fig. 4.2.). The IPC targets in the Investigator® Quantiplex® Pro kit and InnoQuant® HY were the most tolerant to hematin as they did not flag the concentrations of hematin tested as inhibited (until the highest concentration). However, the DNA concentrations reported by these two kits gradually decreased as the concentration of hematin increased, similar to the other two kits (Quantifiler® Trio and PowerQuant®) that flagged inhibition (Fig. 4.3.). Furthermore, there was a statistically significant difference between kits as determined by one-way ANOVA ($F_{3,36}$ = 6.4779, p = 1.28E-03). The Investigator® Quantiplex® Pro kit reported more accurate quantity results (average 0.4359ng/µL when compared to 0.5ng/µL and a variance of 0.00451) for all hematin samples tested (Fig. 4.3.).

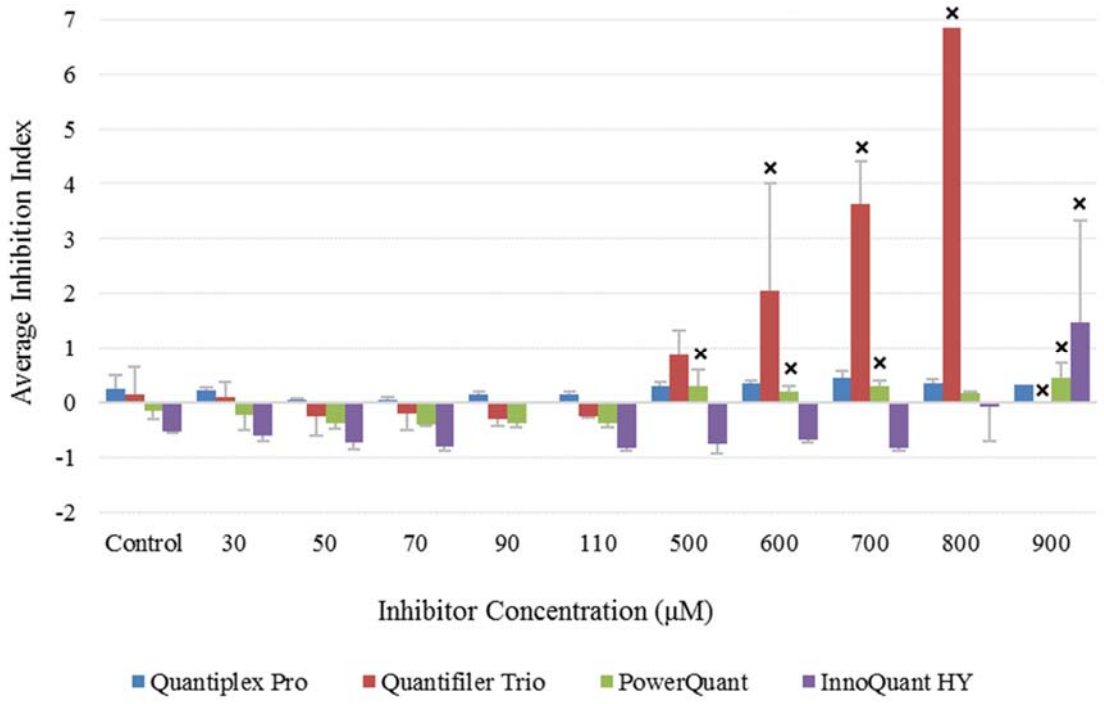


Fig. 4.2. Average inhibition indices for 0.5ng/uL standard DNA spiked with varying concentrations of hematin. Data represents mean ± Std Dev. An **x** denotes when inhibition was flagged by a kits' default setting (as listed in Table 4.1.)

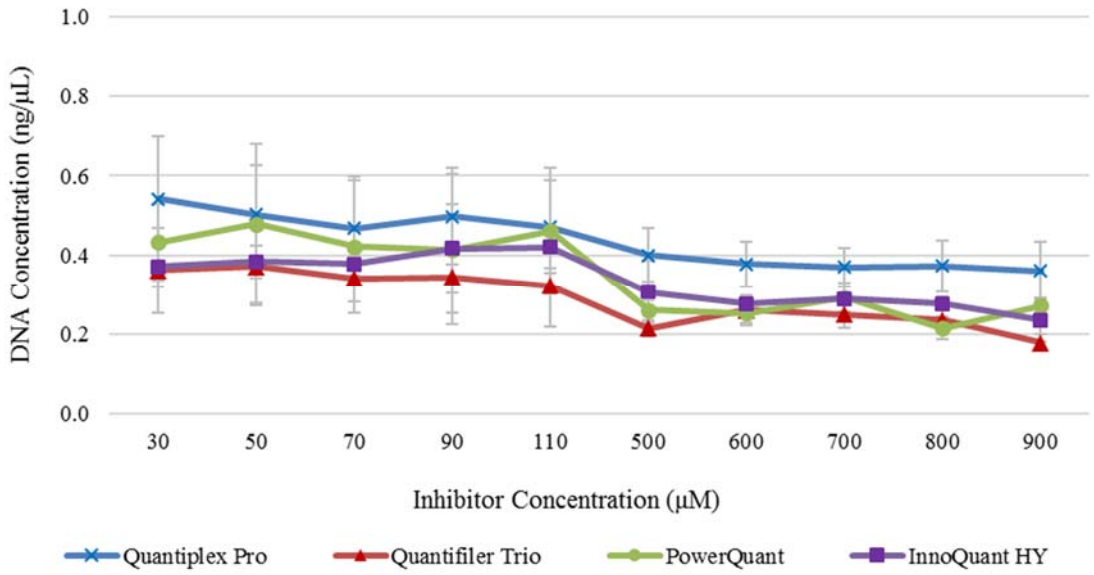


Fig. 4.3. Average DNA concentrations for 0.5ng/uL standard DNA spiked with varying concentrations of hematin. Data represents mean ± Std Dev

Humic acid samples were not flagged for inhibition until 200ng/µL with the PowerQuant® System and InnoQuant® HY kit, 250ng/µL with Investigator® Quantiplex® Pro, and 400ng/µL with Quantifiler® Trio (Fig. 4.4). Quantifiler® Trio results are consistent with Holt et al. [7]. A statistically significant difference in DNA concentration between kits was observed, as determined by one-way ANOVA ($F_{3,36}$ = 14.2939, p = 2.7E-06). The DNA concentrations from the Investigator® Quantiplex® Pro Kit were more accurate and the small target more tolerant (average 0.5075ng/µL with a variance of 0.00176), while the InnoQuant® HY kit was the most susceptible to high levels of humic acid (average 0.2493 and a variance of 0.02432; Fig. 4.5.).

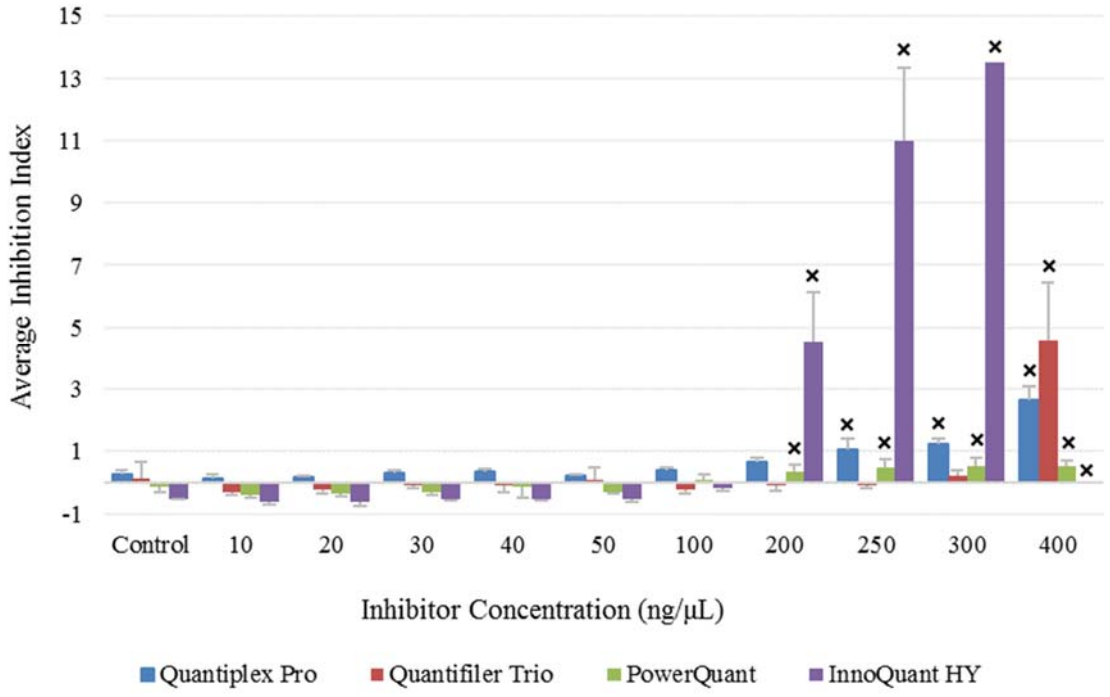


Fig. 4.4. Average inhibition indices for 0.5ng/uL standard DNA spiked with varying concentrations of humic acid. Data represents mean ± Std Dev. An **x** denotes when inhibition was flagged by a kits' default setting (as listed in Table 4.1.)

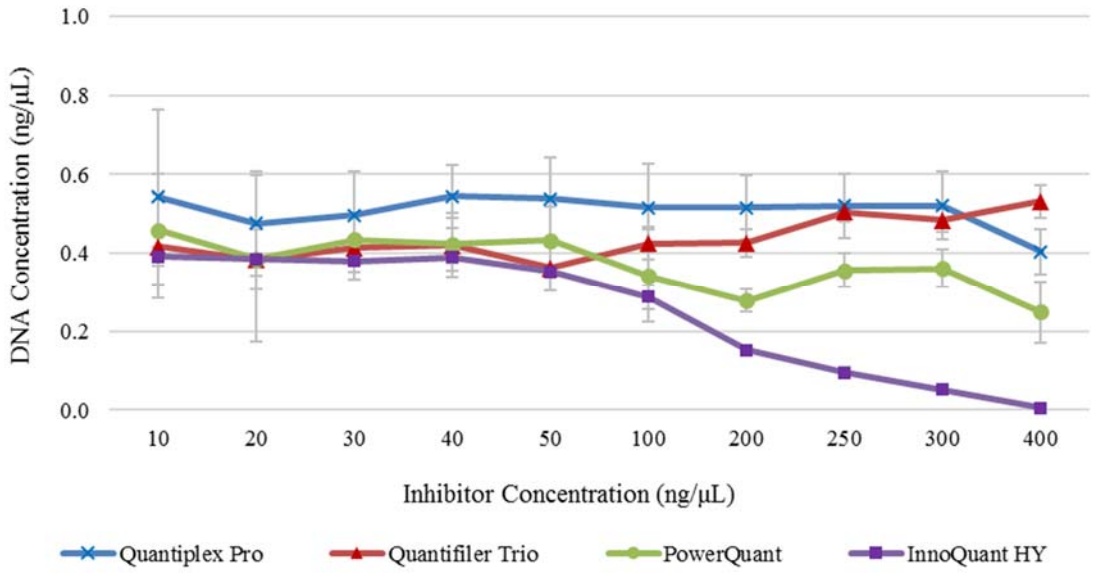


Fig. 4.5. Average DNA concentrations for 0.5ng/uL standard DNA spiked with varying concentrations of humic acid. Data represents mean ± Std Dev

Samples inhibited with calcium were flagged for inhibition starting at 15mM with PowerQuant® and InnoQuant® HY, 25mM with Quantifiler® Trio, and 30mM with Investigator® Quantiplex® Pro (Fig. 4.6.). There was a statistically significant difference in DNA concentration between kits as determined by one-way ANOVA ($F_{3,36}$ = 15.2388, p = 1.5E-06). The DNA concentrations from the Investigator® Quantiplex® Pro kit were more accurate and the small target was more tolerant to this inhibitor (average 0.4038 and smallest variance of 0.00144; Fig. 4.7.). These results suggest that Investigator® Quantiplex Pro kit is suitable for skeletal samples that are often inhibited with calcium from the bone and humic acid from the soil where the bones are often retrieved.

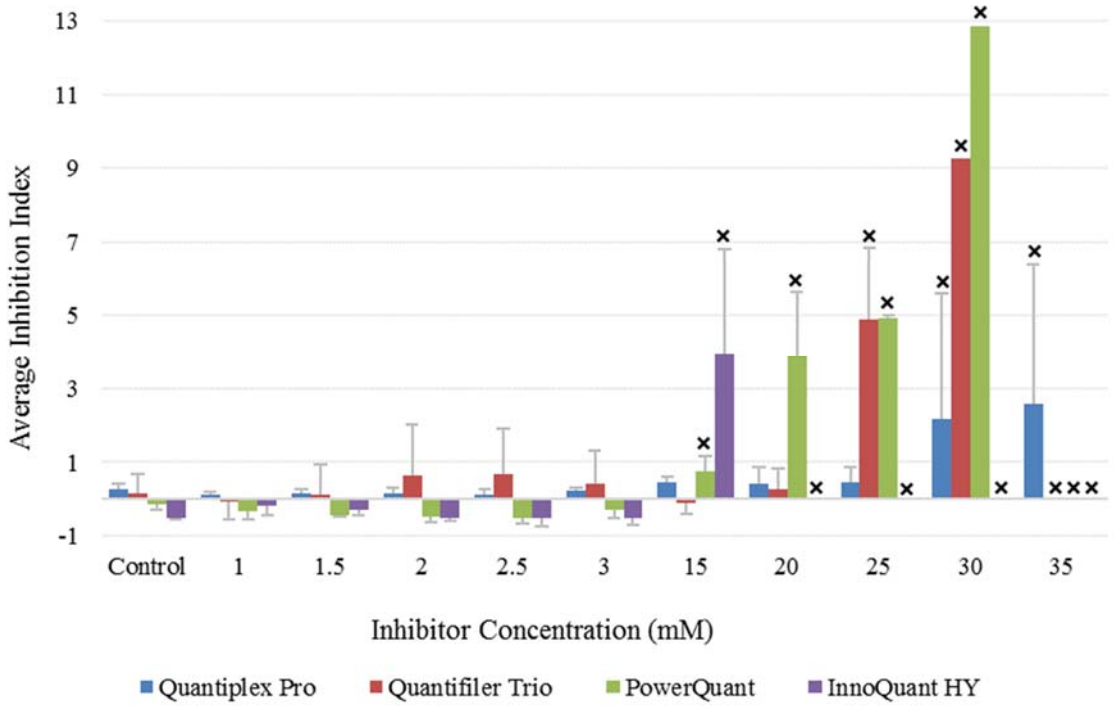


Fig. 4.6. Average inhibition indices for 0.5ng/uL standard DNA spiked with varying concentrations of calcium. Data represents mean ± Std Dev. An **x** denotes when inhibition was flagged by a kits' default setting (as listed in Table 4.1.)

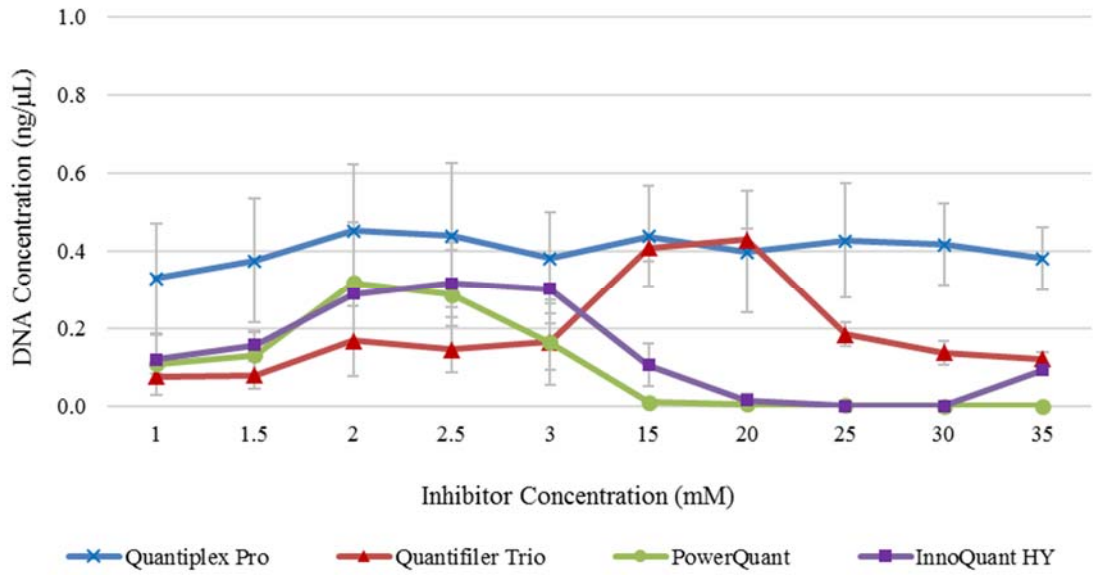


Fig. 4.7. Average DNA concentrations for 0.5ng/uL standard DNA spiked with varying concentrations of calcium. Data represents mean ± Std Dev

Melanin samples were flagged for inhibition at 75ng/µL with the Investigator® Quantiplex® Pro and InnoQuant® HY kits and 175ng/µL with the PowerQuant® System (Fig. 4.8.). The IPC target from Quantifiler® Trio was more tolerant to melanin as none of the samples were flagged for inhibition (Fig. 4.8.). There was a statistically significant difference in DNA concentrations between the four kits as determined by one-way ANOVA ($F_{3,36}$ = 11.4574, p = 2E-05). However, when a separate one-way ANOVA was performed excluding InnoQuant® HY samples no statistical significance was detected between the three remaining qPCR kits. Therefore, the small target from InnoQuant® HY was the most susceptible to these concentrations of melanin compared to the other qPCR kits (Fig. 4.9.).

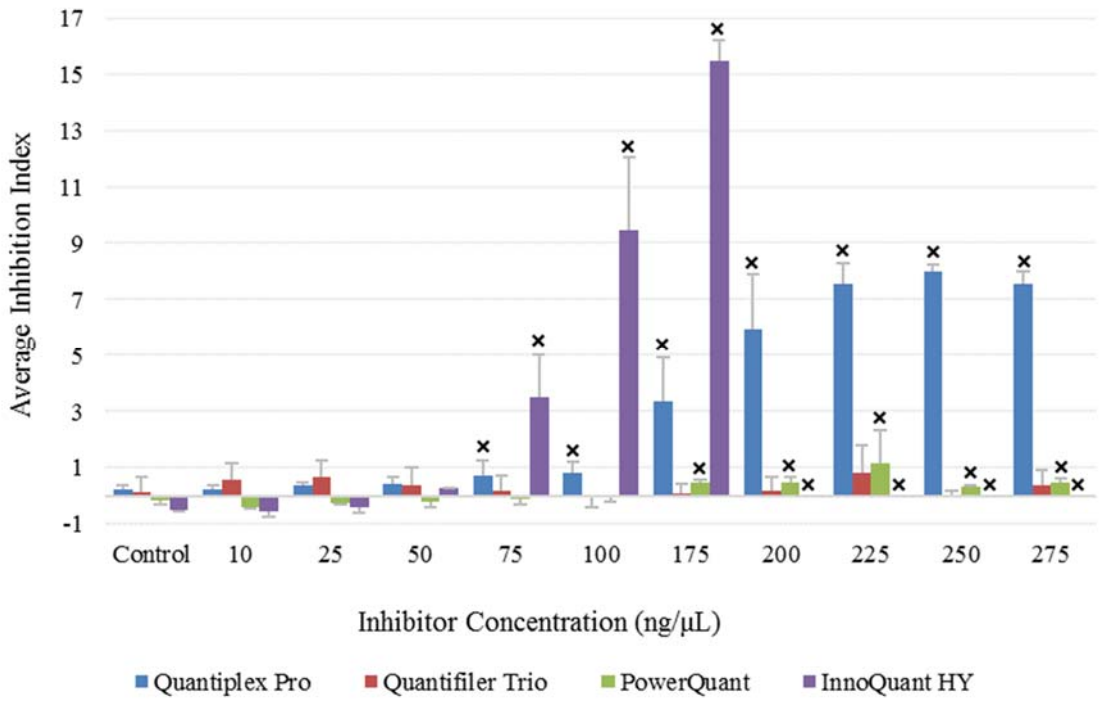


Fig. 4.8. Average inhibition indices for 0.5ng/uL standard DNA spiked with varying concentrations of melanin. Data represents mean ± Std Dev. An **x** denotes when inhibition was flagged by a kits' default setting (as listed in Table 4.1.)

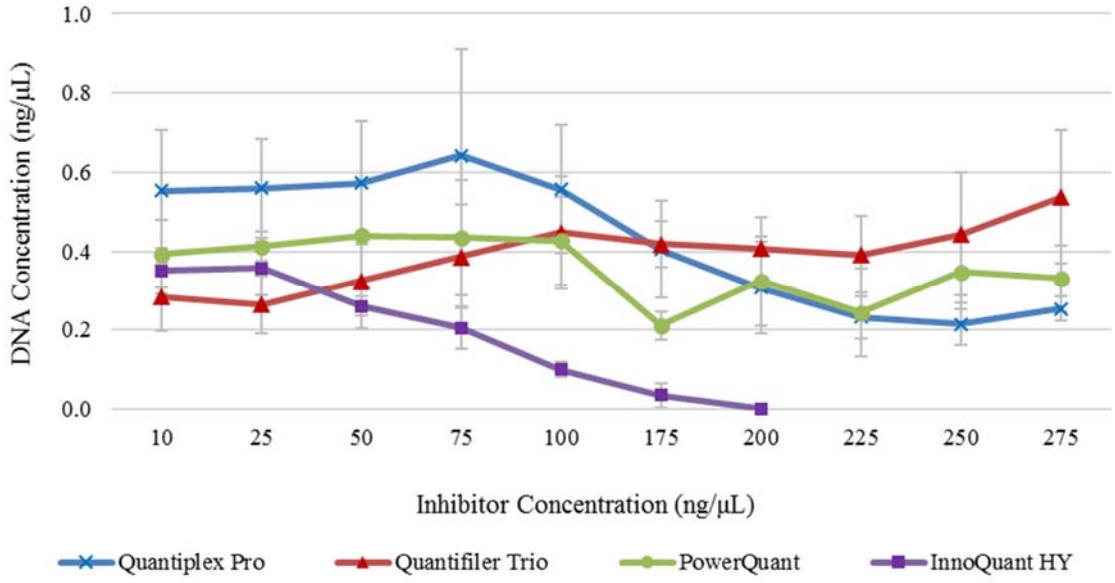


Fig. 4.9. Average DNA concentrations for 0.5ng/uL standard DNA spiked with varying concentrations of melanin. Data represents mean ± Std Dev

Lastly, the samples inhibited with salt were flagged for inhibition starting at 200mM for Investigator® Quantiplex® Pro and PowerQuant® System and 400mM for InnoQuant® HY (Fig. 4.10.). Quantifiler® Trio showed no signs of inhibition before the IPC target failed to amplify at 800mM salt. There was a statistically significant difference in DNA concentration between kits as determined by one-way ANOVA ($F_{3,36}$ = 6.8143, p = 9.4E-04). However, DNA quantification results could only be obtained using Quantifiler® Trio when the concentration of salt in the sample was greater than 500mM (Fig. 4.11.). Therefore, Quantifiler® Trio was more tolerant to these high concentrations of salt (average = 0.3142ng/µL, variance = 0.01264).

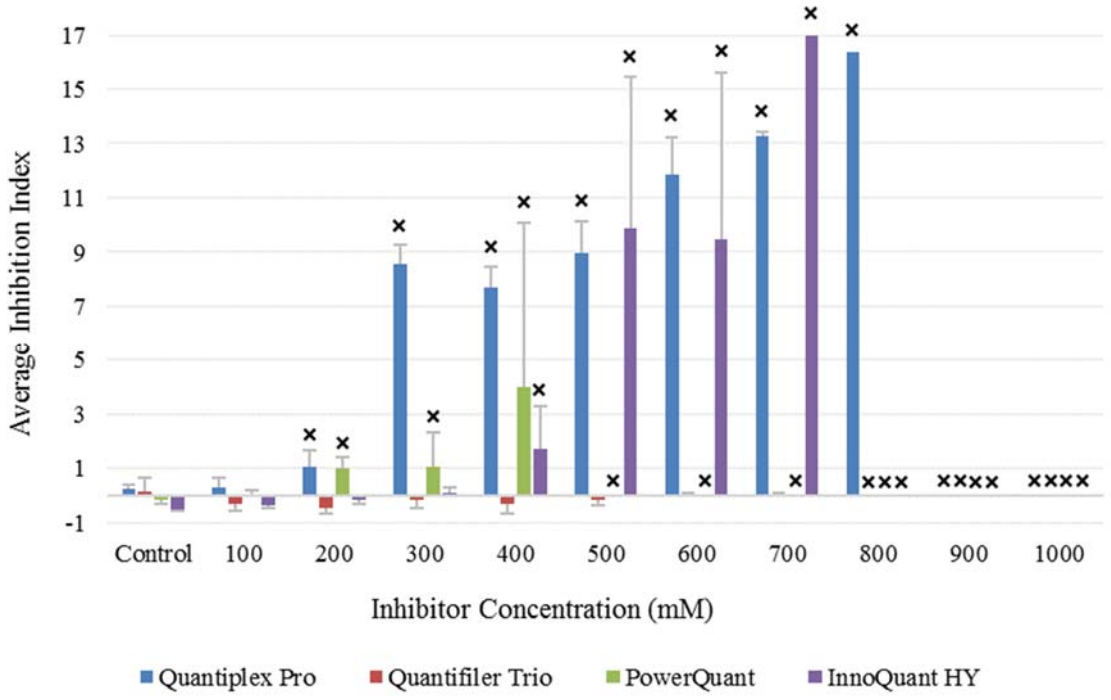


Fig. 4.10. Average inhibition indices for 0.5ng/uL standard DNA spiked with varying concentrations of salt. Data represents mean ± Std Dev. An **x** denotes when inhibition was flagged by a kits' default setting (as listed in Table 4.1.)

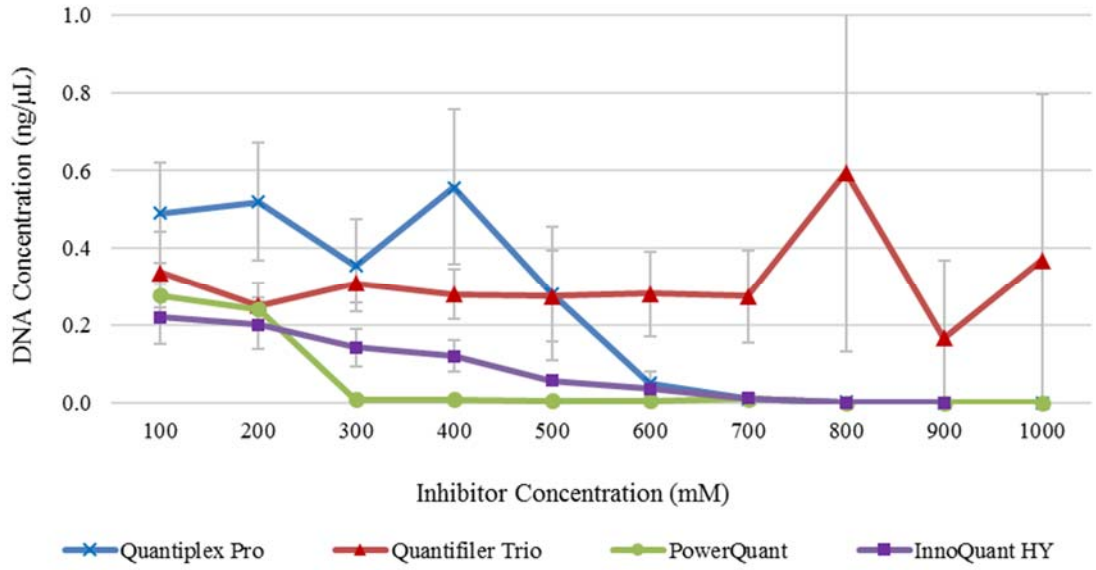


Fig. 4.11. Average DNA concentrations for 0.5ng/uL standard DNA spiked with varying concentrations of salt. Data represents mean ± Std Dev

Our results generally support those of Thompson et al., who also detected inhibition with ~60µM hematin in the reaction (600µM in the sample), 25ng/µL humic acid (250 ng/µL in the sample), and 1.5mM calcium (15mM in the sample for this study) [13]. However, we have found that some kits were more tolerant to certain inhibitors than others. Overall, Investigator® Quantiplex® Pro was more tolerant to inhibitors (except high concentrations of salt), whereas InnoQuant® HY was more susceptible to high concentrations of inhibitors (except high concentrations of hematin; Fig. 4.12.).

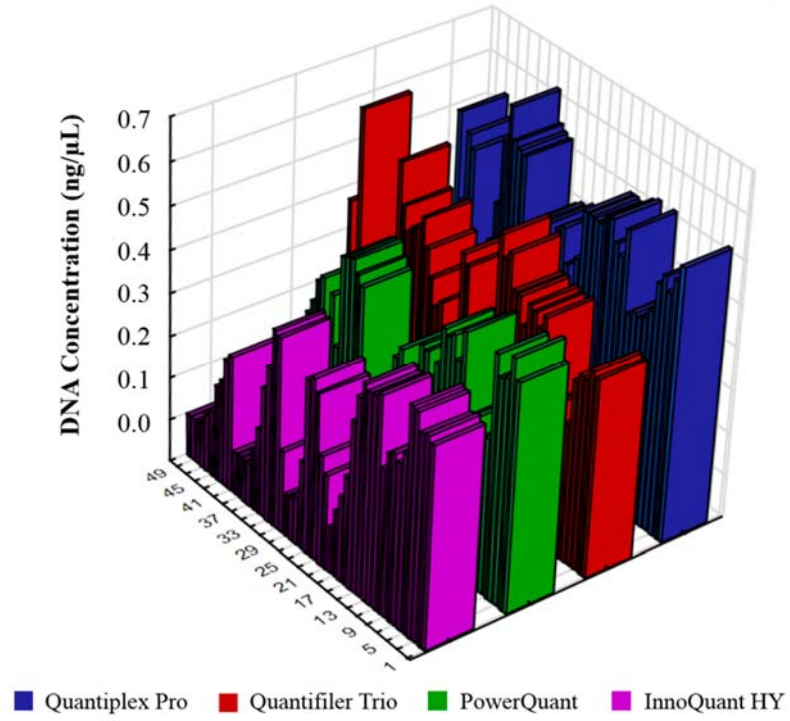


Fig. 4.12. Average DNA concentrations 0.5ng/uL standard DNA spiked with varying concentrations of hematin (bars 1-10), humic acid (11-20), calcium (21-30), melanin (31-40), and salt (41-50)

In general, when the small target DNA concentrations were decreased by half or more (<0.25ng/µL) due to severe inhibition, the IPC target did indicate the presence of inhibitors in all kits. The results of this study support the practice of interrogating the IPC

information to avoid adding more extract volume into the PCR and thereby also increasing the amount of inhibitor. This approach could reduce STR success or result in complete PCR failure.

Degradation

The same volume of neat DNA (2µL) from bones (N=5), decomposed tissues (N=5), and formalin-damaged samples from embalmed tissues (N=5) was quantified with each of the four qPCR kits. For all of the challenging samples tested, the average DNA concentrations based on the small target were similar for each kit, but the large target concentration obtained from Quantifiler® Trio was consistently the highest (Fig. 4.13. A). Thus, Quantifiler® Trio generated the smallest DIs for each set of degraded samples (Fig. 4.13. B). When comparing the degradation flags for each kit, all samples' DIs generated by Quantifiler® Trio were greater than 1 but less than 10 indicating moderate degradation. Investigator® Quantiplex® Pro generated the highest DIs, where all samples were greater than 1 but only 60% of samples indicated degradation (DI >10) (Fig. 4.13. B). The difference between the results generated by these two kits may be explained by the difference in size between the two large targets in each kit (~140bp). In addition, the PowerQuant® System indicated degradation in all degraded samples (DI >2), whereas InnoQuant® HY indicated moderate degradation in 73% of samples (2.5<DI<20) and severe degradation in 27% (>20).

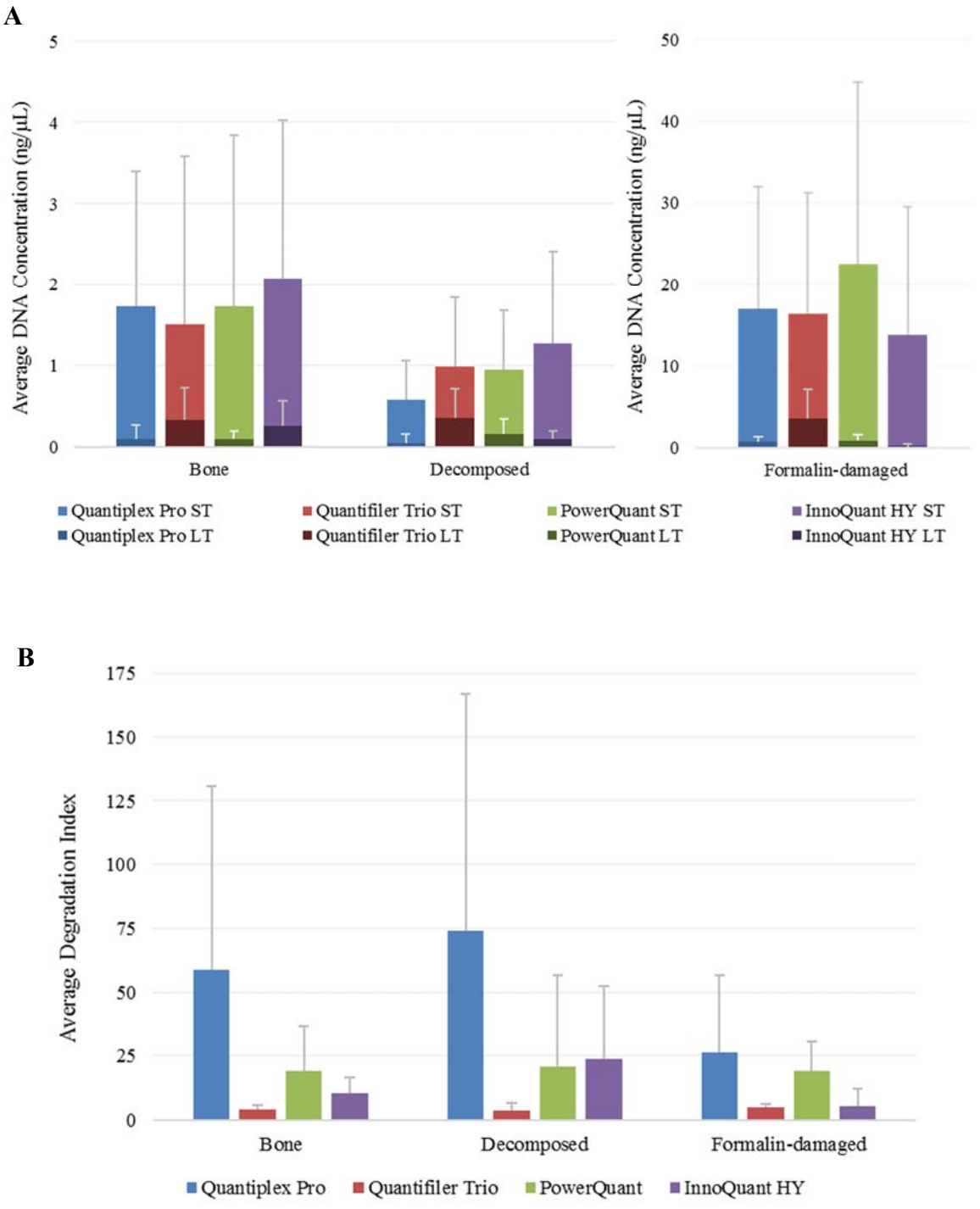


Fig. 4.13. Average DNA quantification results for five bone, five decomposed, and five formalin-damaged samples presented as (A) average DNA concentrations obtained using each qPCR kit (ST = small target, LT = large target) and (B) the average degradation index calculated by each qPCR system. Data represents mean ± Std Dev

All commercial qPCR kits (except InnoQuant® HY) were paired with their respective STR amplification kit. The combinations of Investigator® Quantiplex® Pro with Investigator® 24plex QS and Quantifiler® Trio with Globalfiler® yielded the greatest STR success when using the small target, but the average DIs varied greatly between the two qPCR kits (Fig. 4.14.). Quantifiler® Trio predicted moderate degradation in all samples and Investigator® Quantiplex® Pro generated high DIs. Although degradation was indicated by both qPCR kits, full profiles were produced in 53% of samples for Investigator® 24plex QS and 47% for GlobalFiler® when the small target DNA concentration was used to determine the input volume into PCR. The PowerQuant® System indicated degradation in all samples similar to Quantifiler® Trio, but PowerPlex® Fusion 6C had the highest occurrence of allele drop out when using the small target DNA concentration (Fig. 4.14.). Loftus et al. and Pineda et al. have suggested using the large target DNA concentration from the InnoQuant® assays to assess the amount of sample required for STR typing [3,4]. Due to the lower first pass success of the PowerQuant® and PowerPlex® Fusion 6C combination with degraded samples, the use of the large target DNA concentration for input volume might also be beneficial for the PowerQuant® System. However, routinely using the large target with Quantifiler® Trio and Investigator® Quantiplex® Pro with degraded samples may introduce unwanted artifacts such as drop in and split peaks in the smaller marker region (due to too much DNA) making interpretation more difficult. In this study, using the small target concentrations with Quantifiler® Trio and Investigator® Quantiplex® Pro yielded mostly complete downstream STR profiles. As previously mentioned, Investigator® Quantiplex® Pro contains the longest amplicon for the large target out of the four qPCR kits. If the large target concentration was used to assess input amount

for Investigator® Quantiplex® Pro, eight out of ten bone and decomposed samples would have required maximum volume into the STR reaction compared to only one sample based on the small target. Instead, an alternate approach may be to take the average of the small and large target DNA concentrations to improve STR typing success with these kits. This approach may find a balance between drop out of larger loci and overshooting smaller loci. However, we were not able to test this approach in this study due to the limited sample volume.

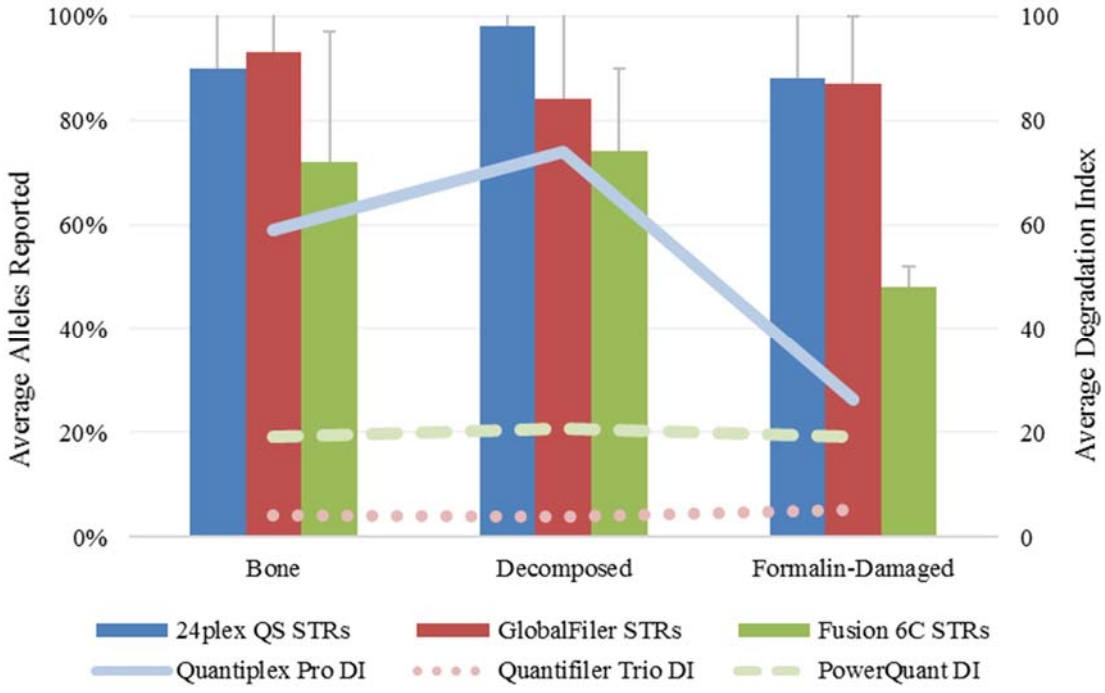


Fig. 4.14. Average STR results and degradation indices for each set of degraded samples

Consistent with previous studies [3,6,7] we also noticed with high concentrations of inhibitors that the large target amplification was preferentially affected, and often indirectly affected the DI value. As a result, these samples may be incorrectly flagged as

severely degraded. Therefore, it is important to note the DI information in conjunction with the IPC data to assess the integrity of the sample prior to STR amplification.

Conclusion

As may be expected, all kits performed similarly with high quality DNA samples and with low levels of inhibitors. All four qPCR kits were able to detect sub-picogram level of DNA and generate reliable, reproducible and accurate DNA quantification data. However, the InnoQuant® HY kit showed the highest precision across the DNA concentration range tested in this study. For the highly inhibited samples, Investigator® Quantiplex® Pro was the most tolerant kit to the inhibitors tested in this study and provided more accurate quantification results (except with high concentrations of salt). Quantifiler® Trio provided more stable DNA quantification results for salt compared to the other three qPCR kits. Generally, the other two kits (PowerQuant® and Innoquant® HY) were more sensitive to high concentrations of the various inhibitors; but the IPC target indicated significant levels of inhibition when the small target DNA concentration was decreased and thereby differentiating them from low template samples.

For degraded samples, each kit generated comparable small target DNA concentrations but predicted varying degrees of degradation. When samples were STR typed with each qPCR kit's respective STR kit (besides InnoQuant® HY), STR results varied with Investigator® 24plex QS and GlobalFiler® generating more complete profiles than PowerPlex® Fusion 6C. Using more input DNA for samples flagged as severely degraded during quantification may increase the chances of first pass successful STR typing for some samples, but might also introduce unwanted artifacts in others. Future research might include a more thorough investigation on increasing first pass STR success

with degraded samples testing various approaches such as using the large target DNA concentration for determining sample input into amplification or taking the average of small and large target DNA concentrations. The results of this study demonstrate the importance of assessing both the degradation and inhibition indicators. However, DI and inhibition flags should be defined within each laboratory to best streamline the laboratory's workflow, as per manufacturers' recommendations [16-19]. With the use of these commercial qPCR kits in the DNA analysis workflow, analysts may better predict the integrity of each sample and triage evidence prior to expensive and time-consuming genetic analyses for human identification purposes.

Role of Funding

This study was partially funded by Graduate Research Fellowship Award #2015-R2-CX-0029 (National Institute of Justice, Office of Justice Programs, U.S. Department of Justice). The opinions, findings, conclusions, or recommendations expressed in this article are those of the authors and do not necessarily reflect those of the National Institute of Justice.

Acknowledgments

The authors would like to thank QIAGEN, Thermo Fisher Scientific, Promega Corporation, and InnoGenomics Technologies for supplying some of the commercial qPCR kits used in this study. The authors would also like to thank the staff at the Applied Anatomical Research Center (AARC) at Sam Houston State University for their assistance, and the individuals and families of those who donated their bodies for scientific research.

Compliance with Ethical Standards

For the degraded samples (bone, decomposed and formalin-damaged tissues) used in this study, Code 45 of US Federal Regulations part 46102(f) exempts the requirement for Institutional Review Board (IRB) approval regarding the use of human cadaveric samples. All procedures were in accordance with the 1964 Helsinki Declaration and its later amendments.

Conflict of Interest

The authors declare that they have no competing interests.

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CHAPTER V

In-field Collection and Preservation of Decomposing Human Tissues to Facilitate Rapid Purification Methods[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1]Holmes AS, Roman MG, Hughes-Stamm S. In-field collection and preservation of decomposing human tissues to facilitate rapid purification methods and STR typing. *Submitted to Forensic Science International: Genetics*

Abstract

Short tandem repeats (STR) are currently the gold standard in human identification for forensic casework purposes, and successful STR typing is dependent on sufficient quantity and quality DNA. In the aftermath of a mass disaster and some forensic cases, human remains are recovered for identification in various stages of decomposition, and ideally these remains are transported to a refrigerated facility in order to halt the decomposition process and preserve the integrity of DNA within the tissue. However, in situations where refrigeration is not available (e.g., after a mass disaster or in rural forensic casework), remains continue to be exposed to environmental insults after collection, causing further DNA damage and degradation. Therefore, successful STR typing is dependent on the time of collection and preservation of the DNA sample. This study aims to test two simple in-field collection and preservation methods for decomposing human tissues that are subsequently stored at room temperature for up to six months either in a tissue preservative solution (modified TENT buffer) or on an FTA® Elute Card. In addition, these collection and preservation methods were tested for their ability to facilitate more direct and faster processing of DNA from preserved tissues or DNA leached into the surrounding TENT preservative solution for STR typing. Pre-PCR methods tested in this study include a quick lysis of FTA® Elute Cards, silica-based purification (QIAquick®), enzyme-based extractions (PDQeX), and simple dilution of liquid preservative. The traditional DNA analysis pipeline, which includes DNA extraction and quantification, will be compared to an alternate direct PCR method, thereby allowing the elimination of these two time-consuming and costly steps.

The results indicate that modified TENT preservative and FTA® Elute Cards both preserved DNA from relatively fresh tissue for up to six months at room temperature. However, mostly partial profiles were produced from decomposed tissues (day 6 – day 14 in this study) when stored for up to six months compared to when tissues were processed immediately following collection. Overall, the modified TENT preservative produced higher DNA concentrations and more successful STR results than FTA® Elute Cards. In addition, a rapid DNA extraction platform (PDQeX) generated the most successful STR typing results from the decomposed tissues stored in TENT for up to six months at room temperature. The direct PCR method used in this study generated comparable STR results to the traditional DNA analysis approach, warranting further investigation of direct PCR methods for forensic casework type samples.

Keywords: DNA collection, DNA preservation, FTA Elute, PDQeX System, STR typing, Disaster victim identification

Introduction

Short tandem repeat (STR) typing is currently the gold standard for human identification. However, successful STR typing is often affected by the presence of inhibitors, low amounts of DNA template, and damaged and/or degraded DNA [1]. Refrigeration or freezing of samples is typically used to prevent further DNA damage and degradation [2, 3]. In the event of a mass fatality event or in rural casework, refrigeration is often not possible due to a lack of facilities and/or limited electricity [2, 3]. If human remains are left exposed to evironmental conditions such as heat, humidity, and microbial activity, then DNA in those tissues begins to degrade [3, 4]. The most common consequence of DNA degradation is the loss of larger alleles during PCR amplification resulting in partial STR profiles [4]. Therefore, various methods have been proposed for simple in-field collection and preservation of biological samples (for a review, see Allen-Hall and McNevin [3]). Several tissue preservatives have been tested for effective ambient temperature storage of biological samples [2-5].

In this study, we assessed the utility of a modified TENT buffer (Tris, EDTA, NaCl, Tween 20) which has been evaluated in previous studies (although with differing salt concentrations) [4-6]. This preservative is ideal for storing tissue samples due to its ability to simultaneously facilitate lysis of tissue and leaching of DNA into solution. The use of this preservative has been coupled with either quick purification and traditional DNA analysis or direct PCR using aliquots of the DNA suspended in preservative solutions. Allen-Hall and McNevin tested the TENT buffer in addition to several other wet and dry preservatives on fresh muscle tissue in hot, humid conditions [5]. They found that TENT preservative containing 100mM salt (NaCl) yielded high quantities of DNA from the

preservative solution but failed to protect that DNA from further degradation during storage [5]. A further study by Sorensen et al. tested TENT and other preservatives for their effectiveness and ability to generate successful STR typing via a direct PCR approach using an aliquot of DNA suspended in liquid preservative [6]. This study used the same TENT buffer (100mM NaCl), in order to facilitate direct PCR without the requirement for dilution to remove inhibitors prior to PCR. The authors reported that all buffers tested except TENT generally produced complete STR profiles after storage for one month or archived at -80°C for 4 years [6]. Therefore, at this low salt concentration, direct PCR was possible, but DNA preservation was not optimal. A later study modified the TENT preservative by increasing the salt concentration to 2M and evaluated this modified TENT buffer in addition to other in-house and commercial preservatives for use with fresh and decomposed skin and muscle tissues [4]. Sorensen et al. found the modified TENT buffer to be the most successful in-house preservative for tissues stored up to three months in heat and humidity [4]. However, Holmes et al. confirmed that the increased salt solutions were inhibitory to quantitative PCR (qPCR) [1]. Therefore, we investigated alternate approaches which would allow for both a higher concentration of salt in the TENT buffer and faster DNA purification methods prior to STR typing.

A new rapid DNA extraction platform, the PDQeX 2400 System (ZyGEM, Hamilton, New Zealand), with enzyme-based buffers was tested for its ability to purify DNA from decomposing tissues preserved in TENT and aliquots of DNA suspended in solution while removing inhibitory compounds. This novel DNA extraction method utilizes the enzymatic activity of Antartic *Bacillus* sp. EA1, which is used to lyse cells and degrade nucleases and proteins at an optimal temperature of 75°C [7]. The extraction

occurs in a closed system with several incubation/heat steps in less than twenty minutes without prior lysis or any transfer steps to minimize contamination or loss of sample.

In addition, we also explored the feasiblity of transferring DNA from decomposing tissues directly onto FTA® Elute Cards in an attempt to preserve DNA for storage and provide a suitable substrate for direct PCR. Whatman® FTA® Cards are commonly used in forensic DNA laboratories to collect and archive blood or saliva for reference samples [3, 8]. Once the samples are allowed to dry, the DNA is preserved, and Cards can be stored at room temperature for long periods of time or be prepared for amplification via direct PCR [3, 8].

The large-scale nature of mass disaster events and rural forensic casework necessitates the development of simple and fast in-field methods to collect and stabilize biological samples for identification in situations when optimal storage conditions are not available. In addition, rapid and more direct high-throughput methods can reduce the cost and time of analysis to help facilitate faster identification for victims' families and the criminal justice community. Therefore, we explored the effectiveness of various approaches for preseverving samples from decomposing human cadavers, coupled with more direct and faster DNA purification and amplification strategies.

Materials and Methods

DNA Collection

Samples were collected from three human cadavers placed in April 2015 at the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, Texas, USA. A 4mm biopsy punch was used to collect tissue samples from the lower leg (skin and gastrocnemius muscle) during various stages of decomposition over a

two-week period (Days 0, 4, 6, 10, and 14). Two biopsy punches (~70mg tissue) were placed in a sterile 2mL microcentrifuge tube and processed immediately as controls (refered to as biopsy controls from here), or two biopsy punches were stored in 800µL of modified TENT buffer (10mM Tris, 10mM EDTA, 1M NaCl, 2% Tween 20; 100mL, pH 8.0) or compressed on an Indicating FTA® Elute Micro Card (GE Healthcare Life Sciences, Pittsburgh, PA). The cards were pre-moistened with three drops of sterile diH_2O to prevent the tissue from sticking and tearing the paper. The tissue was rolled around the defined circle of the FTA® Elute Card for approximately 20 seconds and then discarded. TENT and FTA® Elute Card samples were returned to the lab and stored at room temperature for one, three, and six months.

DNA Lysis and Extraction

The reference tissues (biopsy controls) were lysed overnight at 56°C with 30µL Proteinase K and 270µL ATL buffer from the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany). DNA extraction was performed on the QIAcube® (QIAGEN) using the Casework samples protocol (purification only protocol) [9].

An aliquot (10µL) of TENT buffer containing DNA leached from the tissues was used to make a 1 in 10 dilution for direct PCR. An additional 60µL aliquot of the TENT preservative was purified using QIAquick® PCR Purification Kit (QIAGEN) on the QIAcube® and eluted in 60µL of Buffer EB (QIAGEN) [10]. An aliquot of TENT preservative was also purified using the PDQeX 2400 System (ZyGEM) and the PDQeX *forensic*GEM Tissue Kit (ZyGEM) using the Animal Tissue DNA Extraction Protocol with a minor modification [11]. Liquid preservative (20µL) instead of tissue was added to a ZyGEM 'premium tube' (containing a filter) with 10µL Histosolv, 10µL ORANGE Plus

Buffer, and 2µL *forensic*GEM, then filled to a final volume of 100µL with 58µL distilled H_2O. DNA was also extracted from the preserved tissue (~70mg) using the PDQeX *forensic*GEM Tissue Kit, but in order to fascilitate better lysis of the increased amount of tissue, Histosolv was increased to 15µL, *forensic*GEM was increased to 4µL, and distilled H_2O was reduced to 21µL for a total volume of 50µL. The reduction in volume of water was critical so that the volume of sample expelled from the ZyGEM premium tube would not overflow the collection tube. The tissue was cut into pieces with a sterile scalpel before placing into a ZyGEM premium tube making sure to submerge the tissue in the master mix. The default 'Tissue' program was run on the PDQeX System for both types of samples (aliquot of TENT and the tissue stored in TENT).

FTA® Elute Card samples were prepared for lysis and elution by taking two punches with a Harris Uni-Core Disposable 2.0mm punch (GE Healthcare Life Sciences) and placing them into a sterile microcentrifuge tube. An aliquot of 60µL Investigator® STR GO! Lysis Buffer (QIAGEN) was added to the tube, and lysis was performed at 95°C for 5min with shaking at 1200rpm.

DNA Quantification

Samples were quantified with the Quantifiler® Trio DNA Quantification Kit (Thermo Fisher Scientific, South San Francisco, CA, USA) [12] on a 7500 Real-Time PCR System (Thermo Fisher Scientific) using the manufacturer's recommended parameters [12]. Data were analyzed using the HID Real-Time PCR Analysis Software v1.2, where a R^2 value ≥0.99 on the standard curve was accepted. The internal PCR control (IPC) was monitored for PCR inhibition using the default IPC C_T threshold setting (>2C_T) [1, 12].

Tissue samples extracted with the PDQeX System required dilution due to severe inhibition detected by the IPC target in the Quantifiler® Trio DNA Quantification Kit. Therefore, a 1 in 10 dilution was performed on all tissue extracts prior to re-quantification and normalization of DNA for STR typing.

STR Amplification and Electrophoresis

Samples were amplified with Investigator® 24plex QS Kit (QIAGEN) using the manufacturer's recommended protocol [13]. The DNA concentration of the small target was used to target 0.8ng of DNA for STR amplficiation. When samples were quantified at less than 0.053ng/µL, the maximum volume (15µL) was added to the PCR reaction. The TENT preservative samples (DNA suspended in TENT solution) that were diluted 1 in10 were also amplified using a direct PCR protocol, where 2µL of sample was added to each Investigator® 24plex GO! (QIAGEN) reaction [14]. STR amplification was performed on a ProFlex™ PCR System (Thermo Fisher Scientific) following the manufacturer's recommended cycling parameters [13, 14]; however, a total of 27 cycles was used with the direct PCR TENT samples.

Separation and detection of PCR products was performed on a 3500 Genetic Analyzer (Thermo Fisher Scientific) using a 36cm capillary array and POP-4 polymer. Data were analyzed with GeneMapper® ID-X Software v1.4 (Thermo Fisher Scientific) using the following thresholds for data interpretation: an analyticial threshold of 100 relative flurorescent units (RFUs) and a stochastic threshold of 200 RFUs. The quality sensors in both STR kits were monitored for PCR inhibition.

Statistical Analysis

Data were tested for statistical significance using Statistica 13 (TIBCO S Software Inc., Palo Alto, CA). When a signficance was determined via one-way or factorial ANOVA, Tukey honest significant difference (HSD) post-hoc test was performed. A value of $p < 0.05$ was accepted as the level of significance.

Results and Discussion

Biopsy Controls

Tissue biopsies were processed immediately following each day of collection as baseline controls to determine the approximate quantity and quality of DNA from tissues recovered from the decomposing remains (Fig. 5.1.). As may be expected, DNA concentration decreased while the level of degradation (as measured by the DI; small target divided by large target DNA concentration in Quantifiler® Trio) increased as decomposition progressed over the two week period (Fig. 5.1. A). Nevertheless, complete STR profiles were obtained from all samples but with the average peak heights declining as decomposition progressed, reflecting the reduced DNA quality and quantity (Fig. 5.1. B). The heterozygous peak height ratios remained balanced (~85%) over two weeks of decomposition.

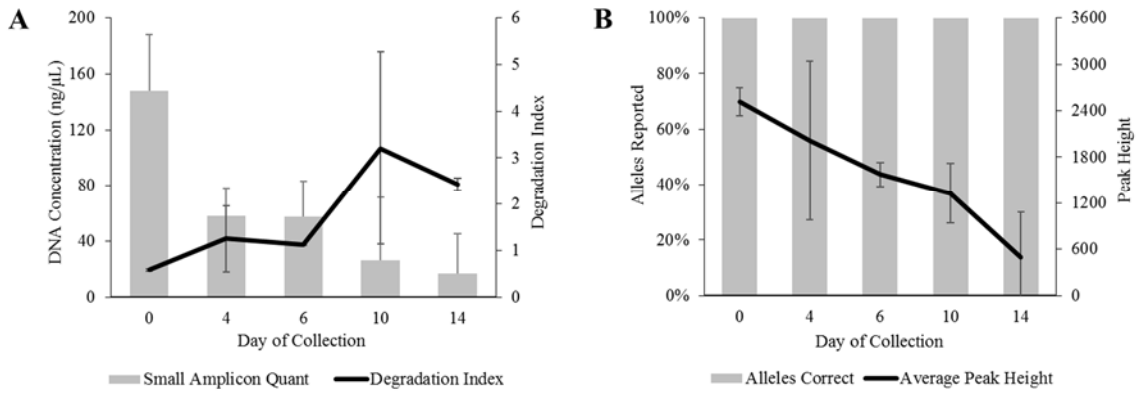

Fig. 5.2. Average DNA quantification results (A) and STR success (B) from the biopsy tissue controls. STR results are from the Investigator® 24plex QS Kit. N = 15. Data represent mean ± std dev

DNA Preservation and Rapid Purification Methods

Biopsies collected in-field were stored in the TENT preservative or compressed on FTA® Elute Cards to test the efficiency of each method for preserving the DNA at room temperature for up to six months. No statistically significant differences in DNA concentrations or alleles reported were observed for all TENT or FTA® Elute Card samples stored for one, three, and six months ($F_{4,432} = 0.378$, $p > 0.05$), indicating that samples did not show any notable DNA degradation after one month regardless of the method. In addition, various methods that facilitate rapid DNA purification and/or direct PCR methods from either the tissue itself or the DNA that has leached into the surrounding TENT buffer were also investigated. Average DNA quantification and STR results for preserved tissue methods and rapid pre-PCR methods for leached DNA preserved in TENT are found in Figures 2 and 3, respectively. Similar to the biopsy controls (Fig. 5.1. A), the DNA concentrations decreased as the degradation index increased for all methods tested (Fig. 5.2.).

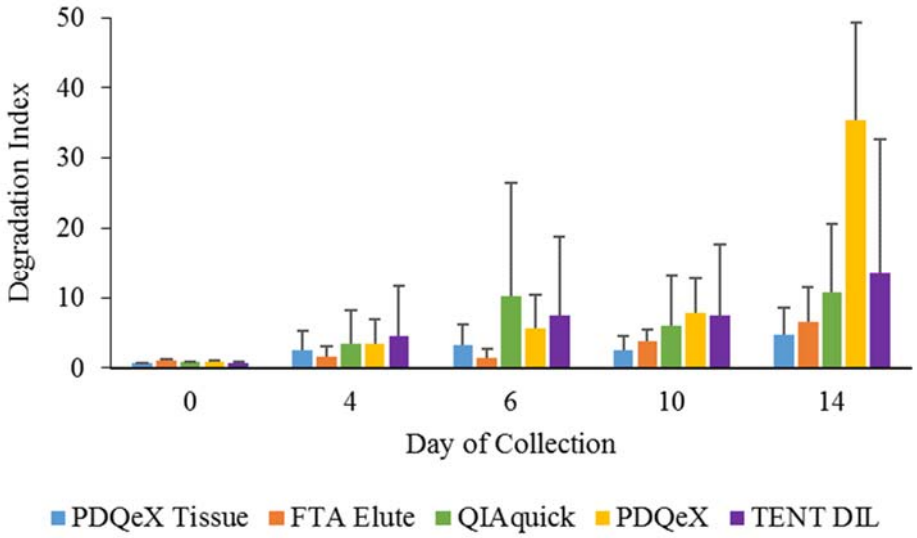

Fig. 5.2. Average DIs for biopsy tissues stored in TENT (extracted with PDQeX) or compressed onto FTA® Elute Cards (lysed in STR GO! Lysis Buffer) and aliquots of TENT (purified with either QIAquick® or PDQeX and diluted 1-in-10). N = 225. Data represent mean ± std dev for 1, 3, and 6 months of storage

Preserved tissue methods

Tissue biopsies collected in-field were stored in the TENT buffer or compressed onto FTA® Elute Cards in order to determine if DNA of sufficient quantity and quality could be recovered from decomposing bodies using these strategies. In general, higher DNA concentrations and more complete profiles were obtained from tissues collected in-field on days 0 and 4 (Fig 5.3. A and B). However, tissue biopsies (~70mg) rapidly purified with the PDQeX System (<20 minutes) provided sufficient DNA concentrations for up to 10 days and more complete profiles for all days of collection, even after a 1 in 10 dilution was performed due to severe inhibition (detected initially via the IPC target in Quantifiler® Trio Kit). It is important to note that all neat DNA extracts showed significant PCR inhibition during quantification, but extraction blanks did not indicate any PCR inhibition. A 1 in 2 dilution was sufficient to overcome PCR inhibition during quantification;

however, when the maximum volume of 15µL was added to the STR amplification reaction, severe inhibition was still detected (via loss of both QS peaks). Therefore, a 1 in 10 dilution was performed for all neat extracts, and samples were re-quantified prior to STR typing.

Due to high amounts of DNA, the majority of samples (60%) required further dilution to target 0.8ng for optimal STR amplification, while the remainder of the samples required the input of the maximum volume of the diluted lysate for PCR. Only 16% of all STR profiles showed evidence of PCR inhibition according to the quality sensors in the Investigator® 24plex QS Kit, but partial profiles were still generated from these samples (4-69% alleles recovered). The authors believe that the inhibition detected in tissue samples purified with the PDQeX System was most likely due to inhibitors co-extracted from the tissue itself, as the PDQeX *forensic*GEM Tissue Kit reagent blanks did not indicate any inhibition via the IPC target in Quantifiler® Trio when 2µL was amplified. When the reagent blanks were diluted 1 in 10 (same as the DNA samples) and the maximum volume was added to the PCR, the quality sensors showed no signs of PCR inhibition. However, when 15µL of neat reagent blanks were amplified, the quality sensors indicated severe inhibition (via loss of both QS peaks). Therefore, additional inhibitors co-extracted from the PDQeX reagents could also have contributed to PCR failure. To note, we increased the enzyme buffers in the reaction and reduced the volume of water to ensure better lysis of the large amount of tissue used in this study, and this may not be the case when the manufacturer's protocols are used.

FTA® Cards are traditionally used for reference and pristine DNA samples, but we explored their effectiveness at storing DNA from decomposing tissues. FTA® Elute Cards

were used in this study because they allow elution of DNA off the card and into a solution, allowing quantification. In general, it appears that insufficient amounts of DNA were transferred from the tissue biopsies to the FTA® Elute Cards for successful STR typing (Fig. 5.3. A and B). Due to severe PCR inhibition from the Investigator® STR GO! Lysis Buffer as detected by the quality sensors in Investigator® 24plex QS Kit (also in the no template control), it was determined that a maximum of 6µL of lysate could be added to each amplification without significant levels of PCR inhibition affecting STR success. We did not investigate further dilution of these samples to overcome the inhibition due to the relatively low amounts of DNA detected (<0.1ng/µL) in the majority (67%) of these samples. Therefore, the FTA® Elute Cards were not a successful strategy for collecting and storing decomposing tissues (performed in this study), as 53% of samples resulted in PCR inhibition and 31% resulted in no alleles called (Fig. 5.3. B), either due to inhibition or too little DNA recovered.

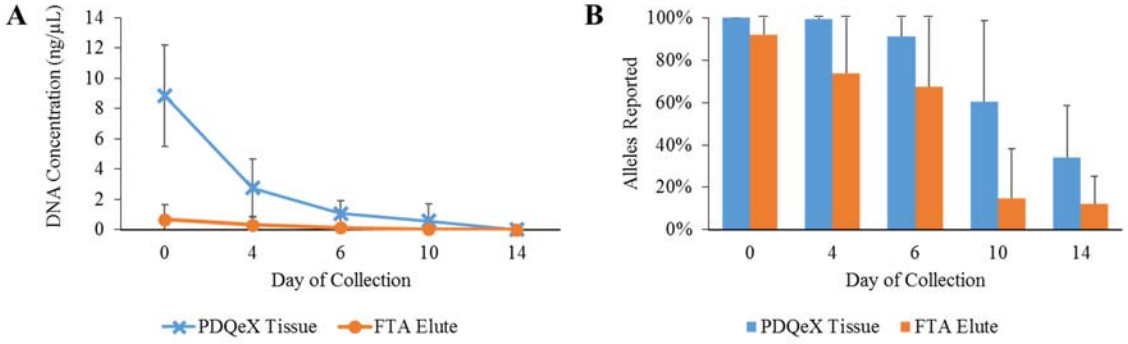

Fig. 5.3. Preserved tissue methods. A) Average DNA quantification results and B) STR success for tissues stored for up to six months in the TENT preservative (tissue extracted with PDQeX) or compressed onto FTA® Elute Cards (punches lysed in STR GO! Lysis Buffer). Quantitation values for the PDQeX Tissue samples reflect 1 in 10 dilutions. DNA was normalized and amplified with the Investigator® 24plex QS Kit. N = 88. Data represent mean ± std dev for 1, 3, and 6 months of storage

Preserved DNA in TENT and rapid pre-PCR methods

The concentration of DNA recovered from the TENT solution surrounding the tissue after rapid purification using the QIAquick® or PDQeX systems, or simply diluted are shown in Figure 5.4.A. An aliquot of the TENT buffer (containing DNA that has leached from the tissue during storage) purified with a QIAquick® column (approximately 30 minutes) produced significantly higher DNA concentrations from DNA preserved in TENT for up to six months ($F_{2,132} = 18.847$, $p < 0.01$) (Fig. 5.4. A). Likewise, the PDQeX System yielded sufficient DNA concentrations in less than 20 minutes (Fig 3A). The difference in the amount of DNA recovered may be explained by the fact that three times more TENT buffer was purified using the QIAquick® method than the PDQeX. Because of severe inhibition (due to the high concentration of salt in the TENT buffer), only 20µL of solution could be added to the PDQeX System, whereas a 60µL aliquot of the buffer was processed during the QIAquick® purification.

According to the Quantifiler® Trio Kit's default inhibition flag (>2 ΔCTs), none of the QIAquick® or PDQeX purified samples indicated PCR inhibition. Holmes et al. previously reported that the Quantifiler® Trio kit is extremely tolerant to high concentrations of salt but was completely inhibited by 1M salt in the neat TENT samples [1]. Therefore, in this study a simple 1 in 10 dilution was also performed on an aliquot of DNA suspended in TENT in order to dilute the inhibition to a level that we expected would facilitate successful quantification and direct PCR (~100mM). In contrast to the purified TENT samples (with QIAquick® and PDQeX), the diluted TENT samples still produced substantial levels of PCR inhibition as indicated by Quantifiler® Trio for samples collected

on days 6, 10, and 14. These samples were not diluted further because they most likely contained low amounts of highly degraded DNA.

Average STR results for first-pass success rates are reported in Figure 5.4.B. Fresh tissues collected on day 0 and stored in the TENT preservative generated complete profiles for up to six months at room temperature. However, mostly partial profiles were obtained from decomposed tissues (days 6-14) with both rapid purification methods (QIAquick® and PDQeX) and after a simple 1 in 10 dilution (Fig. 5.4. B), indicating that TENT can preserve DNA of high quality very well at room temperature but may not preserve degraded and/or damaged DNA from decomposed tissues.

When comparing the two purification methods, QIAquick® resulted in the highest DNA concentrations (Fig. 5.4. A), but STR results were generally comparable to the PDQeX purification of TENT samples for up to six months of storage. Our results are similar to those of Lounsbury et al. where they reported comparable STR results between the enzyme-based and silica-based extractions [7]. However, severe PCR inhibition (determined by the quality sensors in the Investigator® 24plex QS kit) was observed in the PDQeX samples when the maximum volume of 15µL was added to the STR reaction, resulting in no alleles being reported. These samples (38%) could be diluted and re-amplified to help overcome this inhibition; however, these samples contained <0.05ng/µL DNA, and any further dilution could potentially result in insufficient DNA for amplification.

In addition, a simple 1 in 10 dilution of TENT was investigated to dilute the NaCl concentration in the sample prior to direct PCR. Two different approaches were investigated: 1) DNA normalized to 0.8ng was amplified with the Investigator® 24plex QS

kit, and 2) 2µL of a 1 in 10 dilution (regardless of the quantification results) was added directly to each Investigator® 24plex GO! reaction. Average first-pass STR success results were similar to the purified TENT samples (QIAquick® and PDQeX) (Fig. 5.4. B). However, as these samples were diluted to aid in quick removal of PCR inhibitors, the lower amounts of degraded DNA in the decomposed tissues (days 6-14) produced mostly partial profiles. Regardless, it is interesting that comparable STR results were generated for all samples regardless of which STR kit was used, indicating that adding a simple dilution of a small aliquot of sample directly to PCR will likely produce similar results in less than half the analysis time of the traditional workflow (Fig. 5.4. B). In addition, 38% of samples amplified with Investigator® 24plex QS were inhibited, whereas only 4% of samples amplified with Investigator® 24plex GO! showed the effects of inhibition. These results indicate that the QIAGEN direct PCR kit is more tolerant to PCR inhibition than their standard casework STR-typing kit, which is common in most direct PCR kits in order to successfully amplify samples that have not undergone complete DNA extraction procedures [8].

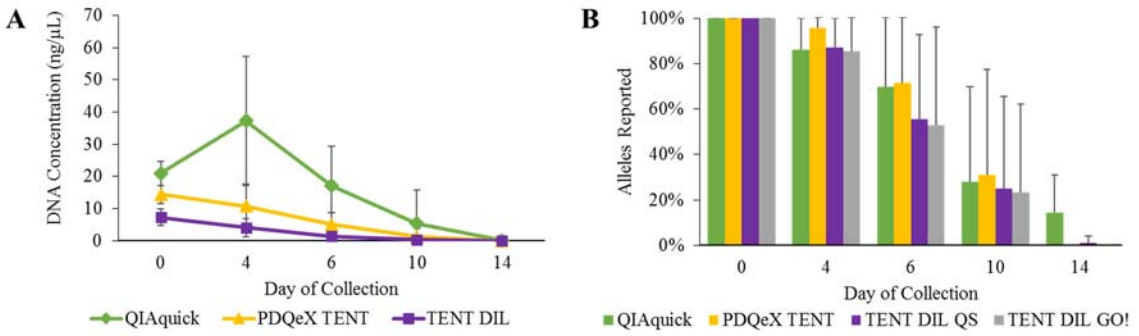

Fig. 5.4. Preserved DNA in TENT and rapid pre-PCR methods. A) Average DNA quantification results and B) STR success for aliquots of TENT preservative stored up to six months and purified with QIAquick® columns, the PDQeX System, or diluted 1 in 10. DNA was normalized and amplified with the Investigator® 24plex QS Kit, or 2µL of the 1 in 10 dilution was added directly to the Investigator® 24plex GO! Kit. N = 180. Data represent mean ± std dev for 1, 3, and 6 months of storage

Overall, none of the rapid purification methods tested in this study for tissues or DNA leached into TENT preservative yielded complete STR profiles from all decomposing tissues (Fig. 5.3. B and 5.4. B), whereas the biopsy controls processed immediately yielded full profiles (Fig. 5.1.B). It should be noted that despite the three bodies being of similar size (body mass), one of the three cadavers seemed to be slightly less decomposed than the other two on each day that tissues were collected. Differences in the rate of decomposition due to biological variation is common when conducting this type of research. As a result, this one body resulted in much higher DNA yields and more complete profiles for each day of collection than the other two cadavers. However, the day of collection and degree of decomposition had a significant effect on STR success for stored samples ($F_{4,198} = 92.346$, $p < 0.01$), where days 6-14 generated significantly lower STR profiling success with all methods tested in this study. This is likely due to a combination of DNA degradation, reduced efficiency of the various purification methods tested, and the amount/type of sample used. For example, a small aliquot of DNA in preservative solution, partially lysed tissue from TENT preservative, and two punches of DNA collected from tissues on FTA® Elute cards was used for the various purification methods compared to a large piece of untreated tissue processed immediately. However, it may be difficult or impossible to process tissues immediately following collection in the field. Therefore, we have demonstrated that tissues can be preserved between one and six months in TENT and on FTA® Elute Cards (Fig. 5.5).

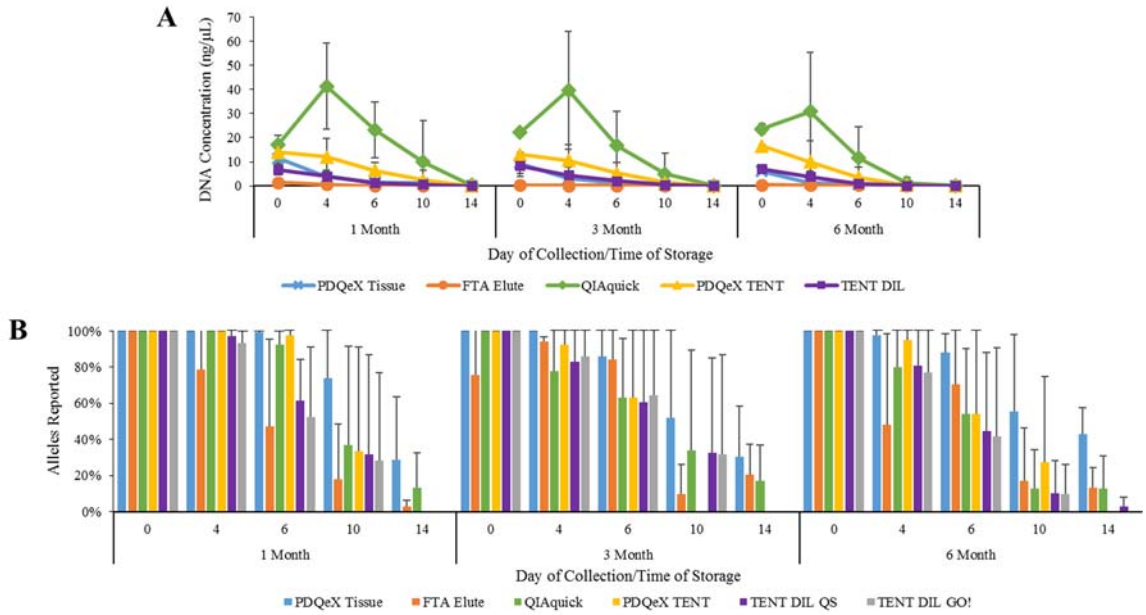

Fig. 5.5. All Stored Samples. A) Average DNA quantification results and B) STR success for tissues stored for up to six months in the TENT preservative and aliquots of TENT preservative purified with QIAquick® columns, the PDQeX System, or diluted 1 in 10

The tissues stored in TENT buffer recovered significantly more quantity and quality DNA than the FTA® Elute Cards for fresh and decomposing tissues ($F_{2,216} = 8.432$, $p < 0.05$). When deciding which TENT method may be the most successful for STR typing of decomposed tissues, the data indicate that the PDQeX purification of the tissue itself (in less than 20 minutes) generates the most complete STR profiles compared to all methods tested in this study ($F_{4,198} = 5.982$, $p < 0.01$) (Fig. 5.6.).

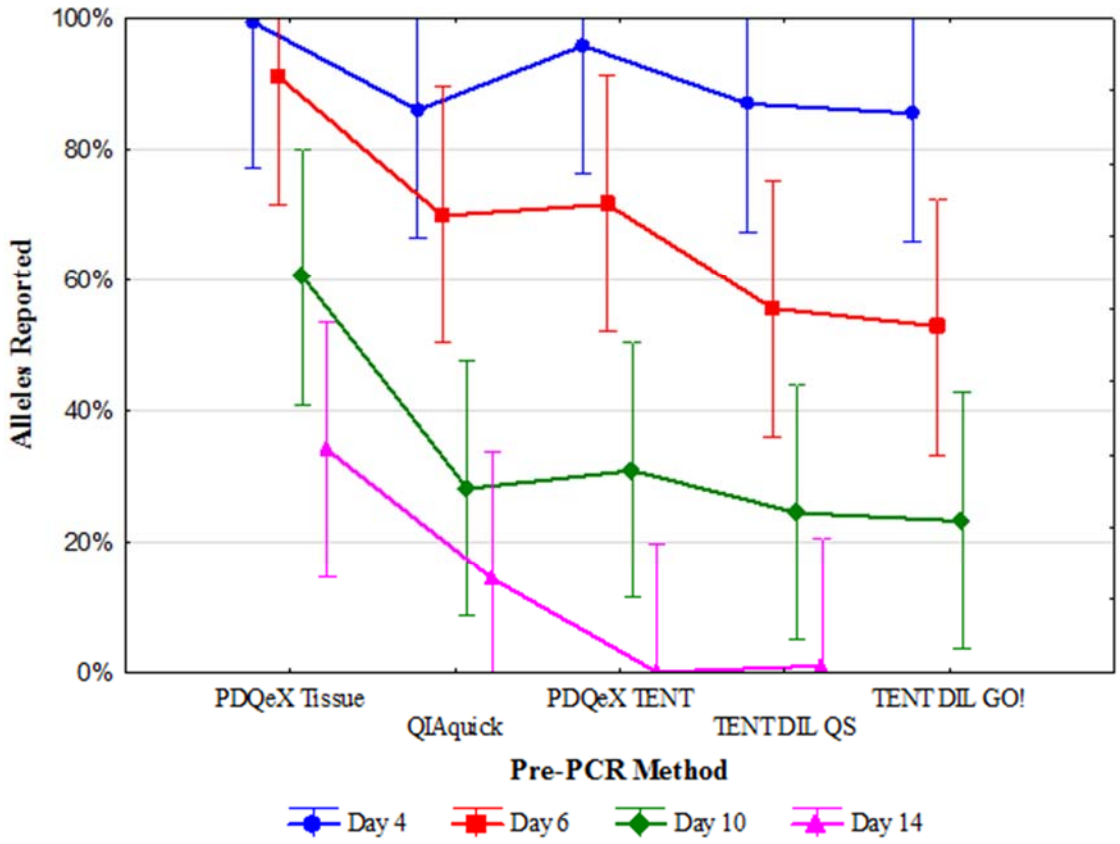

Fig. 5.6. Average alleles reported for biopsy tissues stored in TENT (extracted with PDQeX) and aliquots of TENT purified with QIAquick® or PDQeX and diluted 1 in 10. N = 225.Vertical bars denote 0.95 confidence intervals

Conclusion

This study investigated the effectiveness of a modified TENT tissue preservative and FTA® Elute Cards to collect and subsequently preserve DNA when stored at room temperature for up to six months. In addition, several rapid DNA purification and direct PCR strategies were also evaluated. We first tested the ability of a relatively new, rapid DNA extraction platform (PDQeX System) to purify DNA from tissues preserved in TENT, compared to a quick lysis of DNA recovered from tissues on FTA® Elute Cards.

The PDQeX System produced higher DNA concentrations and more complete STR profiles out of the two methods tested in this study for preserving the tissue itself

Next, we tested two rapid purification methods (QIAquick® and the PDQeX System) to assess their ability to purify the DNA suspended in the TENT buffer and remove PCR inhibitors in under 30 minutes prior to STR typing. Alternatively, we also investigated the effectiveness of a simple dilution of the TENT buffer to produce quality STR profiles using both a traditional STR workflow and a direct PCR approach. In general, QIAquick® purification produced the highest DNA concentrations; however, it resulted in similar STR profiling success compared to TENT purified with the PDQeX System or simply diluted (1 in 10). It is also interesting that the direct PCR approach for diluted TENT samples resulted in similar STR success with a reduction in PCR inhibition effects compared to TENT samples that were normalized prior to traditional STR typing.

Overall, the modified TENT and FTA® Elute Cards successfully preserved DNA from fresh tissues (before day 4) collected in-field and stored at room temperature for up to six months. However, decomposed tissues mostly generated partial profiles. The modified TENT buffer generated significantly higher DNA concentrations and more complete STR profiles for decomposed tissues than from FTA® Elute Cards. In addition, tissues preserved in the modified TENT buffer and rapidly purified with the PDQeX System (<20 minutes) yielded the most successful STR results from all decomposed tissues.

Due to the fact that several different methods were tested on the same sample (in TENT buffer), we could not alter the ratio of buffer volume to the amount (mg) of tissue used. However, this is one factor that could be optimized in future studies to increase DNA

yields for optimal STR typing, especially for decomposed tissues. In addition, even when DNA analysis may be unnecessary due to identification of human remains by other methods such as fingerprint or dental analysis, a biopsy of soft tissue could be easily collected in the field and stored at room temperature in the TENT solution until the sample reaches the laboratory for refrigeration or freezing for future analysis and/or archiving purposes.

Role of Funding

This study was funded by a Graduate Research Fellowship, Award #2015-R2-CX-0029 (National Institute of Justice, Office of Justice Programs, U.S. Department of Justice). The opinions, findings, conclusions, or recommendations expressed in this article are those of the authors and do not necessarily reflect those of the National Institute of Justice.

Acknowledgments

The authors would like to thank the staff at the Applied Anatomical Research Center (AARC) at Sam Houston State University for their assistance and the individuals and families of those who donated their bodies for scientific research. The authors would also like to acknowledge Kayla-Ashley Ehring for her assistance in the laboratory.

Compliance with Ethical Standards

Code 45 of US Federal Regulations part 46102(f) exempts the requirement for Institutional Review Board (IRB) approval regarding the use of human cadaveric samples. All procedures were in accordance with the 1964 Helsinki Declaration and its later amendments.

Competing Interests Statement

The authors have no competing interests to declare.

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CHAPTER VI

In-field Collection and Direct Analysis of Swabs from Decomposing Human Remains for DVI[1]

This dissertation follows the style and format of *International Journal of Legal Medicine*.

[1]Holmes AS, Roman MG, Gangitano D, Hughes-Stamm S. In-field collection and direct analysis of swabs from decomposing human remains for DVI.
A portion of these results will be published.

Abstract

Swabs are one of the most commonly encountered samples in forensic casework due to their ease of use for collecting biological material, low cost, and storage capabilities. This study aims to determine if adequate amounts of DNA for successful STR typing can be retrieved from swabbing decomposing human remains. Cotton, foam, and nylon flocked swabs were used to collect DNA from the skin or muscle of cadavers during various stages of decomposition. Traditional DNA extraction of swabs was compared to a direct PCR approach using the Investigator® 24plex QS and GO! Kits, respectively. Results indicate that nylon flocked swabs were the least effective method, collecting the least DNA and producing the most allele drop-out. Cotton and foam swabs provided successful STR results via direct PCR and traditional processing methods. Thus, we have demonstrated a more efficient method for in-field DNA collection and high-throughput STR typing for forensic casework purposes.

Keywords: Forensic science, DNA typing, DNA collection, Cotton swabs, 4N6FLOQSwabs, EasiCollect device, Direct amplification, Disaster victim identification (DVI)

Introduction

Mass fatality incidents are often complicated by the loss of electricity or lack of facilities to house large numbers of bodies for subsequent identification. Also, when crimes occur in remote or rural areas, it may take several hours or days for transport of remains and/or samples to a crime laboratory. If human remains are not refrigerated immediately, the DNA in those tissues is more susceptible to damage and degradation when exposed to various environmental insults. Therefore, a quick and easy in-field collection method for DNA from decomposing human remains and effective room temperature storage prior to analysis would be beneficial to the forensic community.

Swabs are one of the most common samples processed for routine forensic DNA analysis [1-4]. Various types of swabs are used in forensic DNA laboratories to collect DNA from evidentiary items or as reference samples [2, 5]. Collection of DNA on swabs could enable immediate stabilization of DNA obtained from tissues in the field (when dried) without the need for refrigeration. This is particularly true for the nylon 4N6FLOQSwabs™, which include an anti-microbial agent that produces ideal conditions for DNA preservation and protection during transport and storage [6]. In addition, 4N6FLOQSwabs™ differ from traditional cotton swabs in that the swab tips are coated with short nylon fibers organized perpendicular to the shaft, referred to as flocking [6-9]. This arrangement allows collected cells to remain on the outside of the swab, which could better facilitate complete elution and reduce the loss of DNA sample [3, 6-9].

Furthermore, swabs can also be processed via direct PCR for databasing and reference samples, thereby avoiding DNA extraction and quantification [9-11]. This type of analysis is quicker and cheaper for processing large numbers of samples when the

amount and nature of the biological material is relatively consistent (e.g., buccal swabs) [9]. However, several studies have also investigated direct PCR for touched evidentiary items that are low template [4, 11, 12]. DNA extraction might be avoided specifically for these types of samples due to the high loss of DNA through several wash steps, tube transfers, and/or irreversible binding to silica columns [4, 9, 12]. Therefore, direct PCR of swabs may have potential for use after mass fatality incidents, especially when human remains have been subjected to harsh environmental conditions and the processes of decomposition.

Many factors impact successful short tandem repeat (STR) typing such as the substrate of the swab, the quantity and quality of DNA collected, the time and conditions of storage and transport, and the analysis methods (extraction and amplification chemistries) [5]. Therefore, this study aims to investigate the effectiveness of various swab substrates to collect DNA from decomposing human tissues in sufficient quantity and quality for STR typing using traditional and direct PCR approaches. We also examined whether the swabs were capable of preserving the DNA when stored at room temperature for up to one month. In addition, two sampling approaches (types of tissues) were tested to determine which, if either, would be more suitable for decomposing human cadavers. Swabbing the skin of each cadaver is faster and less invasive. However, this approach has a high risk for contamination due to commingled remains or other sources of exogenous DNA. Alternatively, making a small incision in the arm or leg of a body and swabbing the underlying muscle might reduce the risk of contamination, but it is more invasive and requires more time and field equipment. Swabs were tested using traditional DNA

extraction methods in addition to direct PCR to determine if this direct approach is a viable option for these types of samples.

Materials and Methods

DNA Collection

Samples were collected from three human cadavers placed in close proximity in April 2015 at the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, Texas, USA. Control tissue samples were collected on the first day by excising ~10mg of skin and muscle. Cotton-tipped swabs (Puritan, Guilford, ME, USA), nylon 4N6FLOQSwabs™ for Crime Scene (Thermo Fisher Scientific, South San Francisco, CA, USA), and the foam heads from Whatman® EasiCollect™ Devices (GE Healthcare Life Sciences, Pittsburgh, PA, USA) were used to collect DNA samples from the surface of the skin or the underlying muscle (deltoid or biceps) via a small incision made in the skin. Separate cotton and nylon 4N6FLOQSwabs™ were used to collect DNA for time 0, 1 month storage, and direct PCR samples (N=3), whereas the foam head of each Easicollect™ Device was divided into quarters – one each for time 0, 1 month, and direct PCR analysis (N=1). Collection using all three swab types was performed when the cadavers were fresh (day 0-2) and decomposed (days 4-7). The swabs were moistened with 2 drops of sterile water from AddiPak® Unit Dose Vials (TeleFlex Medical, Research Triangle Park, NC, USA), and swabbing was performed in a circular motion for 10 seconds or until the tissue appeared mascerated. The swabs were allowed to dry in a sterile biosafety cabinent overnight. Swab samples were either extracted immediately after drying (no storage) or stored at room temperature for one month prior to extraction.

DNA Extraction

The control tissues (biopsy controls) were lysed overnight at 56°C with 30µL Proteinase K and 270µL ATL buffer from the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany). Tissue samples were extracted on the QIAcube (QIAGEN) using the "Casework Samples" protocol (purification protocol). The entire cotton or 4N6FLOQSwabs™ head was placed into a sterile tube for processing, while one quarter of the much larger foam head from the Whatman® EasiCollect™ Device was used. All swabs (including blanks) were extracted acccording to the section titled "Isolation of Total DNA from Surface and Buccal Swabs" following the cotton or Dacron swabs part of the protocol in the QIAamp® DNA Investigator Handbook [13]. All samples were eluted in 60µL of QIAamp® ATE buffer.

For the direct PCR approach, the entire cotton or 4N6FLOQSwabs™ head or one quarter of the foam head of the Whatman® EasiCollect™ Device was also prepared by lysis in 300µL of Investigator® STR GO! Lysis Buffer (QIAGEN) in sterile 1.5mL tubes. Swabs were incubated at 95°C for 5 mins with shaking at 1200rpm in a thermoshaker [14].

DNA Quantification

Due to the FBI requirement for quantification of casework samples [15], all swabs (including those lysed with STR GO! Lysis Buffer for direct PCR) were quantified with the Investigator® Quantiplex® Pro Kit (QIAGEN) on a 7500 Real-Time PCR System (Thermo Fisher Scientific) using the manufacturer's recommended parameters [16]. Data were analyzed using the HID Real-Time PCR Analysis Software v1.2, where a R2 value ≥0.99 on the standard curve was accepted. Additional analysis was performed using the QIAGEN Quantification Assay Data Handling and STR Setup Tool v2.01 (QIAGEN).

STR Amplification and Electrophoresis

Samples were amplified with the Investigator® 24plex QS Kit (QIAGEN) using the manufacturer's recommended protocol [17]. The DNA concentration of the small amplicon was used to target 0.8ng for STR amplification. When samples were quantified at less than 0.053ng/µL, the maximum volume of 15µL was added to the PCR reaction. STR amplification was performed on a ProFlex™ PCR System (Thermo Fisher Scientific) following the manufacturer's recommended cycling parameters [17].

Direct PCR swabs were amplified with the Investigator® 24plex GO! Kit (QIAGEN) using the manufacturer's recommended cycling parmeters with slight modifications [14]. Samples with a quantity of less than 5ng/µL determined by Investigator® Quantiplex® Pro Kit were amplified using 5µL of lysate for 29 cycles, and samples with 5ng/µL and greater were amplified using 2µL for 27 cycles. Due to severe PCR inhibition as indicated by the IPC target of the Investigator® Quantiplex® Pro Kit and the quality sensors in the Investigator® 24plex GO! Kit, 4N6FLOQSwabs™ were diluted 1 in 2 and re-amplified using 5µL of the dilution for 29 cycles.

Separation and detection of PCR products were performed on a 3500 Genetic Analyzer (Thermo Fisher Scientific) using a 36cm capillary array and POP4 polymer. Data were analyzed with GeneMapper® ID-X Software v1.4 (Thermo Fisher Scientific) using the following thresholds for data interpretation: an analyticial threshold of 100 relative flurorescent units (RFUs) and a stochastic threshold of 200 RFUs.

Statistical Analysis

Data were tested for statistical significance by one-way and factorial ANOVA with Tukey honest signficant difference (HSD) post-hoc test in Statistica 13 (TIBCO Software Inc., Palo Alto, CA). A value of $p < 0.05$ was accepted as the level of significance.

Results and Discussion

Biopsy Controls

Skin and muscle biopsy samples (~10mg) were collected and processed immediately as baseline controls (Fig. 6.1.). For DNA concentration, we performed an ANOVA with two independent variables (stage of decomposition and tissue type). There was a significant effect of stage of decomposition ($F_{1,14} = 21.835$, $p < 0.01$), tissue type ($F_{1,14} = 14.293$, $p < 0.01$) and an interaction between these two factors ($F_{1,14} = 6.564$, $p = < 0.05$). Complete STR profiles were generated from all fresh tissues (skin and muscle) and decomposed skin samples, but the decomposed muscle samples produced an average of 92% of expected alleles. In general, the skin samples provided both the highest average DNA concentrations and the most complete profiles from both fresh and decomposing human tissues (Fig. 6.1.).

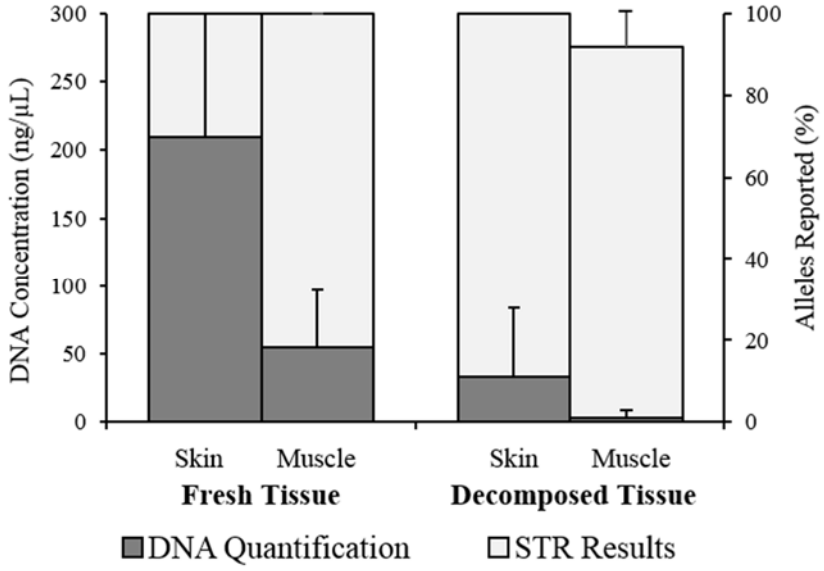

Fig. 6.1. Average DNA quantification results and average STR results for skin and muscle from three human cadavers. N = 18. Data represent the mean ± Std Dev

DNA Quantification Results of Swabs

Cotton, foam (Whatman® EasiCollect™ Device), and nylon flocked swabs (4N6FLOQSwabs™) were used to collect DNA from fresh and decomposing skin and muscle. In addition, two analysis methods were tested to investigate whether a direct PCR approach would yield comparable results to traditional DNA extraction and analysis. Average DNA quantification results for cotton, foam, and nylon swabs processed with traditional DNA analysis immediately after drying overnight (Time 0) or stored at room temperature for one month are shown in Figure 6.2. Swabs used on fresh tissues resulted in sufficient quantities of DNA, which were approximately ten-fold higher than swabs used on decomposed tissues (Fig. 6.2.A and B). A one-way ANOVA analysis showed that the stage of decomposition had a signficant effect on DNA concentrations obtained from all swab samples ($F_{1,106} = 28.754$, $p = < 0.01$). When deciding which swab type is better suited

for decomposed tissues, it appears that cotton and foam swabs provide sufficient quantities of DNA for STR typing (Fig. 6.2.B). A one-way ANOVA analysis showed that swab type indeed had a borderline effect on DNA concentration from decomposed tissues ($F_{2,69} = 3.135$, $p = 0.0498$), and a Tukey HSD post-hoc test showed that nylon swabs significantly differed from cotton swabs ($p < 0.05$). It is also important to note that 72% of the swabs used to collect DNA from decomposed tissues and processed with traditional DNA extraction methods were assessed as being highly degraded (degradation index, or DI, >10 in the Investigator® Quantiplex® Pro Kit).

In addition, the three swab types were tested for their ability to store DNA at room temperature for up to one month because samples collected following mass disasters or rural forensic casework may take weeks to transport back to a laboratory to be refrigerated. When cotton, foam, and nylon swabs were used to collect DNA from fresh and decomposed tissues, then stored for one month at room temperature (Fig. 6.2.A and B), DNA concentrations were significantly affected ($F_{1,106} = 6.430$, $p = 0.0127$). Although stored samples generated lower DNA concentrations than those without storage, sufficient DNA quantities for successful STR typing were produced from cotton and foam swabs after one month of storage.

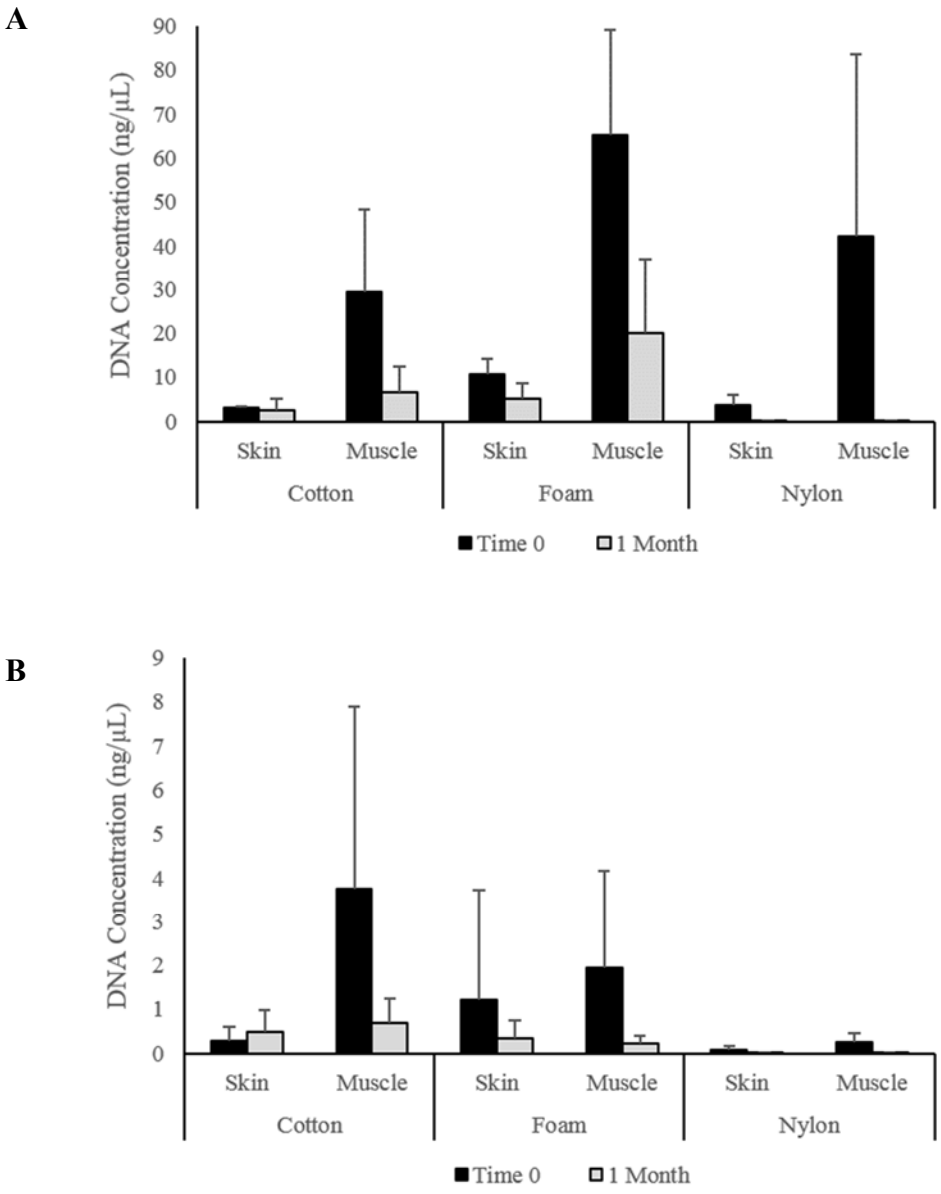

Fig. 6.2. DNA quantification results for traditional processing of swabs. Results for DNA collected from A) fresh, and B) decomposed tissues on cotton, foam, and nylon swabs without storage (Time 0) and after storage for one month. N = 108. Data represent the mean ± Std Dev. *Note: The Y axis on Graph A is 10x the scale of graph B

Swabs processed using the direct PCR approach (lysis in STR GO! Lysis Buffer) were also quantified. Average DNA quantification results for cotton, foam, and nylon swabs used to collect DNA from fresh and decomposing tissues are displayed in Figure

6.3. As expected, fresh tissues provided higher DNA concentrations than decomposed tissues for all swab types. DNA concentrations from fresh tissues were substantially lower when swabs were lysed in STR GO! Lysis Buffer in preparation for direct PCR than when the swabs were processed using a full extraction procedure (Fig. 6.2. and Fig. 6.3.). This difference in DNA concentrations could be explained by the fact that the DNA was diluted in 300µL of STR GO! Lysis Buffer for direct PCR, compared to a 60µL elution off a column after purification. Almost all swabs (from fresh and decomposed tissues) produced high DIs when analyzed with the Investigator® Quantiplex® Pro Kit. This observation could also be due to low DNA concentrations artificially increasing the DI. However, the IPC targets also indicated severe PCR inhibition. Therefore, as has been previously reported with commercial qPCR kits [18], a likely reduction in the amplification of the large target due to inhibition could have artificially increased the DI values. This phenomenon was mainly observed in the nylon flocked swab samples, in which every large target DNA concentration was undetermined or zero. Furthermore, when considering which swab may collect DNA from decomposed bodies more efficiently and be processed with a direct PCR approach, no significant statistical difference was seen between cotton and foam, but the nylon flocked samples showed significantly lower DNA concentrations ($F_{2,33} = 3.647$, $p < 0.05$).

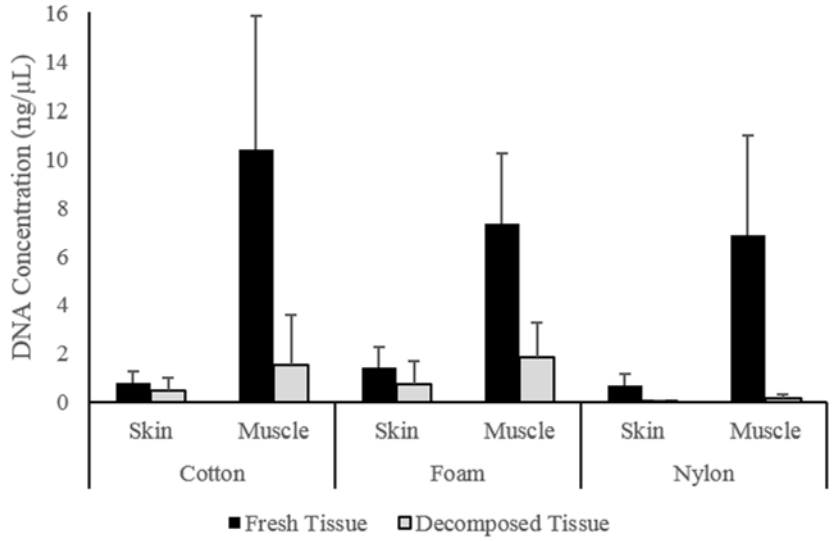

Fig. 6.3. DNA quantification results for direct PCR approach for swabs. Average DNA quantification results for cotton, foam and nylon swabs collected from skin and muscle of decomposing human cadavers. Swabs were lysed in Investigator® STR GO! Lysis Buffer prior to amplification. N = 54. Data represent the mean ± Std Dev

Overall, factorial ANOVA analysis showed that the interaction between analysis method, stage of decomposition, and swab type had a significant effect on DNA concentrations recovered ($F_{2,84} = 4.321$, $p < 0.05$). A Tukey HSD post-hoc test showed that the DNA concentration from fresh muscle for cotton, foam, and nylon swabs processed with traditional analysis was significantly different from other tissue types (decomposed muscle and fresh or decomposed skin). This was to be expected because fresh tissue would have the most pristine DNA, and blood (in muscle) commonly produces high DNA concentrations. In addition, DNA extraction purifies the DNA, removing cellular debris and any inhibitors while concentrating in 60µL. Although DNA can be lost with full DNA extraction methods, this would more likely affect samples with already low amounts of DNA, such as decomposed tissues. Furthermore, the purpose of this investigation was to

test if a particular swab and tissue type would provide higher DNA quantities for successful STR typing from decomposed tissues, and if direct PCR was a viable approach for these types of samples. A two-way ANOVA showed that swab type ($F_{2,60} = 4.936$, $p < 0.05$) and tissue type ($F_{1,60} = 7.402$, $p < 0.01$) both had significant effects on DNA concentrations obtained from decomposed tissues regardless of the analysis method. In general, a post-hoc test showed that DNA concentrations from muscle were significantly higher than concentrations from skin and that nylon swabs were less effective than cotton and foam swabs ($p < 0.05$). The type of analysis method (traditional versus direct) had little impact on the DNA yield from decomposed tissues ($F_{1,60} = 1.338$, $p > 0.05$).

STR Results of Swabs

Complete or near complete (≥99% alleles) STR profiles were obtained from all fresh tissues with all swab types after traditional DNA analysis. However, DNA samples collected with nylon swabs from fresh muscle tissue and stored for one month showed an average of only 73% alleles. Average STR results for swabs used to collect DNA from decomposed tissues, processed with traditional extraction and amplified with the Investigator® 24plex QS Kit are shown in Figure 6.4. As expected, more allelic drop-out was observed in samples from decomposed tissues (Fig. 6.4.). Cotton and foam swabs appeared to generate more complete profiles than nylon swabs from skin samples, while nylon swabs generated comparable profiles from muscle tissue when processed immediately (time 0) (Fig. 6.4.). Although the tissue type was found to affect the amount of DNA recovered, tissue type did not have a significant effect on downstream STR success as measured by the percentage of alleles reported ($F_{1,60} = 0.900$, $p > 0.05$). This

demonstrates that both skin and muscle swabs provided sufficient quantities of DNA for STR typing.

When considering the effect of storing swabs at room temperature for one month, the STR profiling quality did not decrease for any samples except the nylon swabs (with 29% - 65% fewer alelles on average) (Fig. 6.4.). Indeed, a factorial ANOVA analysis showed that swab type ($F_{2,60} = 17.107$, $p < 0.01$), storage time ($F_{1,60} = 7.804$, $p < 0.01$), and the interaction between these two factors ($F_{2,60} = 13.291$, $p < 0.01$) had significant effects on STR results from decomposing tissues, and a Tukey HSD post-hoc test showed that the mean percentage of alleles for the nylon swabs stored for one month significantly differed from the means for cotton and foam, with and without storage ($p < 0.01$). These data suggest that regardless of whether skin or muscle was sampled, DNA may not be preserved as well after one month of storage at room temperature when collected using the nylon 4N6FLOQSwabs^{TM} (Fig. 4.5.).

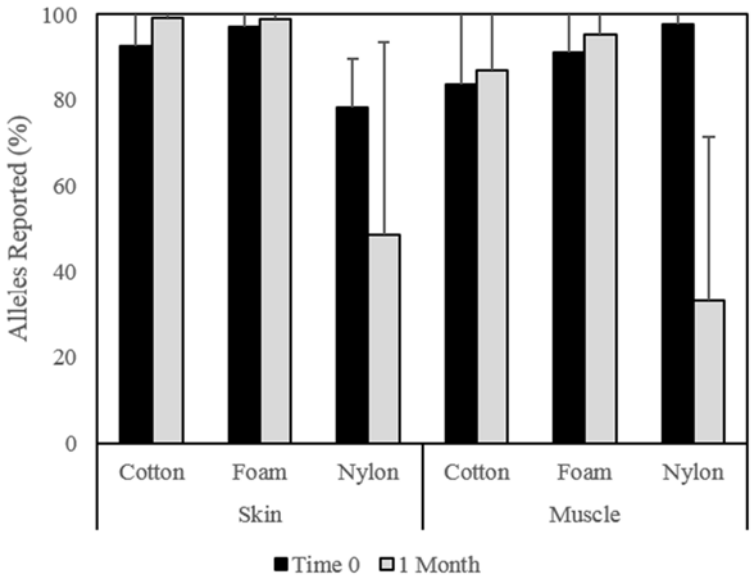

Fig. 6.4. STR success for traditional processing of swabs. Average STR results for DNA collected from skin and muscle of decomposed bodies on cotton, foam, and nylon swabs processed immediately. N = 54. Data represent the mean ± Std Dev

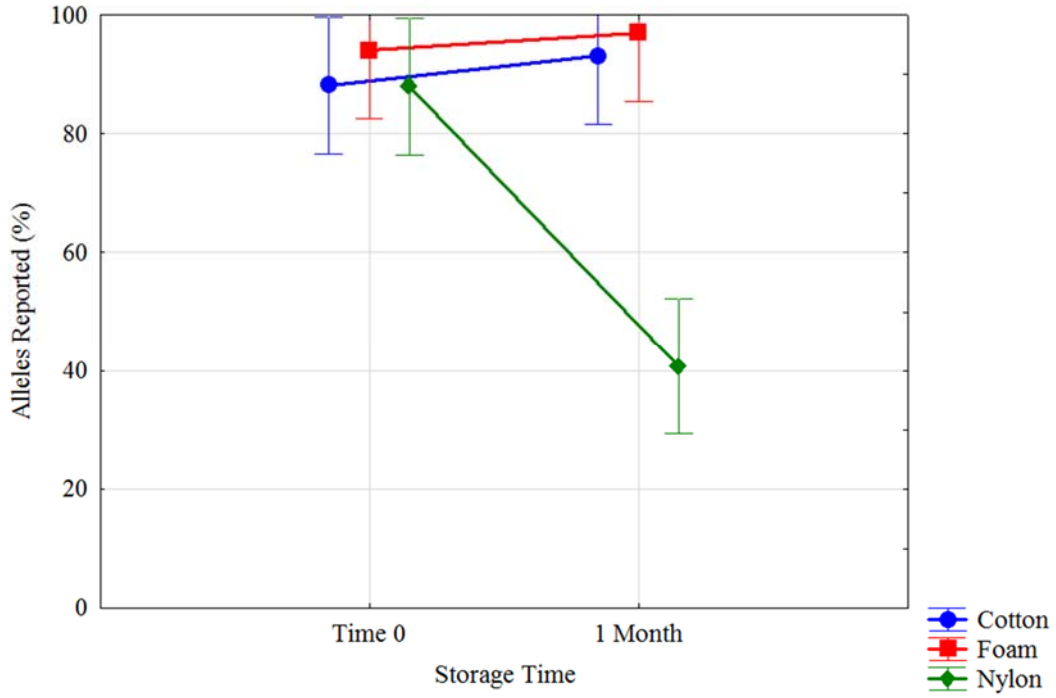

Fig. 6.5. Average STR success for cotton, foam, and nylon swabs used to collect DNA from decomposed tissues (skin and muscle) and processed immediately or stored at room temperature for one month prior to traditional DNA analysis. N = 54. Data represents mean and vertical bars denote 0.95 confidence intervals

The STR results from direct analysis of swabs with Investigator® 24plex GO! are displayed in Figure 6.6. Similar to the traditional processing of swabs, complete STR profiles were generated from cotton and foam swabs for all fresh tissues and from nylon swabs used on fresh skin, while nylon swabs used to collect DNA from fresh muscle resulted in an average of 91% alleles reported (Fig. 6.6.). Decomposing tissues generated mostly partial profiles with all tissues and swab types. Due to severe degradation detected during quantification, the swab samples with less than 5ng/µL of DNA (85% of samples) were amplified using 5µL instead of the usual 2µL sample input (following manufacturer's protocols), and the number of cycles was increased from 27 to 29. This resulted in more alleles being reported and increased RFUs for degraded and low template samples.

However, most nylon swabs resulted in PCR failure due to PCR inhibition (according to the IPC target in Investigator® Quantiplex Pro and the loss of both quality sensors in the STR kit). This observation was also previously reported by Dadhania et al. and Habib et al., where nylon flocked swabs in DNA IQ™ Lysis Buffer and STR GO! Lysis Buffer, respectively, were shown to inhibit PCR [6, 11]. In our study, only one nylon sample generated a full STR profile, but significant levels of inhibition were still indicated based on the severe imbalance between the 'S' and 'Q' quality sensors (Fig. 6.7.). Three other nylon samples resulted in partial profiles (2-33% alleles reported), even when inhibition was indicated. Therefore, all nylon samples were diluted (1 in 2) and re-amplified (5µL and 29 cycles) in order to overcome inhibition without overdiluting the small amounts of DNA present. The amended protocol for nylon swabs slightly improved the STR results, producing five full profiles from fresh tissue and seven partial profiles (Fig. 4.6. – Nylon). However, a factorial ANOVA showed that only swab type had a significant effect on the STR results from decomposed tissues using this direct PCR approach ($F_{2,30} = 14.340$, $p < 0.01$). In addition, samples collected with nylon swabs showed significantly fewer reportable alleles than the samples collected with cotton and foam swabs ($p < 0.001$). Therefore, in this study nylon flocked swabs were the least effective swab type for collecting DNA from decomposing tissues for direct PCR analysis due to too little DNA recovered and severe PCR inhibition. In contrast, Templeton et al. found nylon flocked swabs to be the most successful swab type for direct PCR compared to cotton and foam [4]. These conflicting conclusions are likely due to the difference in sample type (fingerprints on glass slides versus decomposing tissue) and the size of the swab ($2mm^2$

portion) in their study compared to the entire swab (cotton and nylon) and a quarter of the foam swab used in this study.

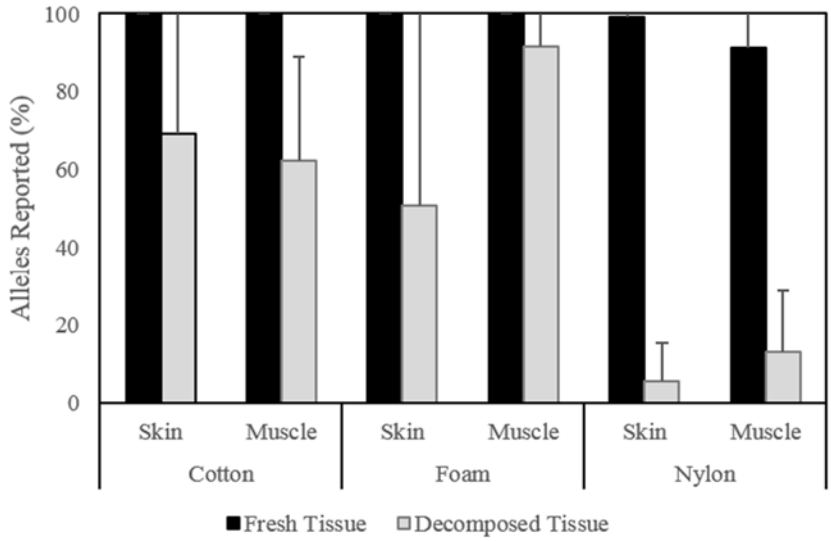

Fig. 6.6. STR success for direct PCR approach for swabs. A) Average DNA quantification results and B) average percentage of alleles reported for cotton, foam and nylon swabs collected from skin and muscle of decomposing human cadavers using a direct PCR protocol. Swabs were lysed in Investigator® STR GO! Lysis Buffer prior to amplification. N = 54. Data represent the mean ± Std Dev

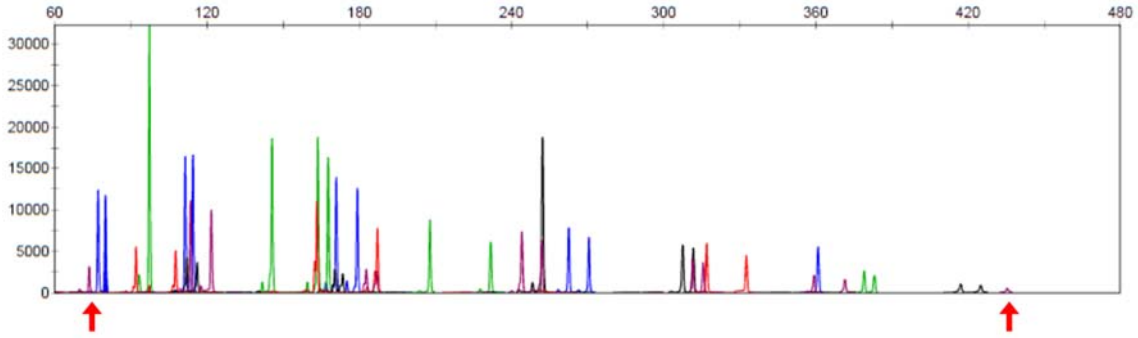

Fig. 6.7. Electropherogram for the single sample that generated a full STR profile and indicated severe PCR inhibition. Red arrows indicate the Q and S quality sensors (Q – 3312 RFUs, S – 633 RFUs)

When comparing the analysis methods for swabs used to collect DNA from decomposing human remains, factorial ANOVA analysis showed that analysis method

($F_{1,60} = 53.519$, $p < 0.01$), swab type ($F_{2,60} = 13.833$, $p < 0.01$), and the interaction between these variables ($F_{2,60} = 10.911$, $p < 0.01$) indeed had a significant effect on STR success, but when a Tukey HSD post-hoc test was performed, nylon direct PCR samples significantly differed from cotton and foam regardless of analysis method and tissue type used (Fig. 6.8.). Therefore, results indicate that direct PCR of cotton and foam swabs used to collect DNA from decomposing remains has potential for forensic applications. The direct PCR protocols developed for this study allow extraction to be eliminated; however, the FBI requires all non-reference samples to be quantified. Therefore, we favored the approach of lysing swabs with STR GO! Lysis Buffer prior to amplification, as it was a faster method and the resultant lysis buffer could also be quantified. The quantification results could also be used to triage degraded and lower template samples with one protocol and samples with high amounts of DNA with another protocol for the best first-pass STR success.

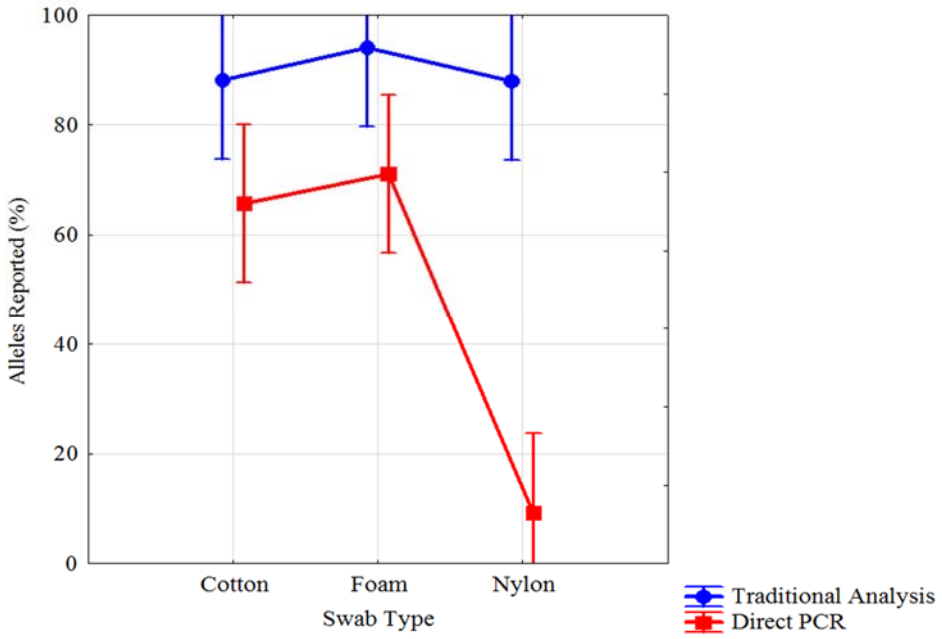

Fig. 6.8. STR success for cotton, foam, and nylon swabs used to collect DNA from decomposed tissues and processed with traditional DNA analysis or a direct PCR approach. N = 81. Data represent the mean, and vertical bars denote 0.95 confidence intervals

Although we were initially concerned that swabbing the skin of cadavers in close proximity could lead to contamination, no cross-contamination was observed in any of our DNA samples. However, this could still be an issue when dealing with commingled remains and less controlled environments. For this reason, the authors believe that cotton and foam swabs show the most potential when used to swab the underlying muscle tissues of remains for collection of DNA.

Conclusion

Cotton, the foam head of the Whatman® EasiCollect™ Device, and nylon 4N6FLOQSwabs™ were used to test if sufficient amounts of DNA could be collected from the skin surface or the underlying muscle (via a small incision) from decomposing remains for human identification. All swabs collected enough high quality DNA from relatively fresh bodies for successful STR typing. However, when human remains were more

decomposed, higher DNA concentrations and more complete STR profiles were generated from the cotton and foam swabs. In addition, when the swabs were stored at room temperature for one month prior to extraction, DNA seemed to be well preserved and generate comparable STR profiles for the cotton and foam swabs but not the nylon 4N6FLOQSwabs™. Overall, cotton swabs and the foam heads from Whatman® EasiCollect™ Devices seemed to generate the most consistent results regardless of the tissue type sampled.

Furthermore, our results indicate that direct PCR of swabs taken from the surface of the skin or an incision in the muscle has great potential for use following mass fatality situations or in rural forensic casework. In general, the cotton and foam swabs prepared for direct PCR analysis generated sufficient quantities of DNA for STR typing. Nylon swab samples required an additional dilution step due to severe inhibition; therefore, this type of swab is the least desirable, especially for decomposed tissues that are degraded and contain low amounts of DNA. In addition, STR success was comparable when cotton and foam swabs were processed using either amplification approach (traditional DNA extraction method versus direct). Future investigations to optimize the DNA quantity and quality might include concentrating the DNA sample on the tip of the cotton swab and using a smaller portion of foam swab to perform lysis in a reduced volume of lysis buffer.

In conclusion, the authors suggest that making a small incision in the muscle to collect DNA with cotton or foam swabs from human remains has the greatest potential to obtain the best quantity and quality of DNA while minimizing the risk of contamination for successful downstream STR typing. In addition, direct PCR holds some potential for faster processing but warrants further investigation prior to use in forensic casework.

Role of Funding

This study was funded by Graduate Research Fellowship Award #2015-R2-CX-0029 (National Institute of Justice, Office of Justice Programs, U.S. Department of Justice). The opinions, findings, conclusions, or recommendations expressed in this article are those of the authors and do not necessarily reflect those of the National Institute of Justice.

Acknowledgments

The authors would like to thank QIAGEN for donating some of the Investigator Quantiplex® Pro kits used in this study. The authors would also like to thank the staff at the Applied Anatomical Research Center (AARC) at Sam Houston State University for their assistance, and the individuals and families of those who donated their bodies for scientific research.

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CHAPTER VII

Conclusions

Natural and man-made disasters are unexpected events that often leave widespread destruction and numerous fatalities. It is the responsibility of forensic personnel to recover and identify victims in a timely manner to provide closure to the victims' families and communities. However, human remains may take days or weeks to be recovered, transported back to a laboratory, and placed in cool/freezing temperatures (if available). Furthermore, tissue samples for DNA identification may not even be collected until after the other primary identification methods (dental and fingerprints) are found to be unsuccessful in securing an identification. Consequently, the DNA in those decomposing tissues is exposed to harmful environmental conditions, fragmenting the DNA into smaller and smaller pieces. Therefore, immediate in-field collection and subsequent preservation methods to halt the harmful processes of DNA degradation as soon as possible, coupled with more direct and faster DNA-based identification methods is imperative.

If human tissues can be sampled and stored in a chemical preservative solution, especially in situations where refrigeration is not possible, the rate of DNA damage and degradation can be greatly reduced. We investigated the efficacy of several tissue preservatives to preserve DNA from fresh and decomposing tissues at ambient temperatures for long-term storage. We chose three in-house (modified TENT, DESS, and LST) and two proprietary preservatives (RNA*later* and DNAgard®) to assess the quantity and quality of DNA preserved in soft tissue or released into solution after storage in 35°C and high humidity (to mimic harsh tropical climates) over a three month period. Results indicated that DNA from human tissues could leach into the surrounding preservative and

be processed directly, thereby eliminating the need for a long tissue digestion process, minimizing the handling of tissue, and therefore providing faster DNA-based identification. We have demonstrated that all five preservative solutions (modified TENT, DESS, LST, DNAgard®, and RNA*later*) preserved adequate amounts of DNA in fresh and decomposed skin and muscle for successful STR typing after storage for up to three months. In addition, the LST, modified TENT, DESS, and DNAgard® have the added advantages that DNA is released into the preservative solution during storage for direct purification prior to STR typing. However, of the five preservatives tested, the modified TENT buffer and DNAgard® most consistently yielded DNA of high quantity and quality from both the tissues and the liquid preservatives stored for up to three months at 35°C with high humidity.

When preservatives were investigated for their effectiveness and ability to perform successful direct amplification from an aliquot of DNA suspended in preservative solution, the original TENT buffer (100mM NaCl), DESS, DNAgard®, and DNA Genotek generated complete or partial profiles after a simple dilution (to remove PCR inhibition), thereby skipping DNA extraction all together. The TENT buffer yielded the highest DNA concentrations in fresh tissue, but generated mostly partial profiles, whereas DESS, DNA Genotek, and DNAgard® generated mostly full profiles from fresh tissue archived at -80°C for four years. However, DNAgard® and DESS yielded the highest DNA concentrations in decomposing tissues stored at ambient temperature for one month.

For ease of use, availability, cost-effectiveness, and maximum performance, we suggested that out of the five preservatives tested in our study the modified TENT buffer may be the best candidate for application in DNA-based DVI operations. The modified

TENT buffer (2M NaCl) preserved decomposing tissues better than the original TENT buffer (100mM), but was unable to facilitate direct PCR due to the excessive salt. Therefore, we reduced the amount of salt in the buffer (1 M) to balance the need to preserve DNA in solution and also facilitate direct PCR approaches. In order to assess which DNA quantification kit was best suited to our needs and unique sample type, we compared four forensic DNA quantification kits for their ability to detect PCR inhibition and DNA degradation in a sample. Overall, Investigator® Quantiplex® Pro was the most tolerant to inhibitors (except salt) and in general provided a more accurate indication of DNA degradation in the sample, whereas Quantifiler® Trio was the most tolerant to high concentrations of salt but still indicated severe PCR inhibition with 1M salt based on the IPC targets.

The effectiveness of this new method of preserving tissue using the modified TENT buffer was compared to using FTA® Elute cards to preserve DNA from decomposing tissues. A tissue biopsy punch was used to collect skin and muscle tissues from decomposing bodies in the field for storage in the TENT buffer or compressed onto an FTA® Elute Card, and stored at room temperature for up to six months. Neither preservation method was capable of consistently producing complete STR profiles from all decomposing tissues, but both methods did successfully preserve DNA from all fresh tissues (up to 4 days of decomposition) for up to six months of storage. Overall, the pre-PCR methods for the TENT samples produced higher DNA concentrations and more complete STR profiles than FTA® Elute Cards.

We tested two methods to rapidly purify DNA from aliquots of TENT buffer; a silica-based purification (QIAquick®) in under 30 minutes and an enzyme-based extraction

(PDQeX System) in under 20 minutes. The QIAquick® method resulted in the highest DNA yields from aliquots of TENT but generated comparable STR results to the PDQeX System. A simple dilution (1 in 10) of the TENT buffer was also performed prior to normalization of DNA into the standard casework STR kit compared to a direct PCR approach of adding an aliquot of the same TENT dilution directly to the direct PCR kit. Interestingly, comparable STR profiles were produced by both methods, but still mostly yielded partial profiles for highly decomposed tissues. Therefore, the PDQeX System was tested on the tissue biopsy itself that was preserved in the TENT buffer, and generated the most complete profiles from decomposing tissues. The results of this study indicate that tissues can successfully be preserved at room temperature in a chemical tissue preservative and rapidly purified (or directly amplified), provided that tissues have not experienced severe DNA degradation as observed in bodies that are highly decomposed (after days 4-6 in this study).

Lastly, a separate approach for directly collecting DNA from decomposing skin and muscle using various types of swabs was also investigated. Foam (Whatman® EasiCollect™ devices), nylon (4N6FLOQSwabs™), and traditional cotton swabs were evaluated based on whether sufficient DNA could be collected for traditional DNA extraction or a direct PCR approach, and whether each swab type was capable of storing DNA at room temperature for one month without significant loss or degradation of DNA.

In general, cotton and foam swabs provided higher DNA concentrations and more complete profiles than nylon flocked swabs, regardless of the analysis method used (traditional DNA analysis vs direct PCR). In addition, no significant difference in STR success was observed when cotton and foam swabs were stored for one month, but a significant decrease in the number of reportable STR alleles was observed from samples

stored on the nylon swabs, suggesting that the nylon swabs did not adequately protect the DNA from further degradation.

Cotton, foam, and nylon flocked swabs were prepared for direct PCR by eluting cells off the substrate using a lysis buffer that also allowed for quantification prior to STR typing. This facilitated a triaging protocol that increased first pass success for cotton and foam swabs with high quantities of DNA compared to those with extremely low quantities of DNA. Nylon swabs were the least successful due to severe PCR inhibition and too little DNA collected. In addition, swabs were used to collect biological material from the skin of remains or the underlying muscle tissue (via a small incision in the skin). Results indicate that swabbing muscle may yield higher DNA concentrations, but both tissue types resulted in comparable STR profiles. However, there is a greater risk of contamination when swabbing the dermal surface of human remains, especially when bodies are in close proximity to one another, or severe fragmentation and commingling has occurred. Therefore, swabbing the underlying muscle with cotton and foam swabs have the greatest potential for collection of DVI samples and capable of faster and more direct STR typing.

Final Recommendation for DVI-DNA Based Methods

In conclusion, we have demonstrated that in-field sample collection using simple biopsy punches or cotton and foam swabs have the potential to immediately collect DNA for room temperature storage and delayed sample processing. As expected, there was a noticeable decline in DNA quantity and quality as decomposition progressed over a two week period. Therefore, the immediate collection and preservation of DNA samples from human remains in such circumstances are important considerations for the success of DNA-based identification. Tissue samples that have not already undergone severe

decomposition or putrefaction could be preserved in the modified TENT preservative to suspend DNA degradation until refrigeration or DNA analysis is possible. Cotton and foam swabs also have the potential for collecting biological material from decomposing bodies in the field and storing samples at room temperature until refrigeration or DNA analysis is possible.

In addition, direct PCR methods have been proven to be a viable option for both sample types (TENT and swabs). The direct PCR approach used for swabs in this study generated STR profiles in approximately three to four hours. This approach takes more time due to various steps including cutting cotton or foam swabs, five minute incubation and lysis, quantification in order to adequately triage samples for amplification (2µL and 27 total cycles or 5µL and 29 cycles). However, the final modified version of the TENT buffer facilitated direct PCR after a quick and simple 1 in 10 dilution generating comparable STR results to the traditional workflow (full DNA extraction, quantification, and normalization of DNA prior to PCR). This method only took approximately an hour for dilutions and PCR amplification. Therefore, this method is the most rapid approach for human identification following a mass disaster, provided that tissues have not already undergone severe decomposition, in which case DNA typing from bones and teeth would be required for successful identification.

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ZyGEM. Instruction manual PDQeX 2400.

APPENDIX A: Cadaver Information for Chapters II and III

This section contains figures displaying decomposition for cadavers A, B, and C and the weather information for the two week period of sample collection in October 2013.

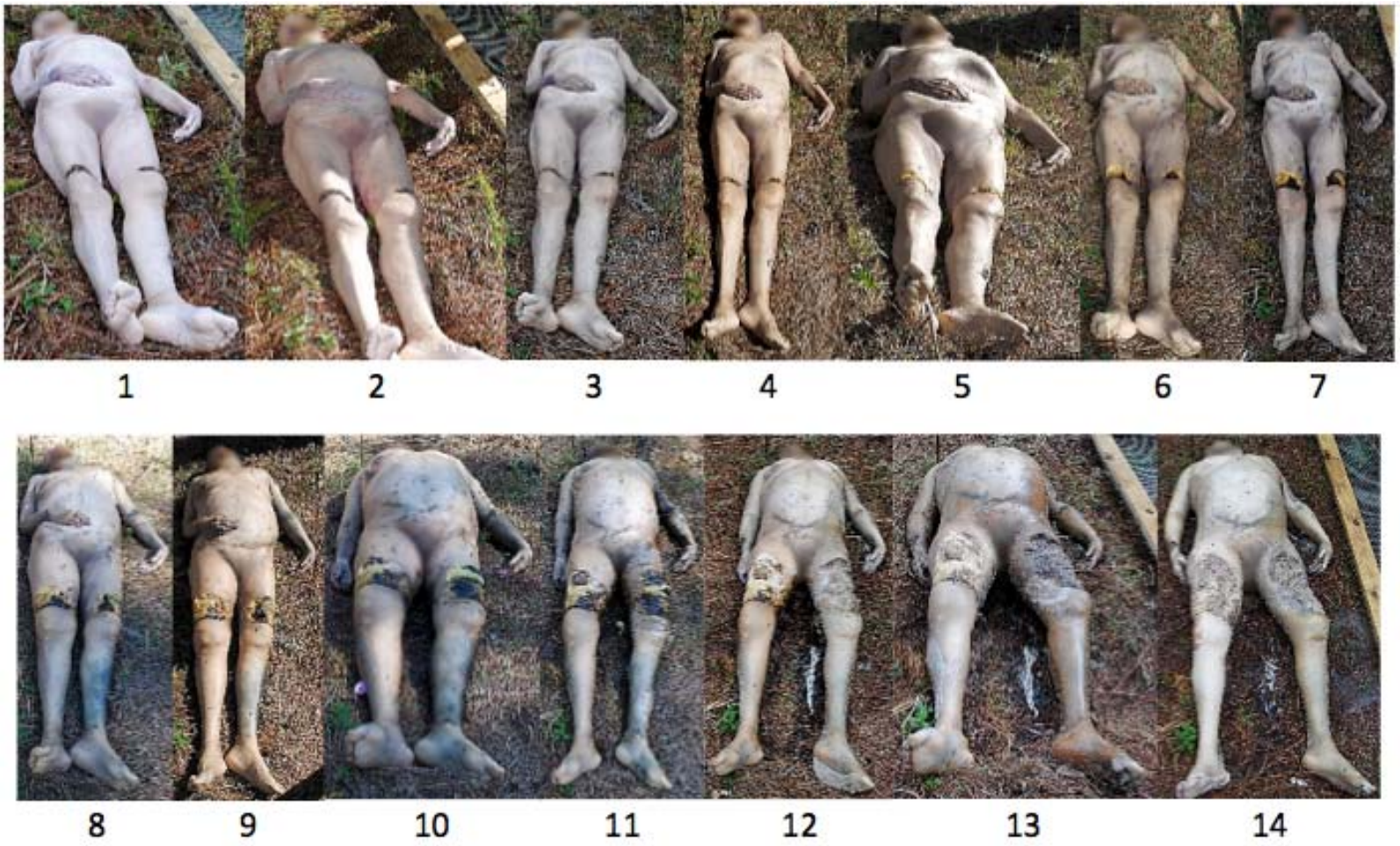

Fig. A1. Days of decomposition for Cadaver A

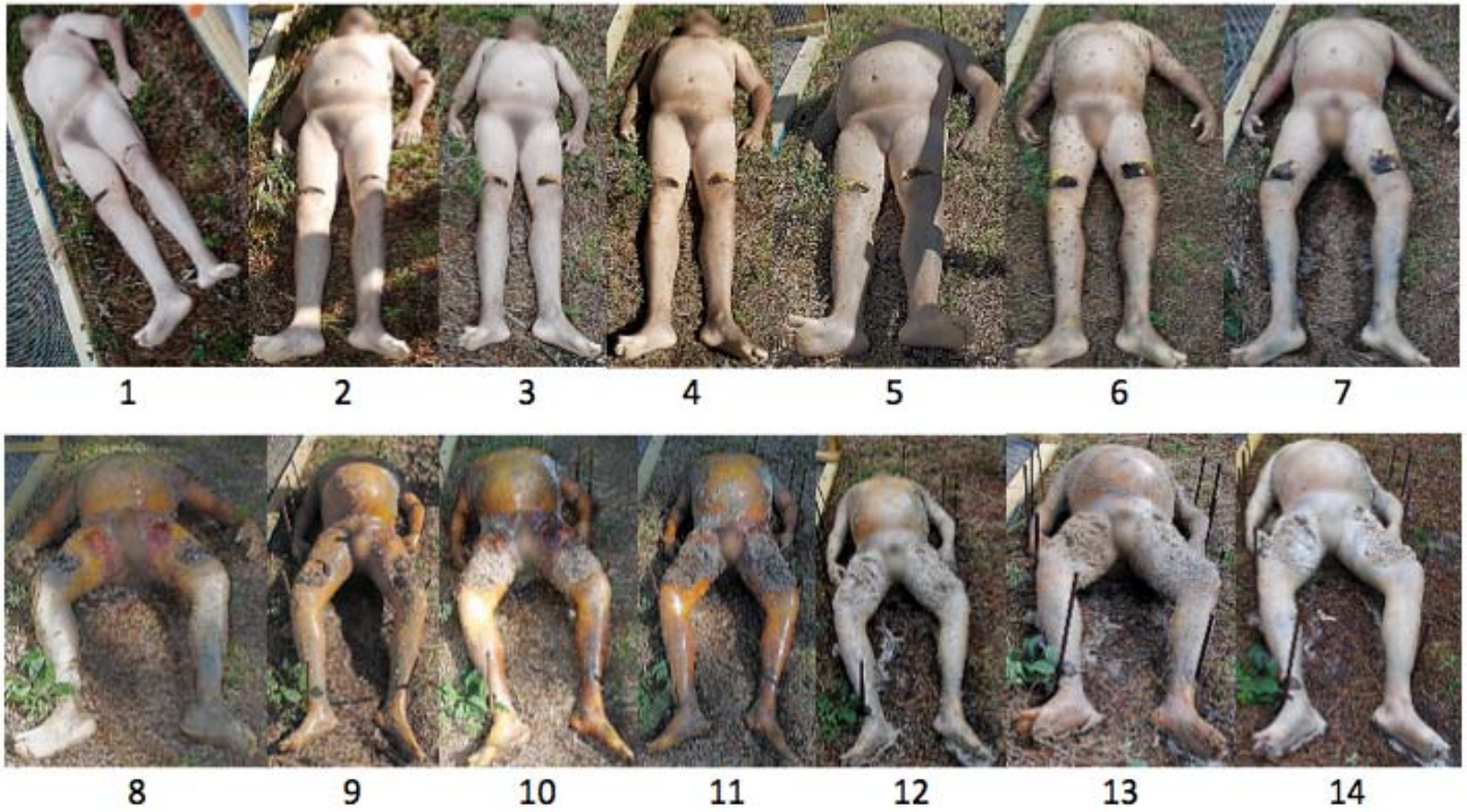

Fig. A2. Days of decomposition for Cadaver B

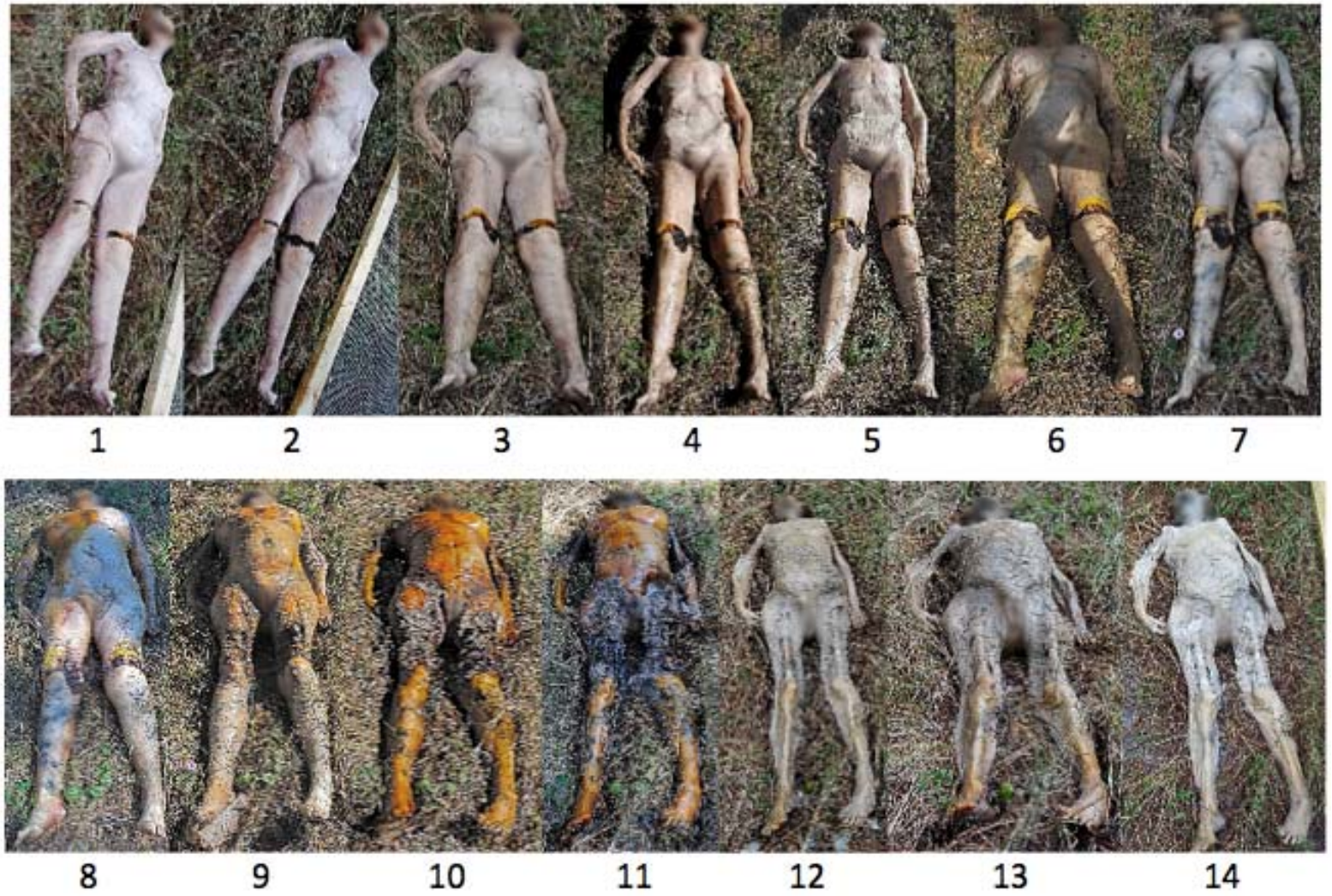

Fig. A3. Days of decomposition for Cadaver C

Table A1. Weather data for the two weeks of photographs and sample collection from Cadavers A-C in October 2013

2013	Temp. (°F)			Dew Point (°F)			Humidity (%)			Sea Level Press. (in)			Visibility (mi)			Wind (mph)			Precip (in)	Events
October	high	avg	low	high	avg	low	high	avg	low	high	avg	low	high	avg	low	high	avg	high	sum	
15	86	78	71	73	70	68	96	83	57	30	29.96	29.87	10	7	2	10	5	16	0.35	Rain
16	72	66	59	70	62	57	100	95	88	30.05	29.98	29.91	10	6	1	10	5	~	0.19	Rain
17	73	64	54	57	53	49	94	78	44	30.11	30.04	29.99	10	10	7	8	3	~	0.01	
18	70	62	54	58	55	52	93	78	60	30.03	29.97	29.91	10	9	2	5	1	~	0	Rain
19	66	57	48	58	48	39	93	74	37	30.15	30.08	29.98	10	10	10	17	6	28	0	
20	72	57	43	54	46	41	96	70	38	30.11	30.03	29.95	10	10	9	8	1	~	0	
21	79	69	60	64	60	55	90	75	54	30.01	29.97	29.92	10	9	2	7	4	16	0	
22	75	64	54	64	55	48	94	75	40	30.12	30.06	30	10	10	10	12	6	20	0	
23	82	66	51	53	48	45	86	60	28	30.15	30.11	30.08	10	10	6	5	1	~	0	
24	82	68	54	54	50	45	87	60	28	30.23	30.18	30.11	10	10	9	8	3	~	0	
25	73	64	55	53	52	50	93	74	49	30.38	30.29	30.23	10	10	8	6	2	~	0	
26	80	66	51	62	55	44	84	68	47	30.25	30.18	30.09	10	10	10	10	4	17	0	
27	72	66	60	63	60	57	96	88	68	30.12	30.07	30.02	10	6	2	13	5	28	1.81	Rain, Thunderstorm
28	81	70	60	68	64	58	96	79	62	30.14	30.07	30.03	10	10	9	5	1	~	0	
29	82	76	69	68	67	66	90	79	60	30.12	30.08	30.03	10	10	8	13	5	20	0	

APPENDIX B: Minimum Time Study

Tissues were incubated in DNA preservatives (LST, Modified TENT, DESS, and DNAgard) and extracted after 1 hour, 8 hours or 1, 3, 7, and 14 days to determine the minimum incubation time required for sufficient quantities of DNA to leach into preservative solution. Fresh and decomposed tissues leached sufficient DNA quantities into all preservatives after 1 hour of incubation generating complete STR profiles.

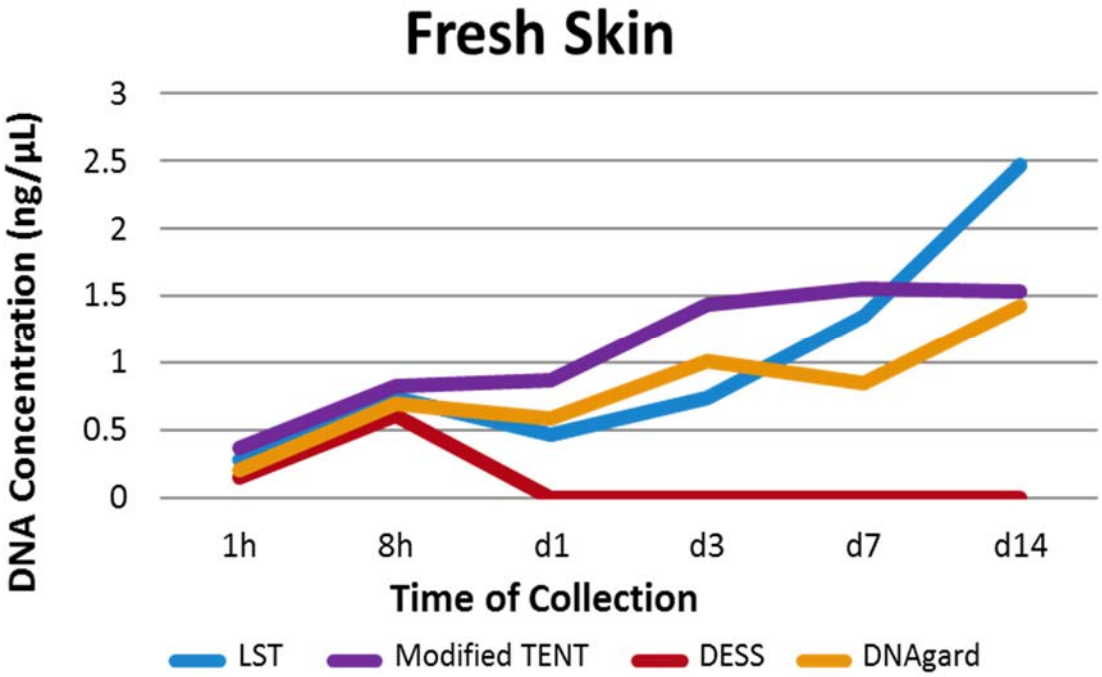

Fig. B1. DNA quantification results for minimum time study for preservatives surrounding fresh skin (1h = 1 hour, d1 = day 1)

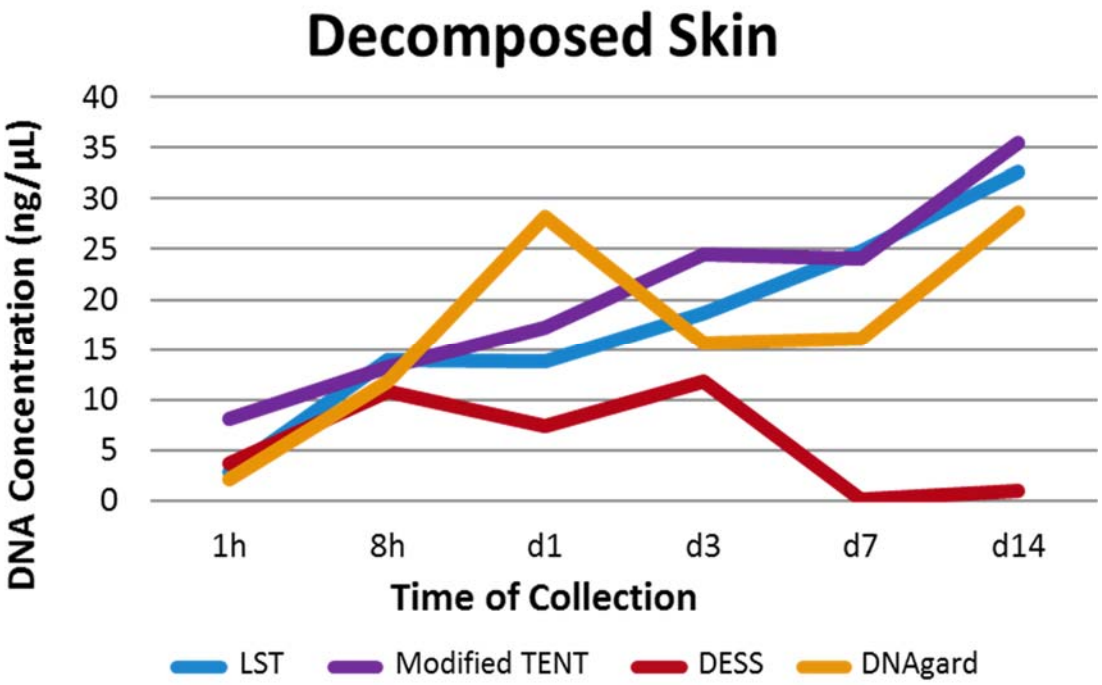

Fig. B2. DNA quantification results for minimum time study for preservatives surrounding decomposed skin (1h = 1 hour, d1 = day 1)

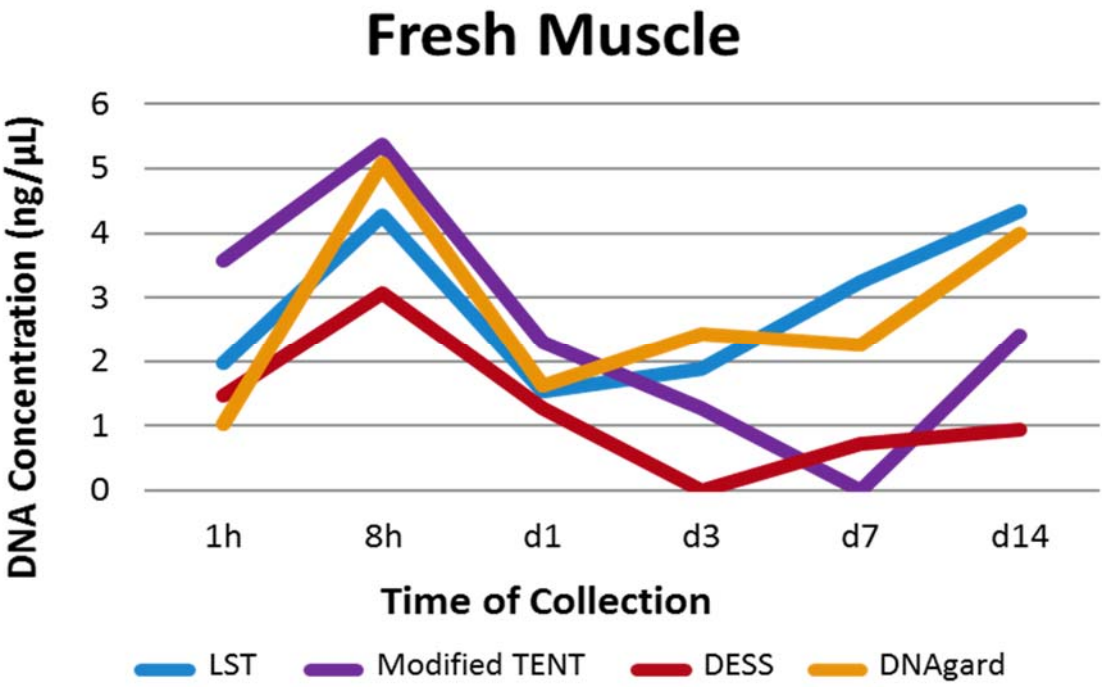

Fig. B3. DNA quantification results for minimum time study for preservatives surrounding fresh muscle (1h = 1 hour, d1 = day 1)

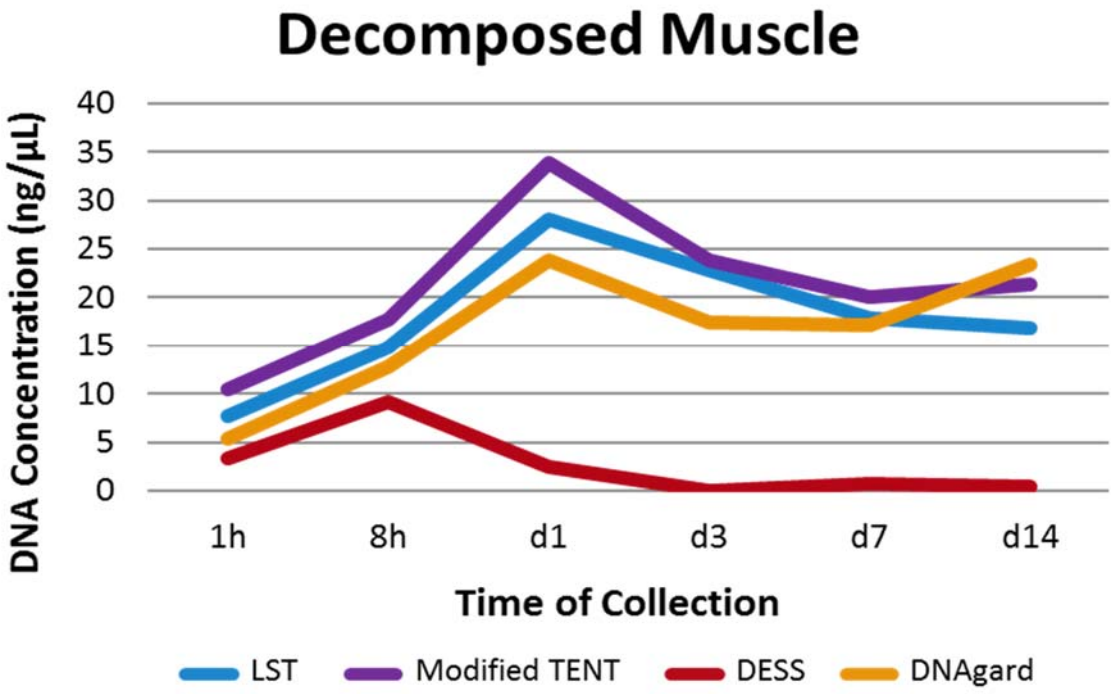

Fig. B4. DNA quantification results for minimum time study for preservatives surrounding fresh muscle (1h = 1 hour, d1 = day 1)

APPENDIX C: Application Note

Chapter IV was also significantly revised and printed as a commercial application note for QIAGEN's Investigator® Quantiplex® Pro Kit. To find more information, visit the following link:

https://www.qiagen.com/us/resources/resourcedetail?id=873477a8-a5a4-45b6-87e7-9a6e13fa844e&lang=en

APPENDIX D: Cadaver Information for Chapters V and VI

This section contains figures displaying decomposition for cadavers D, E, and F and the weather information for the two week period of sample collection in April 2016.

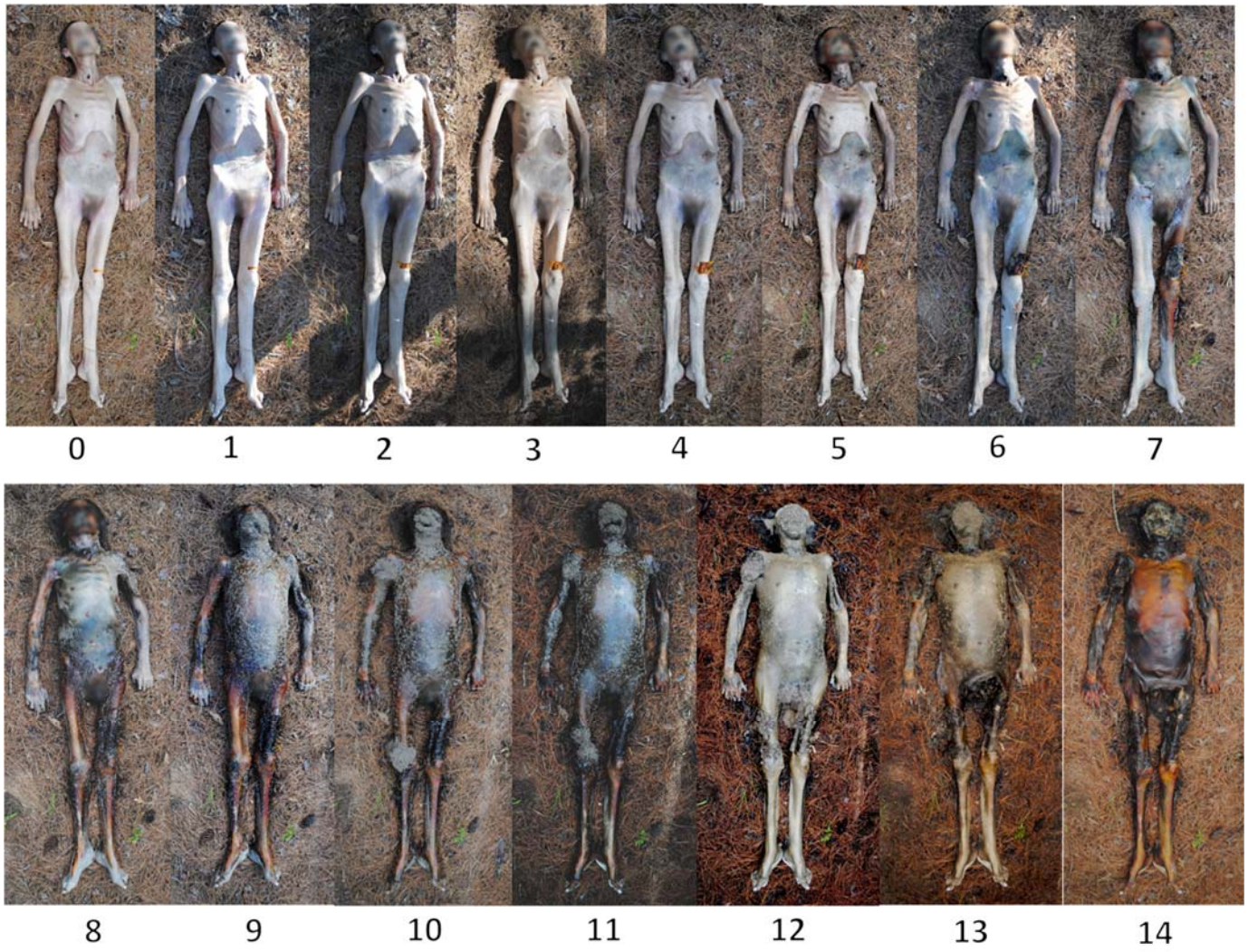

Fig. D1. Days of decomposition for Cadaver D

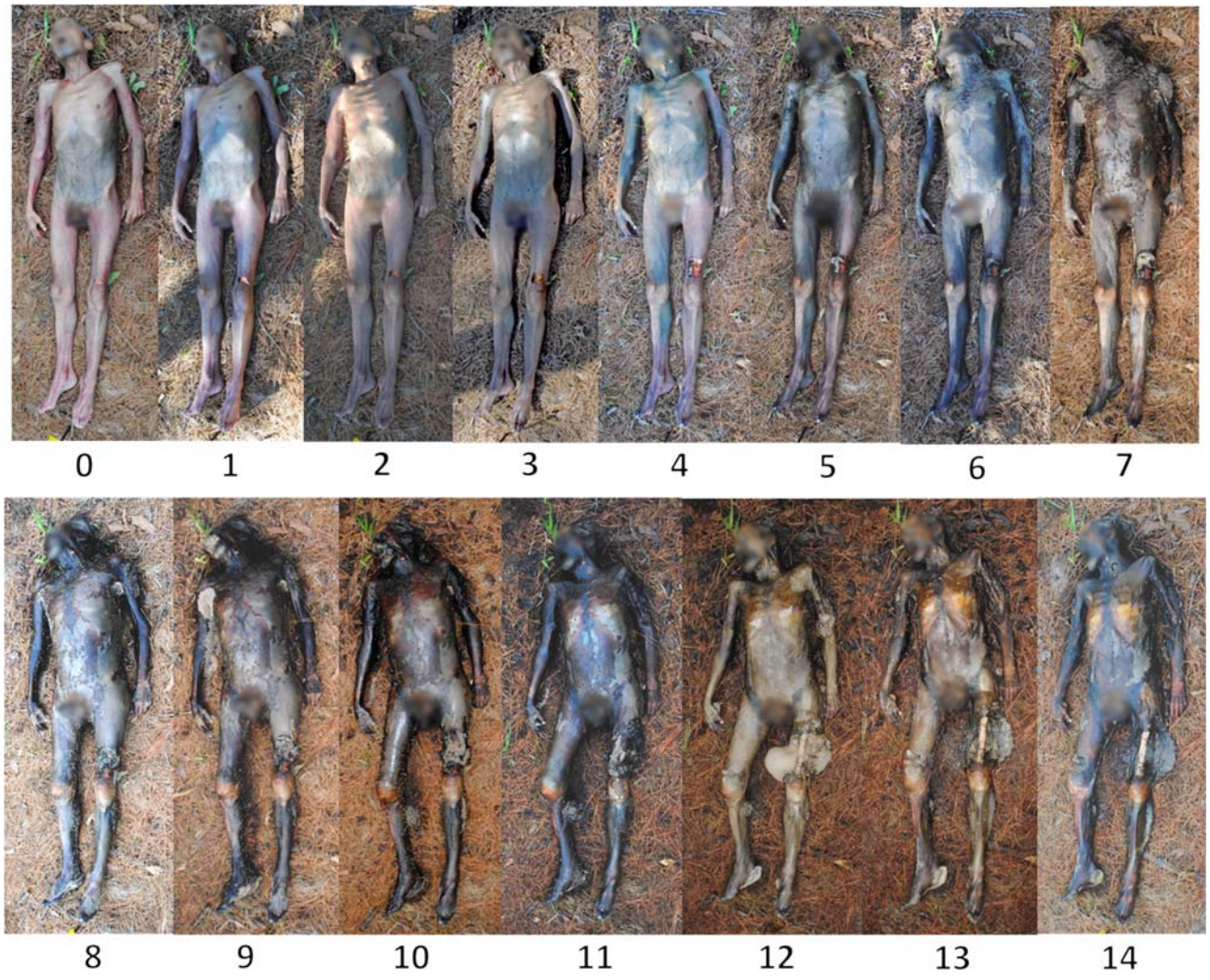

Fig. D2. Days of decomposition for Cadaver E

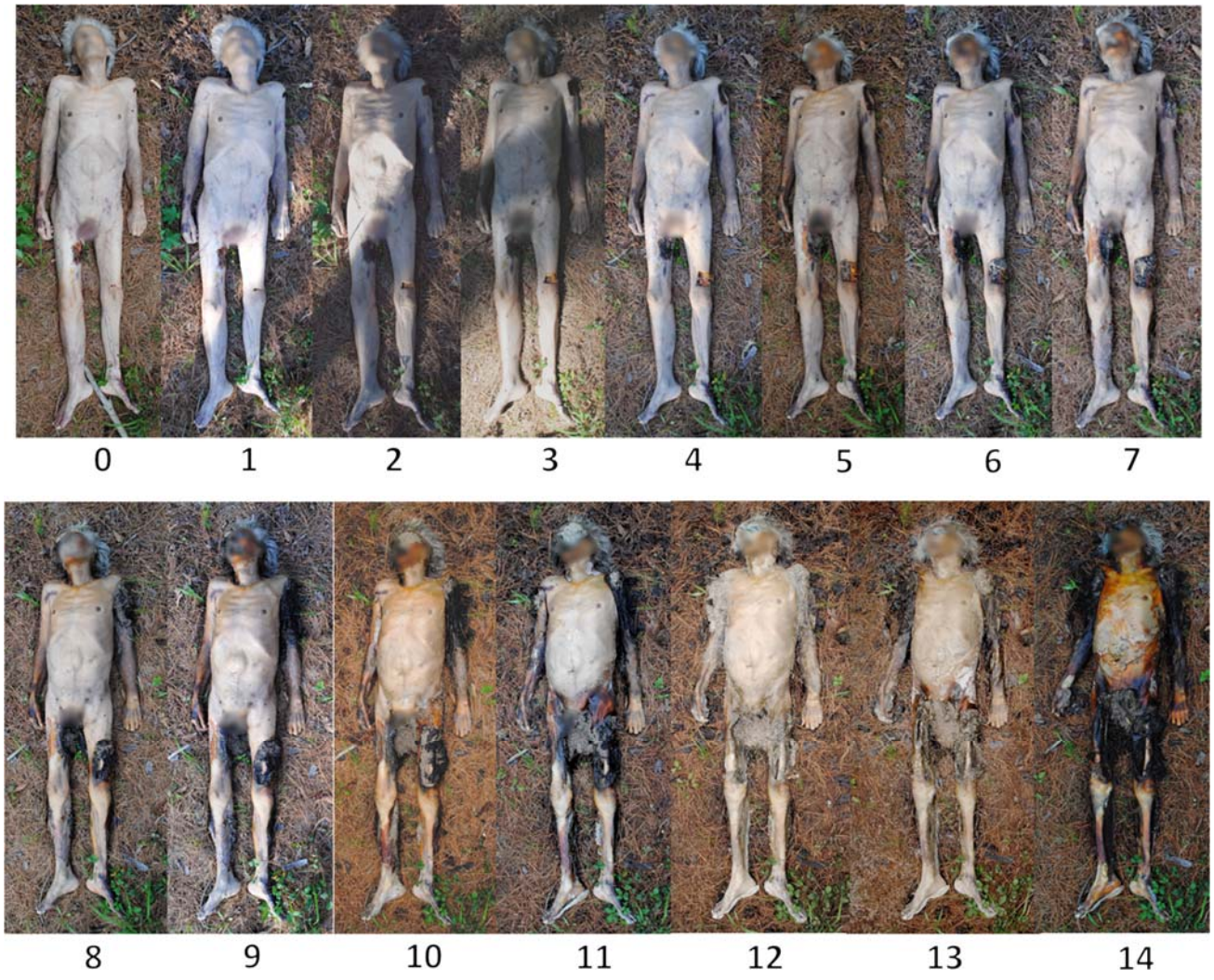

Fig. D3. Days of decomposition for Cadaver F

Table D1. Weather data for the two weeks of photographs and sample collection from Cadavers D-F in April 2016

2016	Temp. (°F)			Dew Point (°F)			Humidity (%)			Sea Level Press. (in)			Visibility (mi)			Wind (mph)			Precip (in)	Events
April	high	avg	low	high	avg	low	high	avg	low	high	avg	low	high	avg	low	high	avg	high	sum	
1	68	57	46	48	45	42	90	63	40	30.12	29.94	29.78	10	10	10	14	6	26	0.03	Rain , Thunderstorm
2	71	56	42	44	39	34	92	58	26	30.26	30.19	30.12	10	10	10	17	4	25	0.00	
3	75	58	42	46	38	30	82	52	23	30.26	30.18	30.10	10	10	10	8	2	-	0.00	
4	81	67	53	51	48	45	86	56	29	30.21	30.16	30.12	10	10	8	13	7	18	0.00	
5	82	68	53	51	47	44	72	49	30	30.23	30.15	30.06	10	10	8	14	6	18	0.00	
6	80	68	57	59	51	38	87	62	25	30.10	30.03	29.96	10	10	8	15	7	25	0.00	
7	84	66	48	50	43	37	77	47	19	30.05	30.00	29.94	10	10	5	9	3	-	0.00	
8	81	66	52	47	41	30	77	46	17	30.18	30.12	30.03	10	10	10	8	3	-	0.00	
9	77	64	54	55	46	36	73	52	30	30.18	30.11	30.04	10	10	10	9	2	20	0.00	Rain
10	82	72	61	65	60	56	90	71	47	30.05	29.99	29.92	10	10	10	17	9	31	0.00	
11	79	74	69	72	69	66	91	85	76	29.97	29.92	29.82	10	8	2	20	10	34	0.02	Rain
12	72	68	64	68	64	60	96	87	73	30.12	30.05	30.00	10	9	4	10	4	-	0.00	Rain
13	68	64	61	63	61	58	97	91	81	30.12	30.01	29.93	10	7	2	6	3	-	0.50	Rain , Thunderstorm
14	73	66	61	62	61	58	97	86	68	30.01	29.96	29.90	10	8	3	10	4	-	0.02	Rain
15	79	66	54	61	57	53	97	81	48	29.95	29.89	29.82	10	7	0	6	1	-	0.00	Fog

VITA

Related Work Experience

•Sam Houston State University, Huntsville, TX
•Forensic Science Department—Graduate Teaching Assistant, June 2015-Present
•Forensic Science Department—Research Assistant, Jan-Aug 2014
•Impaired Driving Initiatives—Graduate Assistant, Aug-Dec 2013
•Houston Police Department, Houston, TX (now Houston Forensic Science Center)
•Crime Lab, DNA/Biology Section—Intern, May-Aug 2013

Relevant Graduate Coursework

- Instrumental Analysis
- Forensic Biology
- Advanced Forensic DNA
- Statistical Genetics for Forensic Science
- Quality Assurance and Ethics
- Law (including moot court)
- Stats and Evidence Interpretation
- Research Methods
- Scientific Communications
- Advanced Topics in Forensic Biology
- Forensic Laboratory Management
- Non-human DNA Forensics
- Bioinformatics

Relevant Undergraduate Coursework

- Biochemistry I and II
- Cell Biology
- Molecular Biology
- Genetics
- Advanced Genetics
- PCR Methods and Applications
- Technical Writing
- Criminology

Instrumentation and Technical Experience

- Robotic Extraction Platforms (QIAcube, EZ1 xL, AutoMate, PDQeX System)
- PCR Thermal cyclers (AB9700, ProFlex, Veriti)
- Real-Time Quantitative PCR (StepOne, AB7500)
- Capillary Electrophoresis (AB3500), Genemapper® ID-X Software
- Agarose Gel Electrophoresis
- Ion PGM™ System, S5 System, and Ion Chef™
- Rapid DNA Analysis Systems (RapidHit™ and DNAscan)
- Qubit Fluorometric Quantitation
- Stereo, Polarized Light, Digital, and Comparison Microscopes
- UV-Visible Spectroscopy
- In-field DNA collection from human remains
- Stryker Autopsy Saw

Independent Research Experience

- Alternative methods for collection, room temperature storage, and processing of DNA samples from human remains: A new DVI approach.
- Evaluation of four commercial quantitative real-time PCR (qPCR) kits with inhibited and degraded samples.
- Improved sample collection and preservation of DNA from decomposing human remains; A direct approach for faster disaster victim identification (DVI).
- Direct-to-PCR tissue preservation for DNA profiling.
- Preservation and high throughput methods for human tissue samples in tropical climates, An improved DVI approach.
- DNA preservation for disaster victim identification in tropical climates: A pilot study.

Other Skills

- Supervision, mentorship, teaching, and leading junior graduate research students
- Conducted training on the QIAcube, StepOne and 7500 qPCR, and 3500
- Project design, report/technical writing, literature reviews, and SOP writing
- Technical troubleshooting/problem solving
- Word, Excel, Powerpoint, and Apple equivalent softwares, Adobe Photoshop, Illustrator, and InDesign
- R Statistical Software, Statisica Software
- Geneious Software
- Primer design and multiplex assay design

- STRmix Software

Manuscripts in Peer Reviewed Journals

- Amy S. Holmes, MS; Madeline G. Roman, BS; Sheree Hughes-Stamm, PhD. In-field collection and preservation of decomposing human tissues to facilitate rapid purification and STR typing. *In peer-review (Forensic Science International: Genetics)*
- Amy S. Holmes, MS; Madeline G. Roman, BS; David Gangitano, PhD; Sheree Hughes-Stamm, PhD. In-field collection and direct analysis of swabs from decomposing human remains for DVI. *In peer-review (Journal of Forensic Science)*
- Amy S. Holmes, MS; Rachel Houston, BS; Kyleen Elwick, BA; David Gangitano, PhD; Sheree Hughes-Stamm, PhD. Evaluation of four commercial quantitative real-time PCR kits with inhibited and degraded samples. International Journal of Legal Medicine (2017, Accepted for publication)
- Amy Sorensen, MS; Elizabeth Rahman, MS; Cassandra Canela, MS; David Gangitano, PhD; Sheree Hughes-Stamm, PhD. Preservation and Rapid Purification of DNA from Decomposing Human Tissue Samples. Forensic Science International: Genetics (2016)
- Amy Sorensen, MS; Clare Berry, BS; David Bruce, PhD; Michelle Gahan, PhD; Sheree Hughes-Stamm, PhD; Dennis McNevin, PhD. Direct to PCR Tissue Preservation for DNA Profiling. International Journal of Legal Medicine (2015)

Conference Presentations

- Amy S. Holmes, Ryan Gutierrez, Madeline Roman, David Gangitano, **Sheree Hughes-Stamm**. In-field Collection of DNA from Decomposing Remains for More Direct Analysis: Experiences with Investigator Quality Sensors. 7th QIAGEN Investigator Forum. Lisbon, Portugal (2018) *Oral Presentation*
- **Amy S. Holmes**, Madeline G. Roman, David Gangitano, Sheree Hughes-Stamm. Alternate methods for collection, preservation, & processing of DNA from decomposing human remains. Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy. Orlando, FL (2018) *NIJ Poster Presentation*
- **Madeline Roman**, Amy Holmes, David Gangitano, Sheree Hughes-Stamm. Effect of Body Mass and Cadaveric Bloat on DNA Quantity and Downstream STR Success. Seattle, WA (2018) *Poster Presentation*
- **Amy Sorensen**; David Gangitano; Sheree Hughes-Stamm. Alternative methods for collection, room temperature storage, and processing of DNA samples from human remains: A new DVI approach. 28th International Symposium on Human Identification. Seattle, WA (2017) *Poster Presentation*

- **Amy Sorensen**; David Gangitano; Sheree Hughes-Stamm. Alternative methods for collection, room temperature storage, and processing of DNA samples from human remains: A new DVI approach. 27th Congress of the International Society for Forensic Genetics. Seoul, South Korea (2017) *Poster Presentation*
- Amy Sorensen; Rachel Houston; Kyleen Elwick; Carrie Mayes; Kayla Ehring; David Gangitano; **Sheree Hughes-Stamm**. Alternate methods for the collection, preservation, & processing of DNA samples from decomposing human cadavers; A DVI strategy. 6th QIAGEN Investigator Forum. Prague, Czech Republic (2017) *Oral Presentation*
- **Amy Sorensen**; Kyleen Elwick; David Gangitano; Sheree Hughes-Stamm. Comparative evaluation of three commercial quantitative PCR kits with inhibited and degraded samples. The 26th International Symposium on Human Identification. Minneapolis, MN (2016) *Poster Presentation*
- **Amy Sorensen**; Clare Berry; David Bruce; Michelle Gahan; Sheree Hughes-Stamm; Dennis McNevin. Direct to PCR Tissue Preservation for DNA Profiling. American Academy of Forensic Sciences (AAFS)—68th Annual Scientific Meeting. Las Vegas, NV (2016) *Oral Presentation*
- **Amy Sorensen**; Clare Berry; David Bruce; Michelle Gahan; Sheree Hughes-Stamm; Dennis McNevin. Direct to PCR Tissue Preservation for DNA Profiling. The 25th International Symposium on Human Identification. Grapevine, TX (2015) *Poster Presentation*
- **Amy Sorensen**; Elizabeth Rahman; David Gangitano; Sheree Hughes-Stamm. Improved Preservation and Purification Methods for DNA in Decomposing Human Tissue Samples; A DVI Application. American Academy of Forensic Sciences (AAFS)—67th Annual Scientific Meeting. Orlando, FL (2015) *Poster Presentation*
- Amy Sorensen; David Gangitano; **Sheree Hughes-Stamm**. Room Temperature DNA Preservation and Rapid Purification of Decomposing Human Tissue Samples; A DVI Application. The 25th International Symposium on Human Identification. Phoenix, AZ (2014) *Poster Presentation*
- Amy Sorensen; David Gangitano; **Sheree Hughes-Stamm**. Room Temperature DNA Preservation and High-Throughput Purification of Decomposing Human Tissue Samples; An Improved DVI Approach. The 22nd International Symposium on the Forensic Sciences. The Australian and New Zealand Forensic Science Society. Adelaide, Australia (2014) *Oral Presentation*
- Amy Sorensen; David Gangitano; **Sheree Hughes-Stamm**. DNA Preservation and Rapid Purification of Decomposing Human Tissue Samples; A DVI Application.

Association of Forensic DNA Analysts and Administrators (AFDAA). Houston, TX (2014 Summer Meeting) *Oral Presentation*

Other Work Products

- Amy Sorensen Holmes, Rachel Houston, Kyleen Elwick, David Gangitano, Sheree Hughes-Stamm. Comparison of four commercial qPCR kits for analyzing inhibited and degraded forensic samples. QIAGEN Application Note
- **Amy Sorensen,** Rachel Houston, Kyleen Elwick, Sheree Hughes-Stamm. Evaluation of four commercial quantitative real-time PCR (qPCR) kits with inhibited and degraded samples. August 2017. Invited talks to QIAGEN Asian Pacific Team in Singapore, Department of Chemistry Malaysia, and National Forensic Service Seoul Institute
- **Amy Sorensen, Rachel Houston**, Kyleen Elwick, **Sheree Hughes-Stamm**. How do modern quantification kits STACK-UP? June 2017. Forensic Magazine webinar sponsored by QIAGEN.

Research Grant Funding

- National Institute of Justice (NIJ) Graduate Research Fellowship Grant Award, 2015-present (Award # 2015-R2-CX-0029)

Honors/Activities

- LTC Michael A. Lytle ’77 Academic Prize in Forensic Science Recipient (2018)
- Traveled to Singapore, Malaysia, and South Korea to visit with QIAGEN customers and present my research using the latest commercial qPCR and STR kits
- American Academy of Forensic Sciences, Forensic Sciences Foundation (FSF) Student Scholarship, 2016
- American Academy of Forensic Sciences, Student Affiliate 2012-present
- Sam Houston State University Society of Forensic Science, 2012-present
- ΑΕΔ Premedical Honor Society, 2009-2012
- Lambda Sigma National Honors Society — Head of Service Committee, 2009-2010
- President’s List/Dean’s List Honors Student, 2008-2009

Continuing Education

- Bloodborne/Airborne Pathogens Training
- Physical Science Responsible Conduct of Research
- SOP Writing for ISO 17025 Accreditation
- Answering the NAS: The Ethics of Leadership and the Leadership of Ethics

- To Hell and Back: The Ethics of Stewardship and the Stewardship of Ethics
- Introduction to Uncertainty in Forensic Chemistry and Toxicology
- Advanced Word and Excel Training