ALTERNATE DNA EXTRACTION AND STR PROFILING STRATEGIES FOR SKELETAL AND OTHER CHALLENGING SAMPLES

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by

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DEDICATION

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ABSTRACT

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Skeletal remains are often submitted for DNA analysis for human identification (HID) purposes, to either supplement or substitute other forensic identification methods, such as anthropological and odontological analyses. However, the identification of skeletal samples via DNA testing is often challenging and alternate sample processing methods may offer some effective solutions. Current protocols for processing skeletal material involve crushing the bone into a fine powder, which requires specialized equipment, reagents, training, and can pose an increased risk of contamination. Although methods that involve powdering bone tissues for extraction often yield sufficient results for statistical comparisons, bypassing this step can eliminate many of these risks, save time and resources, and also make bone extractions easier for forensic DNA laboratories to implement. Several issues, such as PCR inhibition and DNA degradation, can also make identification more difficult. Therefore, products that provide analysts with more information about sample quality at various stages of the HID process could greatly improve the genotyping process and assist with sample triage and workflow decisions.

The main aim of this research was to evaluate the effectiveness of various protocols that eliminate the need to powder bone tissue prior to DNA extraction. A secondary aim of this study was to investigate the benefits of internal STR quality controls for assessing sample quality and determining rework strategies when challenging samples fail to produce complete STR profiles. Several non-powdering DNA extraction methods were tested with human skeletonized remains of varying quality to identify the most efficient protocol to

achieve the highest genotyping success. In addition, a broader scope of forensically relevant sample types were genotyped with the Investigator® 24plex QS and GO! kits that include quality controls to test their effectiveness in identifying issues that negatively impact STR success, and guide the most efficient sample rework strategies.

As a result of this work, an effective powder-free DNA extraction workflow was identified and shown to be successful with a variety of environmentally challenged skeletal samples. Additionally, we have demonstrated that internal STR quality sensors can simplify STR profile interpretation, help reduce the number of sample reworks, and generate more complete STR profiles when samples are reworked based on the information provided by the quality sensors. These alternate methodological approaches can reduce overall processing time and costs for a wide range of challenging samples subjected to STR typing for human identification purposes. This research has also been used as the scientific basis to amend recommended protocols for some commercial HID products, which will directly benefit the forensic community.

KEY WORDS: Forensic science, Forensic biology, DNA, Bone, Skeletal, Human identification, Low-template DNA, LCN, Short tandem repeats, Automation, Challenging samples, Degradation, Inhibition, Quality sensors

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ABBREVIATIONS

AFDIL	Armed Forces DNA Identification Laboratory
BP(s)	Basepair(s)
CE	Capillary electrophoresis
CODIS	Combined DNA Index System
DI	Degradation Index
DNA	Deoxyribonucleic acid
DPAA	Defense POW/MIA Accounting Agency
DTT	Dithiothreitol
DVI	Disaster Victim Identification
EDTA	Ethylenediamine tetra-acetic acid
EPG	Electropherogram
FBI	Federal Bureau of Investigation
HID	Human Identification
IC/IPC	Internal control/Internal PCR control
ICMP	International Commission on Missing Persons
INDEL	Insertion/deletion
ISO	International Organization for Standardization
LT-DNA	Low-template DNA
LCN	Low copy number
MPS	Massively parallel sequencing
mt-DNA	Mitochondrial DNA
NGS	Next generation sequencing
PCR	Polymerase chain reaction
qPCR	Quantitative PCR
SNP	Single nucleotide polymorphism
STR	Short tandem repeat

CHAPTER I

Introduction

Decomposed or skeletonized human remains may be identified using fingerprint analyses or dental record comparisons, but when these strategies cannot be used, DNA analysis is relied upon for identification (1-3). DNA isolated from biological material can be used for paternity testing, human identification (HID), and forensic investigations (3), with short tandem repeats (STRs) being the current gold standard for human identification (4-7). Buccal swabs and blood specimens are common sources of abundant nuclear DNA for forensic analysis (3, 8) and provide robust and reliable genotyping of individuals with high discriminatory power (8). However, some biological samples submitted to forensic laboratories prove to be more challenging for DNA extraction and analysis. Biological evidence recovered from crime scenes can be degraded and/or in trace amounts, with potential contamination and/or polymerase chain reaction (PCR) inhibitors due to environmental conditions or the samples themselves (9). In the case of mass disasters, missing persons' cases, severe fires, and mass graves, skeletonized remains (bone and teeth) may be the only elements available for human identification (3, 4, 9-12).

Mass Disasters and Missing Persons

Mass Disasters

Mass disasters are sudden and catastrophic events that result in the death or injury of many people (3, 13-15). They can be natural (epidemics, severe weather events), accidental (industrial, vehicular), or terroristic acts (chemical, biological, or radiological warfare, explosions) (3, 13-15). Notable mass disasters of the early 21st century include the 9/11 World Trade Center attacks in 2001 (2, 13), Madrid terrorist attack 2004 (13), the

Indian Ocean/Southeast Asian earthquake and tsunami in 2004 (2, 3, 13, 16), Hurricane Katrina in 2005, and the Haiti earthquake in 2010, among many others (13).

The process of identifying human remains resulting from a mass disaster is commonly referred to as Disaster Victim Identification, or DVI. There are two main objectives of DVI: 1) identifying recovered remains, and 2) associating fragmented remains to each other (14). The first function is necessary for both familial closure during the grieving process as well as criminal/civil investigations (3, 13). Re-association of remains to each other is necessary when the remains are highly fragmented, such as those recovered from the 9/11 World Trade Center attacks (3), or co-mingled, such as remains exhumed from mass graves. The identification process consists of comparing and matching ante- and post-mortem samples, such as fingerprints, dental records, unique personal or medical items (e.g. medical implants with serial numbers), and/or through direct DNA matching with personal items belong to the victim, or kinship analysis (3, 13, 15). As there are a variety of methods with which victims may be identified, DVI is a multi-disciplinary exercise and generally involve the following disciplines: forensic anthropology, fingerprinting, forensic odontology, radiology, forensic DNA/biology, and forensic pathology/medical examiners (13, 14). In the United States, DVI teams of experts include the Disaster Mortuary Operational Response Team (DMORT), the FBI's Evidence Response Team (ERT), and the Office of Armed Forces Medical Examiner (OAFME) (13). An interdisciplinary approach is vital as multiple modes of identification help strengthen the confidence of matches (14) and in many instances, some of the more traditional identification methods are limited or not possible.

According to Montelius and Lindbolm, the identification process with DNA includes five main steps: 1) collecting the best ante-mortem samples (e.g. 1st degree relatives, archived blood/biopsy samples from potential victim, personal objects from deceased); 2) choosing the most optimal post-mortem samples that are in the best condition; 3) DNA analysis, matching ante- and post-mortem data, and calculating match statistics; 4) prioritizing quality throughout the process; and 5) cooperation between DVI disciplines (16).

Although DNA is frequently used for DVI and technology and processes have improved over time, it still has limitations. Primarily, DNA testing and identification is dependent on suitable reference samples for comparison (3). For example, without direct ante-mortem references, same-sex siblings cannot be distinguished based on parental DNA profiles alone (14). Also, when multiple family members are involved and in the absence of anthropological data, partial profiles make it difficult to fully identify someone within a family without an ante-mortem reference sample, especially when comingling has occurred (14, 16). During the initial recovery phases after mass disasters occur, storage facilities may not be available or adequate enough to slow decomposition at low temperatures (room temperature storage and preservation needed) (3). In contrast to the highly damaged and fragmented remains recovered from the 9/11 attacks, the victims recovered from the December 2004 Indian Ocean Tsunami had little fragmentation but high putrefaction/decomposition (3). This disaster was responsible for the most abrupt death toll in history with over 200,000 people deaths across 10+ countries (3, 13) and resulted in several recommendations for DVI processes (16).

Current DVI standards are based off Interpol and International Society of Forensic Genetics (ISFG) guidelines (16); however, laws regarding DVI processes are ultimately governed by the country of incidence (14). The cross-training of experts such as forensic geneticists, anthropologists, and pathologists is recommended and ideal (14, 17) as consultation with a geneticist during sample collection can guide the pathologist in choosing the proper tissue type based on the state of preservation, and anthropological landmarks should be avoided when cutting bone samples (14). Anthropologists and/or pathologists can also screen and remove non-human remains, which reduces the amount of unnecessary testing performed (14, 17). The preferred sample types for remains are buccal swabs, blood, and deep muscle/soft tissues, but when high decomposition or other insults prevent use of these, bone and teeth are the last resort (3, 13). Although bone and teeth typically have better results than soft tissue when remains are highly decomposed, the sample processing is long and laborious, with steps including de-fleshing (if applicable), cleaning, drying, cutting, drying, powdering, and decalcification (3). Nevertheless, it is recommended to collect and store as many sample types to avoid re-sampling retrospectively (14). As for genotyping, multiplex STRs are still the preferred DVI DNA technology (13, 14), but the main challenge of successfully genotyping highly degraded DNA has led to the development and implementation of alternate technologies, such as mini-STRs (7, 13, 18), mitochondrial DNA (7, 17, 19-22), or SNPs (14, 23-26).

Missing Persons

In addition to those lost during mass disasters, individuals may be separated from their families and loved ones as a result of political conflicts (e.g. wars), mental illness, kidnappings/human trafficking, murder, and other crimes, or during the process of

immigration and seeking asylum in another country. According to the National Missing and Unidentified Persons System (NamUs), over 600,000 people go missing each year in the United States alone (27). Additionally, an estimated 4,000+ unidentified remains will be recovered annually with approximately 25% of those not being identified after a period of one year (27). Currently, there are over 13,000 open cases for unidentified remains and 16,700+ open cases for missing persons across the United States and its surrounding territories (28). NamUs is a federally funded program that offers a database platform and several forensic services, such as fingerprints, DNA analysis, forensic odontology, and forensic anthropology, at no cost to law enforcement, other agencies, and the public in an effort to help clear missing and unidentified persons cases across the country (27).

The Armed Forces DNA Identification Laboratory (AFDIL) within the Armed Forces Medical Examiner System (AFMES) in Dover, Delaware is responsible for processing and identifying US military and civilian remains from current and past conflicts (29), such as World War I&II, Vietnam War, the Battle of the Punchbowl (Korean War), Pearl Harbor, Gulf War, and other recent wars in the Middle East (17, 29). For the past conflict remains specifically, AFDIL collaborates with, and provides testing services for, the Defense POW/MIA Accounting Agency (DPAA) (17, 29). This partnership aided in the identification of over 200 US military remains in 2017 and approximately 160 the previous year (30). To help their identification efforts, the Armed Forces Repository of Specimen Samples of the Identification of Remains (AFRSSIR) was established in the early 1990's to collect and maintain DNA reference samples (blood cards) for all service members (31). As DNA identifications with only familial references can be challenging,

having direct references has allowed for faster and more definitive identifications when other methods (dental and fingerprint comparisons) could not be used (31).

Outside of the United States, international organizations such as Interpol and the International Commission on Missing Persons (ICMP) are also tasked with the goal of finding and identifying missing persons. Initially established in 1996 with the objective of identifying missing persons' remains from the 1990's Yugoslavian conflicts, the ICMP's mandate has been expanded globally as a result of its success (32). Over the past two decades, ICMP has influenced legislature, cooperation between governments, and the development and implementation of forensic practices across 40+ countries including Kosovo, Western Balkans, Iraq, and Bosnia and Herzegovnia (32). Additionally, the Commission has processed over 70,000 post-mortem samples with over 27,000 unique profiles being generated (32).

Challenging Samples

Decomposed Tissues

In general, soft tissues are sources of abundant DNA, simple to collect, and quick and easy to process (2). However, when the tissues begin to decompose, the integrity of the DNA rapidly declines which makes identification via DNA testing difficult (2, 3). The rate of decomposition can be affected by many factors, including temperature, humidity, trauma, disposal conditions, as well as insect, animal, and microbial activity (1, 3, 4, 33, 34). A study by Schwark in 2010 compared the STR success rates between various putrefied soft tissues (2). Samples from the aorta, kidney, liver, and skeletal muscle were collected from 18 human remains in various stages of decomposition (PMI of several days to over 6 months) (2). DNA quantity and quality decreased with increasing decomposition,

as expected, and skeletal muscle yielded significantly lower amounts of DNA compared to the other three tissue types (2). STR typing results showed that DNA extracted from the aorta and kidney consistently generated the most complete profiles (2). These results supported other studies that suggested aorta tissue was useful for the identification of decomposed remains (35, 36). Other studies have used brain cortex (37, 38), lymph nodes (37), skin (39, 40), muscles (37, 39-41), organs (37, 42), and prostate/uterus tissues from decomposed remains (38), with varying success. Although DNA is more stable in hard tissues (bone and teeth), they are generally only used when soft tissues are not available or yield no useable DNA since the DNA extraction methods are lengthy and laborious (2, 3, 14).

Skeletonized Remains

Over the past 15 years, several studies have investigated which bony elements in the human body may provide the best DNA yields and most successful genotyping results for identification purposes (4, 17, 43-45). The highest DNA yields and STR success rates have been reported in bone samples taken from femur and teeth (~82-87%) due to the dense cortical bone of weight-bearing leg bones and the protective enamel coating on teeth (4). In 2007, Milos et al. ranked bones based on their STR success, with the top five being: femur, teeth, tibia, fibula, and vertebra (4). With the exception of the fibula, STR success positively correlated with Galloway et al.'s ranking of long bones based on decreasing density (femur, tibia, humerus, radius, ulna, and fibula) (46). As a general trend, Milos observed that lower limb long bones yielded consistently higher success rates compared to upper body long bones (4),

While these studies and many others have shown that femurs and other cortical bones may give the highest DNA yields, most of these studies do not include smaller, cancellous bones and other atypical bones (i.e. tarsal bones and phalanges) (17, 44). In a 2014 study, the first distal phalynx of the hand, second cuneiform, and maxillary molar tooth ranked the highest with a DNA yield per mass of sample above 420 ng/g bone while the femur ranked #49 (out of 55) with only 25 ng/g bone (44). When categorized by region, teeth ranked the highest in terms of DNA yield followed by cancellous bones in the foot and hand, with the leg being ranked #5 (out of 7) (44). A synchrotron radiation micro-CT scan was performed on the cancellous bones from that 2014 study, which showed potential soft tissue remnants within the marrow spaces that were not visible to the naked eye, which could have accounted for the higher DNA yields in these cancellous bones (43). Finally, a comprehensive study in 2019 showed that element success varied largely depending on the DNA extraction method used, yet weight-bearing long bones usually ranked higher than other parts of the skeleton (17), which is consistent with many other studies (4, 20, 44-49). Metacarpals and metatarsals showed a variable high success rate; however, they were consistently effective for mitochondrial sequencing (17). In general, smaller elements may be useful for when mostly intact skeletonized remains are recovered; however, additional skeletal elements would likely need to be genotyped when partial or fragmented remains are recovered and long bones may be necessary for the sorting and re-association of fragmented remains (17).

Environmentally Challenged Samples

Exposure of biological samples to extreme environmental conditions such as heat, humidity, and burial in soil for long periods of time affects the amount of nuclear DNA

able to be recovered, thereby making traditional STR typing and analyses difficult (1). Other insults that damage DNA include exposure to ultra-violet (UV) radiation, fire exposure, and submersion in water (50). The effects of fire on human bodies are strongly reliant on the level of heat and the duration of the fire (51, 52). Nevertheless, DNA has still been recovered from a variety of burned remains for identification purposes (53-58).

The quality and quantity of DNA recovered from submerged remains are heavily influenced by factors such as the type of water (saltwater vs freshwater), depth, and length of time submerged (1, 59). For example, for a case where remains were found after being at the bottom of the ocean for ~11 years, a complete STR profile was recovered (1). The lack of exposure to light, alkalinity (pH around 7.5-8.5), and low temperature of the water was believed to preserve the DNA (1). In contrast, remains that were found near a river dam after 3 years generated only a partial DNA profile (59). Other factors that can complicate the identification of submerged remains are post-mortem animal predation, tissue putrefaction and decomposition, and drifting/dragging of remains along the ocean floor (1).

Common Forensic DNA Issues and Solutions

When performing STR analyses from bone and other challenging samples, several difficulties may arise due to DNA damage, fragmentation, very low amounts of DNA available for amplification, DNA contamination, and/or inhibition. These conditions may result in the loss of loci and increase of undesired PCR artifacts due to stochastic effects during amplification (60-63). These artifacts must be taken into consideration when genotyping difficult forensic samples, as they can complicate data interpretation.

DNA Degradation/Damage

Multiple studies have reported reduced STR success due to: an increase in post-mortem interval (2, 4, 44), environmental damage (exposure to: increased temperatures, UV radiation, humidity, insects, animals, and microbes) (1, 4, 16, 20, 33, 34, 64), disposal conditions (buried in soil, partial/complete immersion in water, burning, or wrapping in plastic) (1, 4, 20, 33), and chemical damage (mortuary treatments such as embalming) (16, 19, 64). Nucleases such as DNase enzymatically cleave DNA into shorter fragments (65, 66) by hydrolyzing phosphodiester bonds, and are released endogenously from the cell or introduced exogenously from microbes or invertebrates in the surrounding environment (66, 67). The initial digestion of chromatin is facilitated by proteases following cell death, with DNA becoming susceptible to further digestion/fragmentation (66).

DNA can also be degraded spontaneously via non-enzymatic processes, such as through oxidation and hydrolysis; however, this happens at a much slower rate (66). Environmental damage due to high temperatures and humidity lead to strand breakage and base modifications, while chemical damage results in base transitions due to deamination (19, 64, 67). Crosslinking of DNA can occur either between DNA and proteins or between two DNA strands of the same helix (66, 67), the latter of which is temperature dependent (66).

Soil has been reported to affect DNA degradation and genotyping success (33, 68-70). Factors that influence the DNA degradation rates of biological samples recovered from soil include the presence and concentration of bacterial DNases, pH levels, moisture, and mineral content (33). Soils that have a higher humidity level generally have more microorganisms, and therefore increased DNase activity (33), as a majority of soil

microbes have nuclease enzymes (66). Additionally, warmer temperatures tend to increase DNase activity, greatly decreasing DNA quantity and quality (33). With this knowledge, it is not surprising that marshy soil has been shown to be more detrimental than sandy soil as it has a higher moisture content and consequently, microbial activity (33) and that depending on the conditions, buried samples may have a higher rate of DNA loss than surface-exposed samples (20, 70).

These forms of DNA damage may decrease the success of downstream PCR and quality of STR profiles. There are three main effects degradation can have on the amplification process: amplification failure, preferential amplification, and miscoding lesions (due to base modifications) (66). Current STR multiplexes include amplicons ranging from 60-450 basepairs (bp), but degraded DNA is often cleaved into smaller fragments (approximately <250 bp) (67), resulting in a reduced success of longer loci amplification (loci dropout) compared to smaller amplicons (66). Allelic dropout occurs when one of the allele pairs is preferentially amplified (typically the shorter one), making a heterozygote appear to be homozygous (66). Adjusting interpretation guidelines for degraded samples, such as generating composite profiles, can help accommodate allele drop-out (14). Miscoding lesions affect the amplified sequence by inducing the incorporation of the wrong base by the polymerase during extension (66, 67, 71), and if this occurs early in amplification, it can propagate throughout the PCR leading to an abundance of incorrect sequences (66). However, length-based analyses, such as STRs, are generally unaffected by miscoding (66) as long as no bases were inserted or deleted. One solution commonly applied to degraded samples is performing multiple amplifications of the same sample to confirm results and observe any differences (14, 66, 67). Nevertheless,

the storage of DNA in cooler temperatures or preservative solutions can overall help slow degradation processes by reducing or halting enzymatic activity and preventing further DNA damage events (1, 16, 66).

Low-Template DNA (LT-DNA)

Samples with low-template DNA are classified as having less than 100 pg DNA (66, 72) and/or when the allele calls consistently fall below the stochastic threshold (73). Bone samples often contain low amounts of DNA, and depending on the environmental conditions to which they were exposed, may also be highly degraded (7, 9). Other forensically relevant samples that commonly result in low amounts of DNA being recovered are cigarette butts (74, 75), hair (75-77), nails (74, 75), touched items (75, 78), weapons and explosives (78-81), and minor contributors in some DNA mixtures (78, 82, 83). Amplification of LT-DNA samples often results in various stochastic effects during PCR such as preferential amplification, allele/locus dropout, allele drop-in, and exaggerated stutter. Preferential amplification can result in unbalanced STR profiles, with low peak height ratios (PHR) between sister alleles at heterozygous loci (62, 63, 82, 84). Allele and/or locus drop-out is commonly observed in STR profiles from samples with low amounts of DNA and/or when DNA is highly degraded (60, 62, 63, 84-88). Allele drop-out can be attributed to extreme preferential amplification of one allele in a heterozygous pair, resulting in false homozygotes (63, 82), or both alleles can fail to amplify resulting in locus drop-out (63). Allele drop-in can also occur but is also more likely due to sporadic contamination (82, 86). Finally, stutter peaks may also be higher than expected (exaggerated stutter) (62, 63, 89), making profile interpretation difficult, especially with low-level mixtures (82).

Several approaches are commonly used to improve STR results from LT-DNA samples (11, 19, 60, 62, 63, 84, 86-88, 90). These include increasing the Taq polymerase concentration (19), splitting the sample and performing multiple amplifications to generate a consensus profile (19, 63, 66, 87), nested PCR (63, 66, 90), post-PCR cleanup or concentration, and increasing the injection time and voltage during capillary electrophoresis (62, 66). However, the most common strategy is to simply increase the number of cycles during PCR amplification, a process termed low copy number (LCN) typing (11, 19, 60, 62, 66, 84, 86, 87).

The robustness and reliability of results from LCN typing has been a subject of scrutiny in literature being described as unreliable, non-reproducible, and often practiced inconsistently or inappropriately (61, 63, 86, 91, 92). However, varying levels of success using LCN-typing has been reported (11, 19, 60-63, 84, 86-88). In general, it is agreed that PCR artifacts, such as heterozygous peak height imbalance or exaggerated stutter, are often more severe (60, 62, 63, 84, 86, 88). Increasing the number of PCR cycles from 28 to 34 using the AMFlSTR® SGM Plus™ PCR amplification kit enabled Kloosterman and Kersbergen to generate full profiles from bone and teeth extracts that had previously produced partial or no profiles when amplified with only 28 cycles (84). This six cycle increase with the same kit also allowed Gill et al. to generate full profiles from dilutions of control DNA as low as 25 pg with a decrease in stutter events, but an increase in stutter peak area and heterozygote peak imbalance (63). In another study, serially diluted DNA extracts with 100 pg of DNA that were amplified with 32 cycles showed stutter ratios as high as 63.8% (60). When applying this technique in a crime laboratory setting, thorough

validations need to be performed and the potential benefits must be weighed against any issues (technical, analytical, or legal) that may arise.

Contamination

Due to the nature of forensic evidence, items submitted for DNA testing can be contaminated with exogenous DNA from a variety of sources. Contamination may occur during sample collection, transportation from the scene to the laboratory, and/or handling processes due to improper personal protective equipment (PPE) and techniques (16, 93). DNA contamination can also originate from the collection source itself. For example, the comingling of multiple human remains introduces the potential for cross-contamination which compromises sample integrity (14).

Another source of DNA contamination is during the DNA extraction and pre-PCR sample handling steps (93). In order to reduce DNA contamination, wearing disposable PPE, sound laboratory practices and techniques, frequent cleaning, disposable consumables, appropriate storage conditions, and the physical separation of laboratories (DNA extraction, PCR set-up, and post-PCR) are all routinely employed (16, 86). Furthermore, DNA contamination is monitored within forensic laboratories via the inclusion of negative controls at all stages of testing (86), duplication of PCR amplifications when possible (14, 86), and comparison of results against staff elimination DNA databases (86).

Despite these measures, increased sensitivity in current STR kits has led to more instances of low-level contamination being detected (94). In 2016, the International Organization for Standardization developed a new ISO standard, ISO 18385:2016, which was implemented to regulate the post-production treatment of forensic consumables and

reagents used in pre-amplification workflows. With the goal of minimizing human contamination in these products, “forensic grade” consumables are now being offered by forensic manufacturers and used by laboratories.

PCR Inhibition

PCR inhibitors are substances that interfere with the DNA amplification process in some way, resulting in reduced or no amplification products (65, 69, 95). These agents can be organic (e.g. ethanol, humic acid, melanin) or inorganic (e.g. calcium ions) (65), are abundant in nature (found in foods, environment, and biological matrices), and can have a wide range of inhibitory effects depending on their concentration (65) and other factors. Although inhibition has been reported as the most common reason for failed amplifications when there is sufficient template DNA present (69), severe inhibition may also be misinterpreted as severe degradation in some cases (69, 96, 97).

PCR inhibitors may originate from the sample itself, its surrounding environment (e.g. humic acid in soil), or introduced during sample processing or DNA extraction (65). Common endogenous inhibitors that are often released during cell-lysis and co-extracted with DNA (69, 95, 98) include: proteinases in milk, polyphenol in foods, calcium ions and heavy metals in bones, collagen in tissues, melanin in hair and skin, and hematin in blood (48, 65, 69, 98). Inhibitors that may carry over from DNA extraction include: salts (NaCl), detergents (sarkosyl or SDS), EDTA, ethanol, isopropanol, phenol, mercaptoethanol, and DTT (65).

There have been several proposed mechanisms for PCR inhibition based on the agent (Table 1.1). These generally include the inhibitor binding to the DNA polymerase, interacting with template DNA, interfering with DNA polymerase during extension, and/or

binding to DNA polymerase cofactors (65, 69, 95). The presence of proteases and detergents (e.g. phenol) indirectly result in inhibition by degrading the DNA polymerase, while melanin directly inhibits by forming a reversible complex (65). Melanin also binds to the DNA molecule itself, inhibiting the DNA polymerase during extension. Although larger amplicons are usually preferentially affected by several forms of PCR inhibition, this is not always the case (95, 96). For example, studies have demonstrated that regardless of their inhibitory mechanism, increasing concentrations of inhibitors, such as calcium, collagen, and humic acid, generally had a greater effect on larger amplicons, which visually mimics DNA degradation in STR profiles (96, 99). However, for the inhibitors that bind to the DNA template (e.g. collagen and humic acid), smaller loci were also observed to be affected, with either reduced signal or complete drop out of the allele(s) (96, 99).

Calcium, collagen, hematin, and tannic acid interact with the DNA polymerase and inhibit its activity (65, 95). Humic acid and collagen interacts/binds with DNA, which prevents the enzymatic reaction from occurring, especially when the inhibitor binds at primer sites (65, 95). Higher primer melting temperatures can help decrease primer inhibition as it creates a strong bond with DNA and prevents the inhibitor from binding to the template (65, 95). Calcium in high concentrations competes with magnesium to bind with the DNA polymerase while tannic acid and EDTA chelate magnesium, depleting its availability during amplification (65). However, both result in a reduction of enzymatic activity as magnesium is a vital cofactor for DNA polymerase (65, 95).

TABLE 1.1 – *List of sources and mechanisms for common PCR inhibitors encountered in forensic DNA applications.*

Inhibitor	Source(s)	Mechanism(s)	Reference
Calcium	Bone	Inhibits DNA/Taq polymerase activity	(65, 69, 95, 96)
Collagen	Tissues	Inhibits DNA/Taq polymerase activity; binds with DNA template	(65, 69, 95, 96)
Detergents	DNA extraction	Denatures DNA polymerase	(65, 69)
EDTA	DNA extraction	Inhibits DNA polymerase activity (chelates Mg^{++} ions)	(65)
Hematin	Blood	Inhibits DNA polymerase activity	(65, 69, 95, 96)
Humic Acid	Soil	Binds with DNA template	(65, 69, 95, 96)
Melanin	Hair and skin	Binds with DNA template	(65, 69, 95, 96)
Nucleases	Endogenous, environment	Degrade template DNA	(65, 69, 95, 96)
Phenol	DNA extraction	Denatures DNA polymerase	(65, 69)
Proteinases	Foods	Degrades DNA polymerase	(65)
Salts	DNA extraction, environment	Degrade template DNA	(65)
Tannic Acid	Leather, plants	Inhibits DNA polymerase activity (chelates Mg^{++} ions), binds with DNA template	(65, 69, 95, 96)

Common strategies for inhibitor removal include performing a sample dilution or purification prior to amplification, using hot-start DNA polymerases that are less susceptible to inhibition, and/or adding extra DNA polymerase, bovine serum albumin (BSA), and/or magnesium to the PCR reaction (65, 69, 95). Although some of these removal techniques are effective, they may simultaneously decrease the amount of DNA

recovered or amplified (65, 95). For example, sample dilution reduces both the inhibitors and template DNA, and NaOH or silica column purification often result in a loss of DNA (65, 69, 95). Furthermore, BSA addition is effective against some inhibitors, such as humic acid and melanin, but not for EDTA, NaCl, SDS, calcium, or collagen (65). Despite the potential loss of DNA with silica column purifications, other silica-based extraction methods, such as the use of silica-coated magnetic beads, efficiently remove inhibitors (65, 69). Chaotropic salts, such as guanidinium thiocyanate, interfere with hydrogen bonds to increase the solubility of substances in an aqueous environment and are included in many commercial DNA extraction kits that use silica-based chemistries for DNA purification. They serve a dual purpose as they aid in both the denaturing of proteins and creating an environment for the silica substrate to selectively bind double-stranded DNA via an electrostatic and hydrophobic interaction (69, 100).

Traditional Forensic DNA Workflow

DNA Extraction

Organic DNA extraction methods are traditional, yet still commonly used DNA extraction techniques, as they are efficient at extracting high molecular weight DNA in large amounts (101). However, they involve several harmful chemicals (phenol/chloroform), are labor intensive, and present more opportunities for contamination due to buffer preparation and the transfer of the solution to multiple tubes (64, 102, 103). As an alternative to ethanol precipitation following a phenol-chloroform separation, DNA can be collected and concentrated using a Microcon®-100 filter (MilliporeSigma, Burlington, MA), which can also collect lower molecular weight DNA that may not have been recovered with an ethanol precipitation (104).

Chelex®-100 is an ion-exchange extraction method that uses a chelating resin to bind divalent ions, which are typically cofactors for enzymes that degrade DNA (DNases) (105). While Chelex® is a simple method that reduces contamination opportunities, does not involve toxic reagents, and yields a large quantity of DNA, this process does not include any 'clean-up' step, and therefore any inhibitors other than the chelated ions are not removed from the solution. In addition, the chelating agents (resin) themselves cause PCR inhibition if they are not completely removed prior to amplification due to chelating the Mg^{++} ions required for Taq DNA polymerase (105). Chelex® has been reported as being efficient at extracting more DNA than organic methods in many cases (106) but may not perform as well compared to newer DNA extraction methods such as commercial silica-based chemistries (33).

Solid phase extraction methods using silica-based membranes in spin columns, or silica-coated magnetic beads, are based on the premise that DNA preferentially binds to the silica at a low pH and in the presence of chaotropic salts (9, 103, 107-110). While the DNA is strongly bound, proteins and other cell debris are washed away. Once in a high pH buffer and no chaotropic salts, the DNA no longer binds and is eluted (111). Many commercial kits such as the QIAamp® DNA Investigator Kit (QIAGEN, Hilden, Germany), PrepFiler® BTA Forensic DNA Extraction Kit (Thermo Fisher Scientific, Carlsbad, CA), E.Z.N.A.® Forensic Extraction Kit (Omega Bio-tek Inc., Norcross, GA), and the DNA IQ™ System (Promega Corporation, Madison, WI) have been produced specifically for the forensic market. Several studies have used commercial kits to extract DNA from a variety of forensic samples, such as bones/teeth (9, 100, 103, 112-117), and

body fluids and tissues (2, 3, 33, 105, 116, 118), with many reporting comparable or improved DNA recovery over the standard organic or Chelex methods (33, 100, 105, 112).

DNA Quantification

Quantitative real-time PCR, or qPCR, is the standard method used in forensics that allows for the determination of the concentration of DNA within a sample (119, 120). Several commercial qPCR kits have been designed specifically for the forensic community. These include Quantifiler® Trio (Thermo Fisher Scientific), Investigator® Quantiplex® Pro (QIAGEN), Investigator® Quantiplex® Pro RGQ (QIAGEN), PowerQuant® (Promega), and InnoQuant® kits (InnoGenomics) (Table 1.2). These kits determine several sample quality metrics, such as the amount of human autosomal and male DNA present in an extract, presence of inhibitors, and the degree of DNA degradation (118). The relative concentration of autosomal and male targets can also allow for the screening of female:male mixtures (118), which is especially helpful for sexual assault samples and the determination of the appropriate amplification strategy. For example, if there is a trace amount of male DNA in the presence of a high female DNA background, Y-STR typing may be the most appropriate genotyping method.

TABLE 1.2 – *Summary of commercial qPCR kits commonly used in forensics*.

Kit Name	Manufacturer	Overview of Targets
Quantifiler Trio	ThermoFisherScientific	Short autosomal (80 bp), Long autosomal (214 bp), Short male gonosomal (75 bp), IPC (130 bp)
Quantiplex Pro	QIAGEN	Short autosomal (91 bp), Long autosomal (353 bp), Short male gonosomal (81 bp), IPC (434 bp)
Quantiplex Pro RGQ	QIAGEN	Short autosomal (91 bp), Long autosomal (353 bp), Short male gonosomal (81 bp), Long male gonosomal (359 bp), IPC (434 bp)
PowerQuant	Promega	Short autosomal (84 bp), Long autosomal (294 bp), Short male gonosomal (81-136 bp), IPC (435 bp)
InnoQuant	InnoGenomics	Short autosomal (80 bp), Long autosomal (207 bp), IPC (172 bp)

*IPC = Internal Positive Control

During forensic DNA workflows, the presence of inhibitors is most commonly predicted during DNA quantification (69). This is accomplished using PCR efficiency calculations and/or co-amplifying an internal positive control (IPC) (69). Although most DNA quantification and STR-typing chemistries have been optimized to be tolerant to a wide range of inhibition, some inhibitors may not have the same effect on the IPC target and downstream STR amplicons (69); for example, a sample might flag as inhibited during qPCR, but show no effects of inhibition during STR-typing, and vice versa. For low template samples that contain PCR inhibitors, the volume of DNA extract used for PCR amplification is generally much higher than for qPCR (15 µL vs 2 µL), and therefore, the effects of the inhibitors are compounded during PCR (120). In contrast, heavily inhibited samples that are flagged as such after qPCR are typically diluted prior to amplification, which dilutes out inhibitors, but also DNA as well (65, 69, 96).

Quantifiler® Trio was the first commercially available qPCR kit designed to assess DNA degradation in a sample prior to genotyping (116). Two autosomal targets are included in the reaction: one short (80 bp) and the other long (214 bp) (118). Because longer DNA targets are more susceptible to degradation, the calculation of their relative amplification (short divided by long) gives a degradation index (DI) (116, 118). Several studies have reported DI values as being generally indicative of the degree of DNA degradation (116, 118, 122, 123). The Quantifiler® Trio handbook reports that a sample is likely not degraded with a DI <1, slightly to moderately degraded with a DI of 1-10, and severely degraded with a DI >10 (124). However, Vernarecci further breaks this down into slightly different DI categories: 0-1.5 is non degraded, 1.5-4 is mildly degraded, 4-10 is degraded, and >10 is severely degraded (116). As with the detection of mixtures, knowing the level of DNA degradation prior to genotyping can also help analysts determine the most effective downstream PCR chemistry/workflow to use to generate the most probative information and save time and resources (116, 118).

Investigator® Quantiplex® Pro RGQ is a DNA quantification kit that has been designed to pair with downstream genotyping kits, such as the Investigator® 24plex QS kit. It is a unique expansion of the Investigator® Quantiplex® Pro kit in that it includes a fifth target; an additional male target that can indicate the level of degradation of any male DNA in the reaction. This kit allows for the simultaneous detection of human autosomal and Y-chromosomal degradation, the ability to monitor inhibition with its Internal Control (IC), and maintaining sensitivity, especially for male DNA in the presence of high amounts of female DNA (e.g. post-coital vaginal swabs).

STR Typing and Analysis

The use of STRs for genotyping individuals is commonly known as genetic fingerprinting (54). STR markers are regions of DNA that are highly polymorphic and are abundant in the non-coding regions of the human genome (125). They consist of two to six basepair repeats, with four basepair (tetrameric) markers being most commonly used in forensic analyses due to their higher resistance to strand slippage of the polymerase (stutter) (5). Over the past 26 years, many commercial multiplex PCR kits have been designed to co-amplify 3-27 STR loci ranging from 60-500 bp (7, 126-129). With the expansion of the FBI CODIS core loci from 13 to 20 markers (plus amelogenin) in 2017, forensic companies had to design new STR kits to accommodate the additional core loci (Table 1.3) (130, 131).

TABLE 1.3 – *List of commercially available STR kits with the expanded CODIS core loci.*

STR Kit Name	Manufacturer	No. Amplicons	Special Loci/Features
GlobalFiler/Global Filer Express	ThermoFisher Scientific	24	10 mini-STRs, SE33, Y-INDEL and DYS391
GlobalFiler IQC	ThermoFisher Scientific	25	10 mini-STRs, SE33, Y-INDEL, DYS391, and Internal quality sensors (IQC system)
VeriFiler Plus	ThermoFisher Scientific	26	11 mini-STRs, Y-INDEL, Penta D, Penta E, D6S1043, and Internal quality sensors (IQC system)
Investigator 24plex QS/GO!	QIAGEN	24	SE33, DY391, and Internal quality sensors (QS markers)
Investigator 26plex QS	QIAGEN	26	D6S1043, Penta D, Penta E, DY391, and Internal quality sensors (QS markers)
PowerPlex Fusion	Promega	24	Penta D, Penta E, DYS391
PowerPlex Fusion 6C	Promega	27	SE33, Penta D, Penta E, DYS391, DYS576, DYS570

The GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific) is commonly used in forensic laboratories and is a 6-dye STR kit with 24 loci, of which 10 are mini-STRs (<250 bps) (131, 132). It is highly sensitive with a validated target DNA input of 0.5-1 ng (depending on the cycle number used); however, several studies have reported full DNA profiles being produced from casework-type samples and diluted control DNA (pristine and spiked with inhibitors) with as little as 100 pg DNA (122, 131, 133). In addition, GlobalFiler® also includes several male targets (a Y-INDEL and DYS391) to monitor drop-out of the Amelogenin Y-allele (131, 132) due to mutations that result in a null allele.

The Investigator® 24plex GO! and Investigator® 24plex QS (QIAGEN) kits are designed to STR-type reference (buccal swabs and FTA® cards) and casework samples, respectively (130). In addition to the expanded CODIS core loci, Amelogenin, and two additional loci (SE33 and DY391), these STR kits also include two quality sensor markers that serve as an internal PCR control (130, 134, 135), similar to those included in many DNA quantification kits. Relative to the 74 bp QS1 (‘Q’) marker, the signal of the 435 bp QS2 (‘S’) marker tends to significantly decrease in height (or drop out) with PCR inhibition (130, 134, 135). However, in the presence of extreme inhibitor concentrations, both markers may completely fail to amplify. The observed difference in height or fluorescence (in relative fluorescent units; RFUs) of the QS markers can often assist in differentiating STR profiles that are of poor quality due to PCR inhibition, DNA degradation, failed amplification, or absence of DNA template (130, 134, 135) (Fig. 1.1). The information provided by the QS markers in the 24plex QS kit and the quality flags in the RGQ kit during DNA quantitation should be consistent with each other, and accurately reflect the

quality of the sample and resultant STR profile. Together, these data could ideally direct DNA analysts towards the most appropriate rework strategies as needed. Other commercial STR kits that integrate one or more quality control markers within the PCR multiplex include the Investigator® Argus X-12 QS, Investigator® Argus Y-12 QS, Investigator® ESSplex SE QS, and Investigator® 26plex QS kits from QIAGEN, and the GlobalFiler® IQC, VeriFiler® Plus, and NGM Detect® kits from Thermo Fisher Scientific.

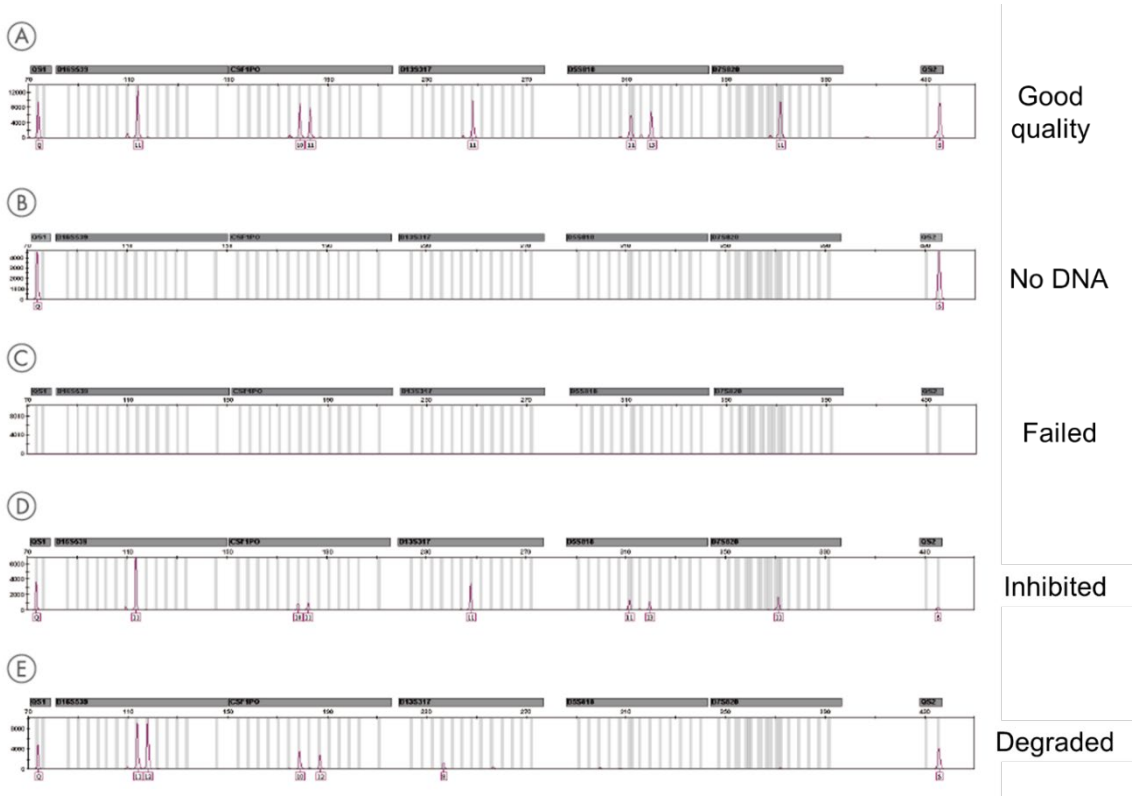

FIG. 1.1 - *Examples of how QS markers behave with different DNA sample types.* ***A) Confirmed successful PCR amplification.*** *Small amplicon Quality Sensor peak (QS1) and large amplicon Quality Sensor peak (QS2) appear at similar heights. Sample allele peaks have balanced height across the profile.* ***B) Confirmed successful PCR amplification but absence of DNA.*** *QS1 and QS2 appear at similar heights. No sample allele peaks appear.* ***C) Failed PCR amplification.*** *Lack of QS1 and QS2. No sample allele peaks appear.* ***D) Inhibited DNA.*** *QS1 with normal peak height and QS2 with decreased peak height can be seen if inhibitors are affecting PCR. Sample allele peaks for the markers show decreasing height towards the larger markers.* ***E) Degraded DNA.*** *QS1 and QS2 appear at similar heights. Sample shows allele peaks for the STR loci with decreasing height towards the larger STR loci. Sourced from: Investigator® Quality Sensor Technical Information (QIAGEN) (136).*

Due to the absence or extremely low amounts of amplifiable nuclear DNA in highly degraded skeletal remains (especially ancient remains), mitochondrial typing is commonly used as a last resort for HID when STR analysis fails (4, 7, 14, 19, 66). Most laboratories that sequence mtDNA focus on either hypervariable regions 1 and 2 (HVI/HVII) in the control region or on the coding region (25, 137). However, due to the maternal inheritance of mitochondrial DNA, the power of discrimination is much lower than STR typing (7, 19, 66). It is therefore preferable to perform autosomal STR typing for HID purposes when possible. When LCN techniques were applied to the remains of missing military service members from WWI, WWII, and the Vietnam War, STR typing was able to provide gender determination, separate co-mingled remains, and resulted in the "first identification from the Vietnam War" using nuclear DNA (19).

Although STR markers are commonly used in forensic analyses, the larger amplicon markers (>200 bp) are susceptible to allelic and/or locus drop-out in degraded samples (138). This results in incomplete profiles and therefore a lower power of discrimination. One of the most common alternate markers used are SNPs as they currently are used to supplement STR data (to aid with mixture interpretation, for example), with the potential of total replacement (23, 26, 139). While small amplicon sizes are attractive for degraded samples (24-26), it has been shown that approximately 50 SNP loci are needed to achieve the same discriminating power as 15 STR loci (23, 24, 140). However, small groups of linked SNPs within STR loci, called microhaplotype loci (microhaps), are a new and powerful type of forensic marker (139, 141). Microhaps are most relevant for lineage determination and mixture deconvolution (139). Another alternative method for typing degraded or low-template samples would be to use insertion/deletion (INDEL)

polymorphisms (138, 142, 143). Similar to SNPs (140), these bi-allelic markers are widely found throughout the genome, have a low mutation rate, are able to be multi-plexed, can have a high power of discrimination for human identification, and have small amplicon lengths (<200 bp), which make them ideal for degraded samples (138, 142, 143).

STR typing is most commonly performed with capillary electrophoresis-based methods (141, 144-146); however, massively parallel sequencing (MPS) is quickly becoming an attractive alternative. MPS technology allows for millions of DNA strands/fragments to be sequenced simultaneously (141, 144-146). Additionally, the use of barcodes allows multiple samples to be pooled together in a single run (146). Compared to Sanger sequencing, MPS increases sample throughput while decreasing the cost per sample (144). With traditional CE-STR kits, only a small number of loci (25-30) are able to be multiplexed, dye artifacts arise, and degraded samples may still result in no or partial profiles, even with mini-STR kits (144, 146). With MPS, alleles of similar/identical lengths can be distinguished based on the template or primer sequence without having to separate loci on different dye channels or moving primer locations (141). This allows multiplexed loci to have overlapping amplicon sizes, resulting in the smallest amplicon length possible, which is most beneficial for degraded samples (141, 144, 145). Also, the sequencing of STRs that contain SNPs increase the discriminatory power of that locus (141, 145). While PCR artifacts such as stutter and heterozygote imbalance are still present (141, 146), sequencing makes their identification simpler (145). For example, sequence variants can help distinguish stutter peaks from minor allele contributor peaks (144). While there are many advantages of MPS and the use of other genetic markers, these alternate methods were not investigated during this research.

Automation

Automation plays a key role in most forensic DNA laboratories due to the demand for a high throughput of samples. Some platforms such as the QIAgility (QIAGEN) are designed as a liquid handling instrument for setting up PCR reactions and CE plates. Other larger platforms such as the Hamilton Microlab® STARlet robot, the QIAsymphony® (QIAGEN), or TECAN systems (e.g. Fluent® or Freedom EVO®), can perform customized combinations of functions such as liquid handling, DNA extraction, and punching samples from FTA® cards, for a fully hands-free setup of databasing samples.

There are several automated DNA/RNA extraction platforms available, including the EZ1 and EZ1 Advanced XL and QIAcube (both QIAGEN), Maxwell 16 (Promega), and the iPrep and AutoMate Express (Thermo Fisher Scientific) (103, 105, 110, 114, 147, 148). These systems allow for multiple samples (up to 16) to be processed in under an hour. Many studies have shown that these platforms routinely generate high amounts of DNA from a wide range of forensically relevant samples for STR-typing (103, 105, 110, 114, 147, 148). However, some studies have also reported substantial losses of DNA when extraction is performed on an automated platform (147). A study comparing the extraction of body fluids on the Biorobot EZ1 platform versus a manual organic method reported a 30-40% decrease in DNA yield with automation, but this did not affect STR success or profile quality (147). In addition, some studies have also reported higher levels of DNA degradation and allelic drop-in in STR profiles of samples extracted using automated methods compared to other manual methods (103, 110, 114). Therefore, precious or LT-DNA samples (such as bone), are traditionally not processed using automated processes due to reduced control and potential DNA loss. Although the ICMP have implemented

automated DNA extractions using the QIAcube for their bone samples (110), most laboratories performing DNA extractions prefer to use manual methods such as a total demineralization followed by an organic or commercial kit DNA purification (10, 107, 149, 150). Even though automated DNA extractions may not always yield higher amounts of DNA (105, 110, 147), automation may greatly reduce the processing time per sample, costs, variation, and the risk of contamination and human error (13, 103, 105, 110, 114, 147, 148).

DNA Extraction from Skeletal Samples

For all forensic samples it is important that DNA extraction methods recover as much DNA as possible (14) while simultaneously eliminating unwanted contaminants and PCR inhibitors. This is especially true for methods extracting DNA from LT-DNA and degraded skeletal material (14). Early DNA extraction procedures were labor-intensive, time-consuming, costly and relatively inefficient (9, 109), but over the past two decades several DNA extraction methods have been developed as faster, simpler, and more effective kit-based technologies, which also have the potential for automation (11, 103, 105, 108, 110, 114, 147, 151). However, despite some advancement in commercial DNA extraction kits, DNA purification is still relatively difficult and time consuming for bone and tooth samples. Many forensic laboratories that process bone samples tend to develop custom in-house protocols for bone preparation, digestion buffer constituents and incubation combinations, and DNA purification methods. However, regardless of the approach, all methods include general bone preparation, digestion/lysis/demineralization of the tissue, followed by purification, precipitation/elution, and concentration of DNA from the lysate.

Sample Processing and DNA Purification

Bone preparation consists of cleaning the external surface of the bone (via washing, UV radiation, and/or sanding), cutting the bone into smaller pieces (if necessary), and crushing the bone into powder with liquid nitrogen (9, 22, 64, 109, 152, 153). While finely-ground bone powder often yields sufficient amounts of DNA (9, 10, 12, 44, 103, 107, 109, 110, 113-115, 154-158), there is a risk of sample contamination and potential hazard to the processor from airborne powder (14, 64, 109, 158). Additionally, this process is more time consuming, requires specialized training (14), and equipment and supplies such as grinding vials, freezer mills, and liquid nitrogen. It must also be noted that powdering the bone tissue also destroys the bone sample. As a result, many studies have explored less-destructive sample preparation and DNA extraction methods from whole bone and teeth (22, 152, 156, 158). This approach is most desirable in ancient DNA applications to preserve museum exhibits and ancestral remains (22, 152), but can also be applied to contemporary samples (156, 158).

One study in 2004 by Rohland et al. demonstrated that digestion of ancient bone and teeth in a guanidinium thiocyanate (GuSCN) buffer for a week, followed by silica purification of the surrounding buffer solution, resulted in a 93% success rate for teeth and 67% for bone with mitochondrial DNA typing (22). It was noted that there was no visible damage to the samples, only that they were cleaner than before (22). Due to the hazardous nature of GuSCN and the lack of amplifiable nuclear DNA in Rohland's 2004 study, Bolnick et al. implemented a previously published complete demineralization protocol (109) with the exception of using whole bone versus powdered bone (152). The demineralization step was performed overnight at room temperature to reduce further DNA

degradation and prevent decalcification to preserve the integrity of the archaeological/museum samples (152). While the teeth tested remained intact, the bone fragment that was tested partially disintegrated and smaller fragments completely dissolved (152). Although this may not be desired for historical or museum samples, the increased DNA yield provided through total demineralization may be considered essential when extracting DNA from challenging skeletal samples for human identification.

Many forensic laboratories that perform DNA testing on human remains, such as the Armed Forces DNA Identification Laboratory (AFDIL) use a version of a complete demineralization digestion protocol (10, 12, 107, 155). Demineralization is the process of removing mineral ions, such as calcium in bone samples to allow cellular material containing DNA to be released (12). DNA is found in the osteocytes of bone, which are housed in a calcified bone matrix with approximately 20,000-26,000 osteocytes/mm3 (12, 43, 159, 160). Approximately 70% of the mineral portion of bone is composed of hydroxyapatite (calcium carbonate, calcium phosphate, calcium citrate, etc.) (4, 10).

While this arrangement protects the DNA from the environment, it also makes it difficult to access the DNA during the extraction process (12). Therefore, decalcification (demineralization) is needed to free the osteocytes by breaking down the matrix into calcium ions and other components (12). Demineralization extraction buffers generally contain high concentrations (0.5 M) of ethylene diamine tetra-acetic acid (EDTA) for this purpose, and to chelate the released calcium cations, which aids in the inactivation of DNAses (10). Although total demineralization releases more osteocytes, it also frees more of the calcium products, which are PCR inhibitors (10). The concentration of the EDTA

and the ratio of bone tissue to buffer volume are thought to influence DNA extraction efficiency (10, 109, 114).

Loreille et al. reported that when total demineralization was performed with 15 mL of 0.5M EDTA per gram of bone power, a significantly higher DNA yield (228 times more DNA/g bone powder), and a lower amount of co-extracted PCR inhibitors was obtained when less bone powder was used (0.2 g vs 1-2 g of bone powder) (10). When AFDIL implemented their new DNA extraction protocol with a reduced amount of powdered bone (0.2 g compared to 2.5 g), an increase in the number of submissions to identify skeletal remains' fragments was observed (17). With this decrease in the minimum amount of bone powder required, DPAA scientists were able to process previously "untestable" materials (17).

In early 2019, Edson reported a comprehensive evaluation of human remains' testing performed at AFDIL from 1990 to mid-2018 and suggested that sampling strategies should take into consideration a greater applicability and more testing platforms, such as next-generation sequencing (NGS) or massively parallel sequencing (MPS), mitochondrial sequencing, and STR platforms (17). During that evaluation study, four main extraction methods were used by AFDIL for fully skeletonized remains with post-mortem intervals (PMIs) of 40-100 years: 1) an incomplete demineralization followed by an organic purification; 2) a complete demineralization followed by an organic purification; 3) a complete demineralization followed by an inorganic purification (QIAquick PCR purification kit); and 4) a protocol designed specifically for NGS testing (17). Additionally, the success of each of these methods was compared across five different DNA typing methods (if/when applicable): 1) mitochondrial DNA sequencing (traditional Sanger

sequencing); 2) modified Y-Filer STR typing; 3) MiniFiler STR typing; 4) PowerPlex Fusion STR typing; and 5) NGS (17).

The overall findings of the study revealed that rather than focusing on which skeletal elements yield the highest DNA amounts and genotyping success, choosing the most effective combination of extraction and DNA typing methods was the most important factor to consider (17), which has also been generally suggested before (114). For example, while the complete demineralization and inorganic purification method generated the best results for all STR platforms, the complete demineralization with an organic purification produced better results when mitochondrial sequencing was performed, regardless of the skeletal element tested (17). Additionally, when evaluating different sample insults, it was shown that both of the complete demineralization protocols resulted in the most complete STR profiles from chemically contaminated remains such as those recovered from the USS Oklahoma (oil soaked remains) (17).

In 1986, Weiner and Price first described the presence of crystal aggregates of bone matrix (161). These fused crystals were shown to be resistant to oxidizing agents, such as sodium hypochlorite (NaOCl) (153, 161). A later study revealed that the DNA fragments contained within isolated crystal aggregates were well preserved and less degraded than DNA extracted from whole bone powder (153). It was therefore suggested that total demineralization may allow for the enhanced release of crystal aggregates that contain large fragments of DNA (10). By using less bone powder, the buffer to sample ratio is much higher, allowing for more efficient demineralization and chelation of calcium ions, and therefore, the extraction of more DNA from the crystals and less inhibitors (10, 109). Other studies have also confirmed that complete decalcification increases DNA yield and

STR success from skeletal samples compared to using an extraction buffer with a much lower concentration of EDTA (9, 70, 109, 110, 114, 155). While these methods are able to extract high quantities of DNA, they are time-consuming (24 to 120 hours) and require a significant amount of sample handling (9, 10, 64, 107, 155).

In 2010, a new bone digestion kit, TBone EX kit (DNA Chip Research Inc., Tokyo, Japan), was developed that releases DNA from whole bone chips to eliminate the need to powder the bone tissue (156). Digestion of a whole bone fragment followed by a silica-based purification yielded approximately three times more DNA than those samples demineralized and purified using an organic method (156). With the non-powdering protocol, three out of four femur fragments resulted in full profiles with a traditional STR kit, and the fourth provided a full profile when a mini-STR kit was used (156). In addition, the bone chips were not totally consumed during the first round of extractions with the TBone kit, and therefore provided an opportunity to perform further extractions from the same chip rather than consuming additional bone samples (156).

A complete demineralization digestion step can be coupled with any DNA purification method. Several studies have shown that silica-based extraction from degraded bone samples reduced the amount of inhibition in the extract and increased the DNA yield and number of full profiles compared to a standard phenol/chloroform method (11, 157, 162). Both silica-based spin columns and magnetic bead filtration extraction methods also have the desired ability to be automated (11, 103, 105, 110, 113, 114, 147).

Statement of the Problem

Since the attacks on the World Trade Center in 2001, the forensic DNA community has focused on improving different methods for the identification of challenging forensic samples and skeletal samples in particular. In general, the entire process of DNA profiling from skeletal samples is time consuming and requires some specialized equipment and training, and therefore some innovative solutions to make these processes faster and easier could benefit forensic laboratories.

Additionally, manufacturers can only access limited types of samples when performing developmental validations of their products, and most forensic laboratories do not have the time or resources to stress-test new products. As a result, published works from independent researchers with access to unique and challenging sample types, and the ability to explore the wider application and performance of commercial products, are relied upon as unbiased data to support the adoption of new methodologies within the medicolegal community.

This research focused on providing alternative solutions to problems commonly encountered throughout forensic DNA workflows by evaluating and optimizing protocols for sample processing, DNA extraction, and STR typing for the identification of human skeletal remains and other challenging biological samples. The overall work was performed in four parts. Phase 1 investigated the efficiency of an alternate bone processing and DNA extraction method (TBone Ex kit) for a variety of bone samples. The TBone Ex buffer eliminates the entire bone powdering process, and also allows for a second round of extractions from the same bone sample. In phase 2, a whole-bone digestion method using a commercial DNA extraction kit was investigated. Parameters modified included: bone

chip size and number and digestion time. Phase 3 examined the effect of increasing the number of PCR cycles in a commercial STR kit when using two different extraction methods to purify DNA from environmentally challenged bone and tooth samples. Finally, Phase 4 explored the application of Quality Sensor (QS) markers in determining the most effective re-work strategies for database and casework samples, including skeletal samples.

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CHAPTER II

Evaluation of a Powder-free DNA Extraction Method for Skeletal Remains[1]

This dissertation follows the style and format of *Journal of Forensic Sciences*.

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Abstract

Bones are often recovered in forensic investigations, including missing persons and mass disasters. While traditional DNA extraction methods rely on grinding bone into powder prior to DNA purification, the TBone Ex buffer (DNA Chip Research Inc.) digests bone chips without powdering. In this study, six bones were extracted using the TBone Ex kit in conjunction with the PrepFiler® BTA™ DNA extraction kit (Thermo Fisher Scientific) both manually and via an automated platform. Comparable amounts of DNA were recovered from a 50 mg bone chip using the TBone Ex kit and 50 mg of powdered bone with the PrepFiler® BTA™ kit. However, automated DNA purification decreased DNA yield ($p < 0.05$). Nevertheless, short tandem repeat (STR) success was comparable across all methods tested. This study demonstrates that digestion of whole bone fragments is an efficient alternative to powdering bones for DNA extraction without compromising downstream STR profile quality.

Keywords: forensic science; forensic biology; DNA; bone; short tandem repeats; automation

Evaluation of a Powder-free DNA Extraction Method for Skeletal Remains

Introduction

In order to generate a high-quality short tandem repeat (STR) profile, it is optimal to extract a sufficient quantity of high quality DNA (>100 pg without low-template techniques (1-3)) while simultaneously eliminating unwanted contaminants and PCR inhibitors. DNA extraction procedures can be labor-intensive, time-consuming, include toxic chemicals, and be relatively inefficient (4, 5). Over the past two decades, several DNA purification methods have developed as faster, simpler, and more effective kit-based technologies with the potential for automation (6-13). However, despite some advancement in commercial DNA extraction kits, DNA extraction is still relatively time consuming for skeletal remains due to lengthy sample preparation including cleaning, processing, and the requirement for crushing bone into a fine powder prior to digestion (4, 5).

Bone preparation consists of cleaning the external surface of the bone (via washing, UV radiation, and/or sanding), cutting the bone into smaller pieces (if necessary), and crushing the bone into a powder with liquid nitrogen in a blender cup or a freezer mill (4, 5, 14-18). While finely-ground bone powder generally yields type-able amounts of DNA (4, 5, 7, 12, 13, 18-28), it destroys the sample, increases the risk of contamination, and creates a potential hazard to the processor from airborne powder (4, 17, 27). Therefore, several studies have explored non-destructive extraction methods from whole bone and teeth (14, 16, 23, 27). This approach is most desirable in ancient DNA applications to preserve museum exhibits and ancestral remains (14, 16), but could also be applied to contemporary and forensic casework (23, 27).

Many forensic laboratories that perform DNA testing on skeletal remains use a version of a complete demineralization digestion protocol (18, 20-22). Demineralization is the process of removing mineral ions, such as calcium in bones, to allow the release of DNA from cellular material (22). Demineralization extraction buffers generally contain high concentrations of ethylene diamine tetra-acetic disodium salt (EDTA) to chelate the free Ca^{++} and Mg^{++} cations, which aids in the inactivation of DNases (18). Weiner and Price (29) first described the presence of crystal aggregates in bone matrix, which were later thought to house and protect sources of DNA (in osteocytes) from environmental degradation (18). These aggregates could therefore be a valuable source of DNA that could be released (15, 18) during a complete demineralization step. Several studies have reported that complete decalcification increases DNA yield and STR profile quality (number of reported alleles, peak heights, peak height ratios, etc.) from skeletal remains compared to using an extraction buffer with a much lower concentration of EDTA (4, 5, 12, 13, 20, 30). A complete demineralization digestion step can be coupled with any DNA purification method such as organic or silica-based methods.

The TBone Ex kit (DNA Chip Research Inc.) is a bone digestion buffer system that was developed to release DNA from whole bone chips rather than finely powdered bone tissue (23). The bone chips may not be totally consumed during the first round of extractions with the TBone Ex kit and therefore could provide an opportunity to perform further extractions from the same chip rather than consuming additional bone (23). Although the TBone Ex kit can be coupled with many commercial DNA extraction kits, the authors chose to use a DNA extraction system commonly used in forensic laboratories

(PrepFiler® BTA™ Forensic DNA Extraction Kit automated on the Automate Express™ platform (Thermo Fisher Scientific).

Automation plays a key role in most forensic DNA laboratories. There are several automated DNA extraction platforms available that allow for multiple samples to be processed in under an hour (6, 7, 11-13, 31). Although automated DNA extractions may not always yield higher amounts of DNA (6, 11, 13), automation can greatly reduce the processing time per sample, decrease run-to-run variation, and minimize the risk of contamination and human error (6, 7, 11-13, 31). Traditionally however, precious bones are not subjected to automated processes due to reduced control and concern for DNA loss.

This project investigated the efficiency of extracting DNA from whole bone chips using a powder-free digestion method (TBone Ex kit). We compared DNA yield and STR profile quality when bone chips were processed using the TBone Ex Kit and when bone powder was extracted using a total demineralization protocol (18) or a commercial DNA extraction kit commonly used in forensic laboratories (PrepFiler® BTA™ Forensic DNA Extraction Kit, with and without automation).

Materials and Methods

Sample Preparation

Bone samples (n=6) were collected from human cadavers exposed to various environmental insults at the Applied Anatomical Research Center (AARC) [previously named Southeast Texas Applied Forensic Science (STAFS) Facility] at Sam Houston State University (Table 1). For bones that were sampled prior to complete skeletonization, desiccated connective tissue was removed directly with a scalpel or via maceration. The surface (1 mm) of each bone was sanded and cut into ~600 mg chips (3-5 mm^2) or bone

shavings (~50 mg each) from the same region using a Dremel® tool (Dremel, Racine, WI, USA) before being washed with a series of 5 min washes of the following: 1% NaOCl three times, autoclaved distilled water three times, and a final 100% ethanol wash. The bone chips were dried in an oven overnight at 30°C. The bone chips were crushed in a SPEX 6750 Freezer/Mill® (SPEXSamplePrep, Metuchen, NJ, USA) using the following conditions: 10 min cooling period followed by two cycles of one min crushing with a two min break between the crushing cycles. The bone shavings (50 mg) proceeded directly to extraction using the TBone Ex kit (DNA Chip Research Inc., Tokyo, Japan). An extraction blank was performed by wetting a cotton swab with sterile water and swabbing the inside of the freezer-mill tubes.

DNA Extraction

All bones (n=6) were extracted in triplicate, yielding a total of 18 extracts for each of the five methods. The swab extraction blank and a reagent blank were both processed in parallel with each extraction procedure to monitor contamination.

One set of bone powders was extracted manually according to the PrepFiler® BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific, Waltham, MA) following the Bone and Tooth protocol with 50 mg bone powder (32). Another 50 mg of the same bone powder was extracted using the PrepFiler® Express™ BTA™ Forensic DNA Extraction Kit on the Automate Express™ platform (Thermo Fisher Scientific) (33).

All 50 mg bone shavings were demineralized and digested according to the TBone Ex Bone Decalcification Kit protocol as previously described (23) followed by DNA purification using the PrepFiler® BTA™ Forensic DNA Extraction Kit manually or automated on the Automate Express™ platform (32, 33).

TABLE 2.1 - *Source information and DNA quantity for the six bone samples used in this study. Data shows the average DNA concentration (ng DNA/mg bone) and the coefficient of variance (CV) for each bone sample. Each sample was extracted in triplicate.*

Sample	Bone	Conditions of Remains	Total Demineralization		PrepFiler® BTA™ Manual		PrepFiler® BTA™ AutoMate		TBone Ex + PrepFiler® BTA™ Manual		TBone Ex + PrepFiler® BTA™ AutoMate	
			Mean (ng DNA/ mg bone)	CV (%)	Mean (ng DNA/ mg bone)	CV (%)	Mean (ng DNA/ mg bone)	CV (%)	Mean (ng DNA/ mg bone)	CV (%)	Mean (ng DNA/ mg bone)	CV (%)
1	Femur	Advanced Decomposition	0.018	3.46	0.013	14.11	0.009	24.76	0.010	17.96	0.004	22.49
2	Tibia	Skeletonized	0.035	9.79	0.025	10.10	0.014	6.22	0.036	85.54	0.007	17.79
3	Femur	Skeletonized	0.054	24.82	0.033	13.92	0.022	13.11	0.039	7.96	0.018	58.95
4	Tibia	Advanced Decomposition	0.361	14.37	0.298	15.88	0.227	2.60	0.182	13.28	0.034	48.76
5	Femur	Severely Burned	0.544	9.31	0.373	22.14	0.270	5.83	0.543	27.17	0.132	64.02
6	Humerus	Embalmed	3.224	15.98	0.756	24.93	0.166	20.59	0.500	95.98	0.010	62.39
Average			**0.71**	**12.96**	**0.25**	**16.85**	**0.12**	**12.19**	**0.22**	**41.32**	**0.03**	**45.73**

For comparison purposes, a final set of powdered bone (50 mg) was extracted according to a previously published purification protocol using complete demineralization and MinElute® PCR Purification columns (QIAGEN, Hilden, Germany) (34) with an incubation time of 20 hours and centrifugation at 2500 x g for 23 mins during the initial concentration step. All extracts were eluted in 50 μL.

DNA Quantification and STR Analysis

The amount of DNA in each extract was determined via quantitative real-time PCR (qPCR) using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific) as per manufacturer's instructions (35) on a 7500 Real-Time PCR System (Thermo Fisher Scientific). Data were accepted with an R^2 value of 0.99 or above.

All extracts were amplified using the GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific) with a target of 0.8 ng (when available) as per manufacturers' protocol on a GeneAmp® PCR System 9700 (Thermo Fisher Scientific) (36). Detection of amplified products was performed using the 3500 Genetic Analyzer (Thermo Fisher Scientific) with a 36-cm capillary and POP-4 polymer (Thermo Fisher Scientific). DNA profiles were generated using GeneMapper ID-X v4.1 software (Thermo Fisher Scientific) with an analytical threshold of 150 relative fluorescent units (RFUs) and stochastic threshold of 600 RFUs.

Statistical Analysis

Parametric assumptions were tested for using a Shapiro-Wilk's W test. Data were tested for statistical significance by ANOVA single factor, followed by Tukey HSD *post hoc* comparisons when appropriate. $p < 0.05$ was accepted as the level of significance.

Results and Discussion

DNA Yield

Overall, the manual total demineralization method yielded the highest amount of DNA per mg of bone with an average of 0.71 ± 0.28 ng DNA/mg of bone (Fig. 2.1). This result supports previous studies (5, 12-14, 18, 20) that also report that the total demineralization protocol by Lorielle et al. consistently yields greater amounts of DNA from bone than other methods. The lowest DNA yield was obtained when the TBone buffer was used in combination with the PrepFiler® BTA™ extraction automated on the robotic platform (0.03 ng ± 0.01 DNA/mg of bone) (Fig. 2.1). In general, the DNA yields in this study are comparable or higher than reported for similar skeletal remains in previous studies (7, 12, 19, 23-25, 28, 34). Variation in DNA quantity between the technical replicates was observed (Table 2.1). As may be expected, more variation was seen when DNA extraction was performed from bone chips versus bone powder (average of 2.4 to 3.7 times greater) (Table 2.1). Parametric assumptions were not met in some cases where it was deemed reasonable to continue with ANOVA statistics. There was a statistically significant difference between the various extraction methods as determined by one-way ANOVA ($F_{4,85} = 3.839$, $p = 0.006$), and therefore, a *post hoc* analysis was performed.

Post hoc comparisons using the Tukey HSD test indicated that the mean score for total demineralization ($M = 0.71$, SD = 1.41) was significantly different than the automated PrepFiler® BTA™ extraction ($M = 0.12$, SD = 0.01) and TBone buffer paired with the automated PrepFiler® BTA™ extraction ($M = 0.034$, SD = 0.003). However, no statistical difference was observed in the average DNA yields when PrepFiler® BTA™ was performed manually with or without the TBone Ex digestion steps (Fig. 2.1). These data may suggest

that the PrepFiler® BTA™ lysis buffer may be equally aggressive in digesting powered bone tissue as the TBone Ex buffer digests whole bone chips.

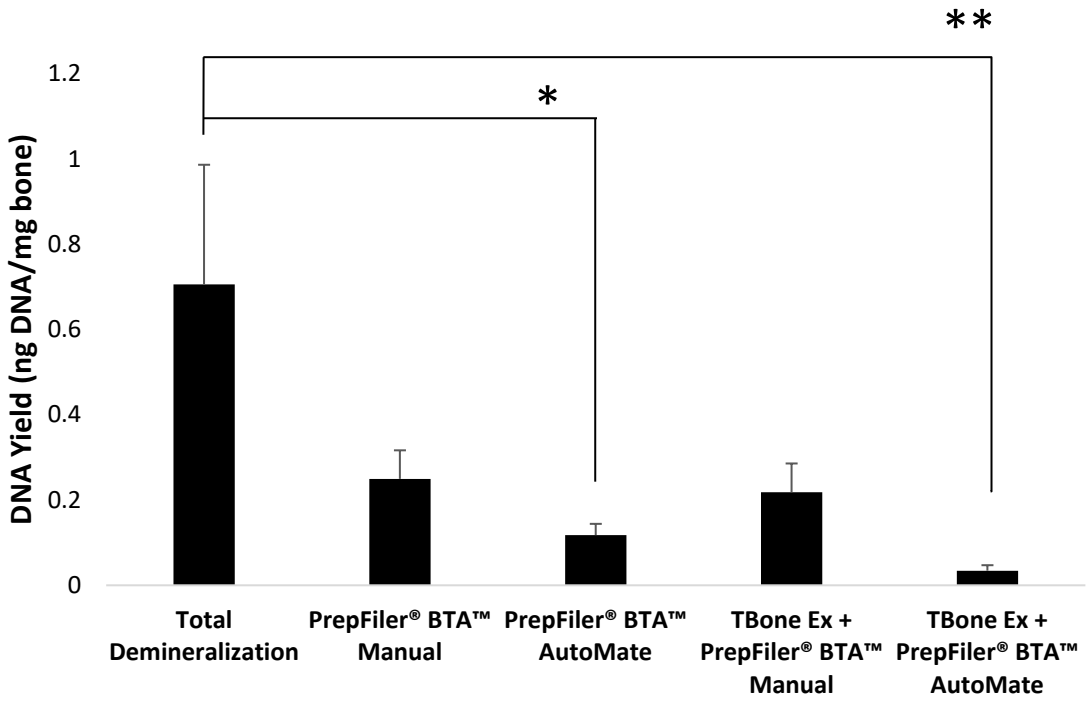

FIG. 2.1 - *Comparison of DNA yield per mg of bone (chip or powder) based on the extraction method used.* n=*18.* *p < *0.05,* **p < *0.01. Data are presented as mean + SEM.*

The decrease in DNA yield we observed when DNA extraction was automated is consistent with previous studies (11-13). Automation may also pose other risks such as the inability to control the pipetting, which led to two extracts in this study having a reduced elution volume (20 µL vs 50 µL) due to bubbles accumulating in the pipette tips. One of the samples was re-extracted, but the other was not due to limited sample. The reported data for this sample reflects the lower elution volume. While an infrequent occurrence, this could be detrimental in cases that have a very small amount of bone available for testing and further demonstrates practitioners' preference of manual methods.

Previous studies have reported high DNA yields when the TBone Ex kit was used to digest bone fragments prior to DNA purification (23, 24) and that multiple rounds of digestion may be performed on a single fragment (23). In that study, digestion of a whole bone fragment followed by silica purification yielded approximately three times more DNA than those demineralized and purified with an organic method using 560 mg bone chips (23). However, in our study, when much smaller fragments (50 mg) were digested with the TBone Ex buffer and PrepFiler® BTA™ extraction, DNA yields were comparable to the powdering methods (Fig. 2.1). This difference may be due to the smaller bone fragments and/or the difference in DNA purification kits used in the two studies. Nevertheless, the TBone Ex buffer provides a simple and efficient alternative to powdering bone tissue, which can reduce the risk of contamination and processing time.

All the bone powder was dissolved using the total demineralization protocol while a slurry of bone powder remained after the initial PrepFiler® BTA™ digestion and lysis step. Although the 50 mg bone shavings incubated in the TBone buffer remained apparently intact, they were smaller in size, showing evident signs of dissolution. A previous study with the TBone buffer (23) has shown that DNA yields from three subsequent extractions were each higher than the first one. However, the previous study used bone chips that were ten-times larger than the 50 mg shavings used in this study. Although a second round of extraction could theoretically be performed using the TBone Ex buffer, the 50 mg chips were too small and were mostly digested in the first round.

According to Loreille at al. (18), more calcium products are released during total demineralization, which may cause some PCR inhibition. However, no PCR inhibition was

detected with any DNA extracts during the qPCR assay. Additionally, all negative controls/reagent blanks showed no signs of contamination.

STR Profile Quality

Although parametric assumptions were not met in some cases, it was deemed reasonable to continue with ANOVA statistics.While there were differences in DNA yield between the various extraction methods, the average number of reportable alleles was comparable as determined by one-way ANOVA ($F_{4,85} = 2.092$, $p = 0.089$). All methods produced STR profiles with >75% average alleles reported (Fig. 2.2A). Although no statistical significance was found, our data support other studies that report a higher percentage of reported alleles when a complete demineralization digestion was performed compared to other DNA extraction methods (12-14, 18, 20). The average percent of alleles reported for the total demineralization method was 96.48 ± 1.72% while the other methods ranged from 75.58 ± 7.75% to 87.70 ± 4.77% (Fig. 2.2A).

No statistically significant difference in average peak height ratio (PHR) was observed in the STR profiles generated from DNA extracted using the various extraction methods tested in this study as determined by a one-way ANOVA ($F_{4,85} = 2.425$, $p = 0.054$) (Fig. 2.2B). However, the extraction method was found to affect the average peak heights (in RFUs) of the STR profiles ($F_{4,85} = 2.774$, $p = 0.032$) (Fig. 2.2C). *Post hoc* comparisons indicate that the average peak heights of the total demineralization ($M = 2662$, SD = 1605) and automated PrepFiler® BTA™ ($M = 2666$, SD = 2159) profiles were significantly higher ($p < 0.05$) than those from the TBone Ex buffer paired with the automated PrepFiler® BTA™ extraction ($M = 1208$, SD = 1268) (Fig. 2.2C).

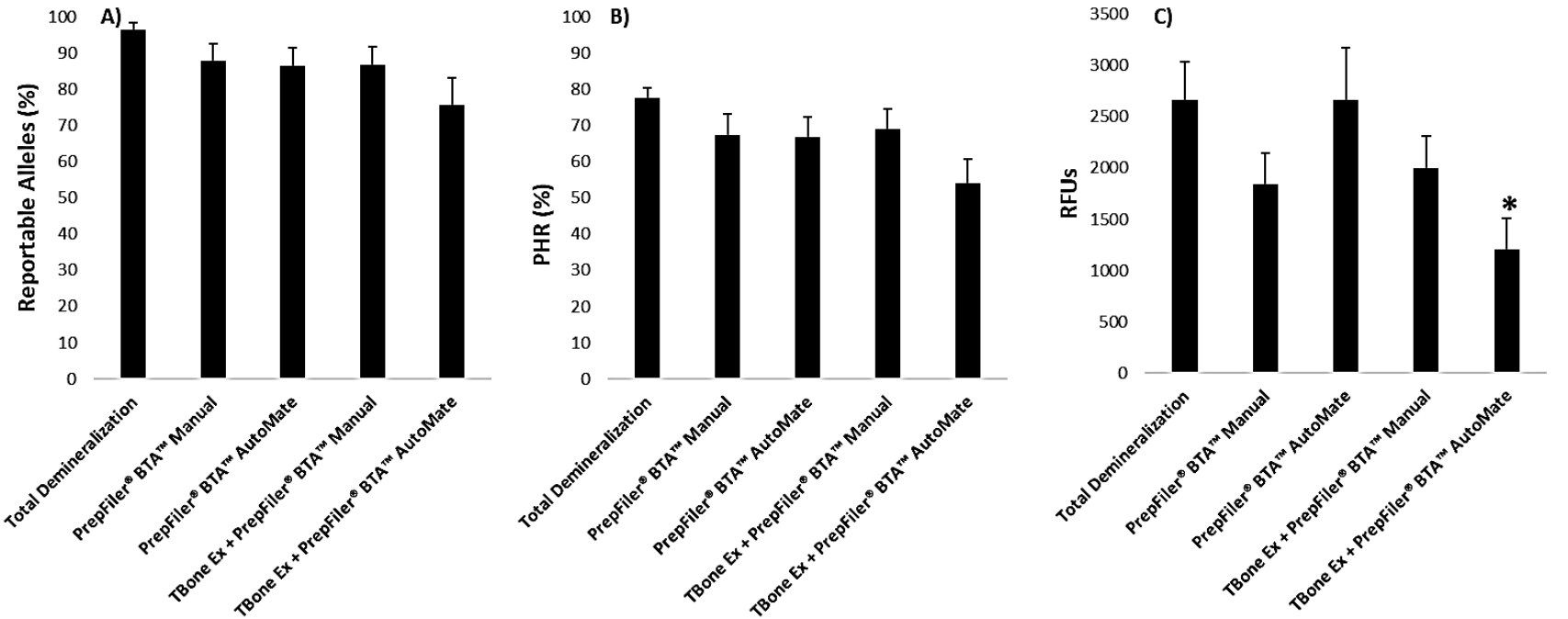

FIG. 2.2 - *Comparison of STR profile quality between the different DNA extraction methods based on (A) percentage of alleles reported; (B) average peak height ratio; (C) average peak height (ANOVA ($F_{4,85} = 2.774$, $p = 0.032$)).* n=*18. *$p < 0.05$. Data are presented as mean ± SEM.*

Conclusions

The results of this study show that powdering of bone tissue may be avoided without significantly reducing DNA yield and STR profile quality. Although the complete demineralization method seemed to extract the most DNA per mg of bone and generate the most complete STR profiles, no statistical difference was observed when compared to the other two manual methods tested in this study (PrepFiler® BTA™, and TBone Ex kit coupled with the PrepFiler® BTA™ kit).

While automation reduces processing time and the potential for human error, some technical issues may still occur with robotic platforms. Overall, a decrease in DNA concentration and STR profile quality was observed in cases when bones were extracted using an automated platform. Sample loss, such as a decreased elution volume, can be detrimental to the success of downstream genotyping, especially in cases when minimal bone tissue is available for analysis. Therefore, manual methods may be preferred.

Our data indicate that manual DNA extraction from whole bone chips (using the TBone Ex kit) is comparable to traditional complete demineralization and the PrepFiler® BTA™ methods that require powdering of the bone tissue prior to extraction. However, results also suggest that the PrepFiler® BTA™ lysis buffer may be equally effective in digesting powered bone tissue as the TBone Ex buffer digests whole bone chips.

Nevertheless, avoiding the powdering steps during bone preparation can reduce the risk of contamination, the initial processing time by approximately 15 minutes per bone (depending on the powdering method used), and the potential for bone powder loss during grinding. In addition, if a sufficient amount of bone chip remains, another round of extraction may be possible from the same bone fragment. This option is not possible when

bones are powdered and consumed during digestion. It should also be noted that the bones used in this study were contemporary (< 6 years). Further work would need to be performed on older bones to determine the efficiency of the TBone Ex buffer with more highly degraded and ancient specimens.

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CHAPTER III

A Powder-free DNA Extraction Workflow for Skeletal Samples[1]

This dissertation follows the style and format of *Journal of Forensic Sciences*.

[1.] Harrel, M. and Hughes-Stamm, S. A Powder-Free DNA Extraction Workflow for Skeletal Samples. J Forensic Sci, 2019 (*Available Online Early Access)* doi: 10.1111/1556-4029.14197

Abstract

The processing of skeletal material poses several challenges for forensic laboratories. Current methods can be laborious, time-consuming, require dedicated equipment, and are vulnerable to contamination. In this study, various sample mass (1x50mg, 3x50mg, and 1x150mg chip(s)) and incubation times (2, 4, and 16hrs) were tested using the PrepFiler® BTA™ Forensic DNA Extraction Kit to digest whole bone chips in lieu of powdering. The most effective method was then applied to bones and tooth fragments collected from contemporary human cadavers exposed to various environmental conditions using an automated platform. Over a third of the samples tested generated full DNA profiles without having to powder the bone/tooth fragment, or further alter the manufacturer's protocol. However, for most samples resulting in incomplete STR profiles due to low amounts of DNA, slightly better results were achieved with powdered tissue. Overall, this work demonstrates the potential use of a faster, non-powdering DNA extraction method for processing skeletal samples as an effective first-pass screening tool.

Keywords: forensic science; forensic biology; DNA; bone; short tandem repeats; skeletonized human remains; PrepFiler® BTA™ forensic DNA extraction kit

A Powder-free DNA Extraction Workflow for Skeletal Samples

Introduction

While the testing of blood, epithelial cells via buccal swabs, or other soft tissues are typically the preferred samples for identifying deceased individuals via DNA typing, these samples may be unavailable or highly decomposed in forensic cases, or in situations such as missing persons and mass disasters (1-3). Hard tissues, such as bones and teeth, are often the only remains available for analysis, which can provide a challenge for the human identification process (2-13). Such considerations include degraded and/or low amounts of DNA, small amounts of bone available for analysis, lengthy and specialized sample preparation, and exogenous DNA contamination from either co-mingled remains or handling during the collection and processing steps (2-9, 14-17).

Many agencies and laboratories traditionally collect or are provided with whole teeth or samples from the cortical portions of weight-bearing long bones (femur, tibia, and humerus) for DNA analysis. This preference and generalized recommendation has been drawn from several studies that have shown these elements to have the highest success rates for achieving the most complete DNA profiles (2, 6, 11, 17-23). However, due to the requirement of large amounts of starting material (0.5–5+ g) for some DNA extraction methods (4, 7, 12-14, 19, 23-26), many of these studies did not include partial tooth fragments or various smaller bony elements such as those from the hands, feet, and skull that do not meet the mass requirement, but may be more commonly recovered from human remains (6). A growing number of more recent studies have provided evidence that higher DNA and allele recovery may be obtained from some smaller and less frequently sampled bony elements such as phalanges of the hands and feet, as well as petrous bones in the skull

(2, 6, 22, 27-31). The increasing efficiency of DNA extraction methods for skeletal remains (with as little as 50 mg starting material required (8, 32-34)) and sensitivity of STR kits have made it possible for a wider variety of sampling options that include these alternate skeletal elements.

Traditional bone processing includes decontamination using various combinations of UV radiation, bleach and ethanol washing steps, and surface removal via sanding prior to grinding into a fine powder (4, 5, 8, 14-16, 24-26, 35). However, powdering bone destroys the sample and requires specialized equipment and laboratory conditions. This additional step in sample handling is commonly performed in a blender cup or a freezer mill with liquid nitrogen (4, 7, 12, 14, 24-26) with a fine, airborne powder posing a possible biological hazard for the processor and increasing the risk of sample contamination (5, 14, 15, 36). Despite these concerns, using powdered bone for DNA extraction has remained the sample processing method of choice due to its reported success in literature and casework, and with very few studies investigating alternate approaches.

Most laboratories that process skeletal samples crush bone into a fine powder and use aggressive digestion buffers with high concentrations of EDTA to demineralize the bone powder through the chelation of mineral ions, such as Ca^{++} (4, 7, 8, 12-14, 25, 35, 37, 38). The complete demineralization of bone powder releases high amounts of DNA into solution, likely from crystal aggregates that may provide protection against environmental degradation (7, 35, 39) and therefore are valuable sources of DNA for human identification.

Although powdering bone is the most common method for forensic laboratories, some studies have demonstrated the use of less destructive methods to leach DNA from bone and teeth into solution and obtain successful DNA profiles (5, 10, 34, 36, 38, 40, 41).

While this approach has been primarily used for ancient remains and other museum samples (38, 40, 41), its application may also extend to contemporary skeletal remains for forensic applications. In a previous study, it has been shown that comparable DNA yields and STR profile quality can be achieved via the digestion and extraction of whole bone chips (50 mg) compared to other traditional methods that use powdered bone (34). However, the digestion of bone chips was performed with a different suite of buffers; the TBone Ex kit (DNA Chip Research Inc., Tokyo, Japan). While these buffers eliminated bone powdering steps, were convenient, and coupled as a pre-treatment for a commercial DNA extraction kit, the lengthy overnight incubation and additional sample handling and reagent costs could not be avoided. Therefore, investigating the effectiveness of a single commercial DNA extraction kit to rapidly process whole bone chips is of value.

Additional strategies for increasing sample through-put for skeletal remains include the use of automation during the DNA extraction steps. Although automation has been shown in several studies to decrease DNA yield from challenging samples (and in some instances also allele recovery downstream), the amount of DNA recovered can still be sufficient to yield full STR profiles (8, 25, 34, 42-45). Despite these concerns, automation can mitigate many human errors, sample contamination, and inter-run variation (8, 25, 42-45).

This project involved two parts: In Phase 1, two variables (the size and number of bone chips and incubation time in the lysis buffer) were evaluated to determine the most effective protocol for extracting DNA from whole bone chips using a commercial DNA extraction kit. In Phase 2, the best combination of experimental conditions from Phase 1 were then applied to twenty bones and five partial tooth fragments collected from nine sets

of contemporary skeletal remains that were environmentally challenged (fire exposure, embalming, burial, and advanced decomposition). These results were compared to traditional sample processing (powdering) and a traditional DNA extraction method (complete demineralization protocol). The efficiency of using an automated platform as a potential screening and/or first-pass processing tool for bone samples within crime labs was also investigated.

Materials and Methods

Sample Preparation

Bone (n=21) and tooth (n=5) samples were collected from nine human cadavers exposed to various environmental insults at the Applied Anatomical Research Center (AARC) at Sam Houston State University (Table 3.1). For bones that were sampled prior to complete skeletonization, desiccated connective tissue was removed directly with a scalpel or via maceration. Broken tooth fragments were gently cleared of any soft tissue by hand and the surface (1 mm) of each bone was sanded and then cut into ~600 mg of moderate-sized pieces (5-8 mm2) or thin bone chips (~50-150 mg each) (Fig. 3.1) from the same region of compact bone using a Dremel® tool (Dremel, Racine, WI, USA). To achieve the appropriate weight of bone chips (± 0.5 mg of target weight), a small piece of bone was cut from the bone source, weighed, and if needed, further shaved down with the Dremel® until the desired weight was reached. Bone cuttings and tooth fragments were washed with a series of 5 min washes of the following: 10% bleach, autoclaved distilled water twice, and a final 100% ethanol wash. All bone cuttings and tooth fragments were allowed to dry in a fume hood overnight at room temperature; however, they were dry in under 3 hours and could therefore be processed the same day if desired. The bone cuttings were then

crushed in a SPEX 6750 Freezer/Mill® (SPEXSamplePrep, Metuchen, NJ, USA) the next day using the following conditions: 10 min cooling period followed by one cycle of 5 min crushing at 15 impacts per second. An extraction blank was performed by wetting a cotton swab with sterile water and swabbing the inside of the freezer mill tubes. The bone chips (50-150 mg) and tooth fragments were not powdered but proceeded directly to extraction using the PrepFiler® BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific, Waltham, MA) (32).

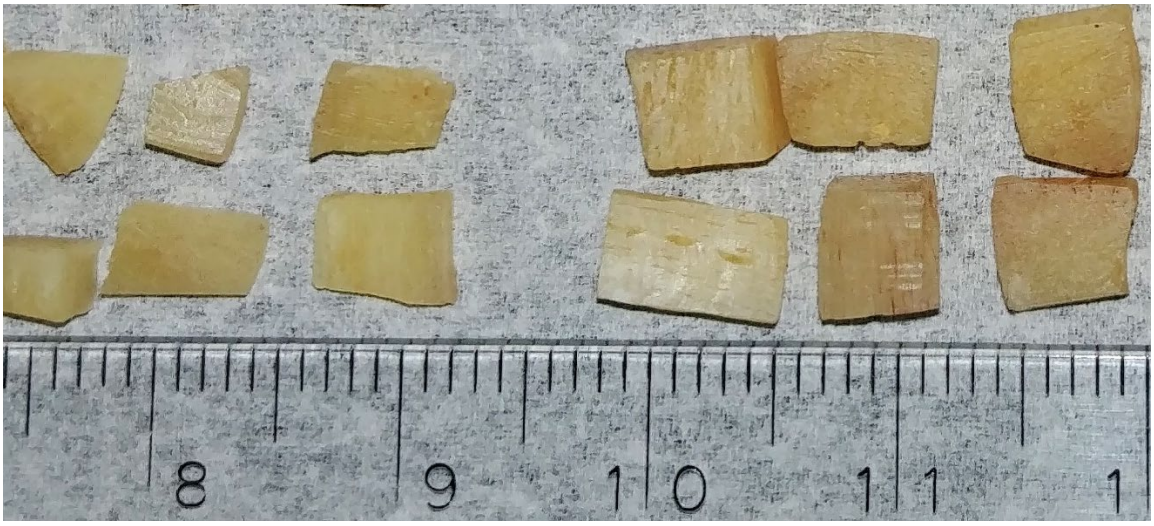

FIG. 3.1 - *Examples of 50 mg (left) and 150 mg (right) bone chips used in this study.*

TABLE 3.1 - *Information for samples used in Phase 1 and 2. LQ = Low DNA concentration, HQ = High DNA concentration.*

Cadaver Code	Sample #	Bone/Tooth	Insult
Phase 1			
A	LQ	humerus & tibia	buried (2 years)
B	HQ	femur	burned*
Phase 2			
C	1 2	femur humerus	embalmed using a formalin-based fixative solution
A	3 4 5 6	rib end humerus femur tibia	buried (2 years)
D	7 8 9 10 11	hand phalanx humerus femur tibia foot phalanx	decomposed remains (6 months on surface)
E	12 13	femur vertebral transverse process	burned*, then further cremated in an outdoor fire pit
F	14 15 16 17 18 19 20	hand phalanx foot phalanx humerus ulna radius femur tibia	burned*
G	T1 T2	root (unknown tooth) premolar fragment	decomposed remains (3 months on surface)
H	T3	incisor fragment	burned*
B	T4	premolar fragment	burned*
I	T5	canine fragment	burned*
* Burnt bodies were doused with accelerant, set alight, and allowed to burn until the fire self-extinguished (approximately one hour)			

DNA Extraction

For Phase 1, three variations of each variable (bone chip mass: 1x50 mg chip, 3x50 mg chips, and 1x150 mg chip; and incubation time: 2, 4, and 16 hrs) were tested in tandem for a total of nine combinations with five replicates each (n=45). As a set of controls, 50 mg and 150 mg aliquots of powdered bone from each sample were also tested in the same manner (n=30). All samples were extracted manually according to the PrepFiler® BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific) following the Bone and Tooth protocol (32) with the exception of the test variables.

For Phase 2, all bone chips were demineralized and digested using the PrepFiler® BTA™ Forensic DNA Extraction Kit automated on the Automate Express™ platform (Thermo Fisher Scientific) (32, 46) with the optimal conditions identified in Phase 1 (50 mg bone chip with 2 hr incubation) (Appendix Fig. B1). For comparison purposes, two sets of powdered bone were extracted manually with either the PrepFiler® BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific) or according to a previously published purification protocol using complete demineralization and MinElute® PCR Purification columns (QIAGEN) (47) (Appendix Fig. B1). The total demineralization protocol was performed with an incubation time of 20 hrs and centrifugation at 2500 x g for 23 mins during the initial concentration step. All DNA extracts were eluted in 50 µL. Swab extraction and reagent blanks were both processed in parallel with each extraction procedure to monitor contamination.

DNA Quantification and STR Analysis

The amount of DNA in each extract was determined via quantitative real-time PCR (qPCR) using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific) as

per manufacturer's instructions (48) on a 7500 Real-Time PCR System (Thermo Fisher Scientific). Data were accepted with an R^2 value of 0.99 or above.

All extracts were amplified using the GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific) with a target of up to 1 ng for 29 cycles or up to 0.5 ng for 30 cycles (when 1 ng was not available with maximum input volume) as per manufacturers' protocol on a ProFlex™ PCR System (Thermo Fisher Scientific) (49). Separation and detection of amplified products was performed using the 3500 Genetic Analyzer (Thermo Fisher Scientific) with a 36-cm capillary and POP-4 polymer (Thermo Fisher Scientific). DNA profiles were generated using GeneMapper ID-X v4.1 software (Thermo Fisher Scientific) with an analytical threshold of 150 relative fluorescent units (RFUs) and stochastic threshold of 600 RFUs.

Secondary Processing

Samples from Phase 2 that did not produce a full STR profile (n=12) were subjected to secondary processing strategies as outlined in Appendix Figure B1 in an attempt to increase the number of reportable alleles. Method A: the remaining DNA eluates from replicate extractions were pooled in a single tube (approx. 70-90 µL) and concentrated in a CentriVap® Concentrator (Labconco, Kansas City, MO) for 25 min at 50°C to reduce the final volume to ~30-50 µL. Method B: duplicate bone chips that were previously digested separately were combined in the same tube, manually crushed with a disposable pestle (if possible), and re-digested and extracted manually with the PrepFiler® BTA™ Forensic DNA Extraction Kit (Thermo Fisher Scientific). DNA quantification and STR typing/analysis were performed as described above.

Statistical Analysis

Parametric assumptions were tested for using a Shapiro-Wilk's W test. Data were tested for statistical significance by Student's *t*-test in Excel or ANOVA single or multi-factor, followed by Tukey HSD post hoc comparisons in Statistica (50) when appropriate. $p < 0.05$ was accepted as the level of significance.

Results and Discussion

Phase 1 - DNA Yield and STR Profile Quality

The two sets of bones used in this phase were selected to represent those within the upper and lower range of sample quantity and quality that might be observed in human remains recovered for identification. Due to the large inherent differences between the samples with high amounts of DNA (> 5 ng/μL) with moderate degradation (degradation index (DI) 3 – 11), and those samples with low amounts (< 0.02 ng/μL) with slight degradation on average (DI < 5), data for each was grouped and analyzed separately.

For the high quantity samples, average DNA concentrations ranged from approximately 8 ng/μL (1x50 mg bone chip) to 24 ng/μL (150 mg bone powder) (Fig. 3.2A). As determined by a Shapiro-Wilks W test, quantification data met the parametric assumption (p(normal) > 0.05)) for ANOVA statistical tests. A single factor ANOVA determined there to be a statistically significant difference between the sample types ($F_{4,70} = 39.4333$, $p = 0.0000$) so a post hoc analysis was performed. Both 50 mg of bone powder and a 50 mg bone chip yielded comparable DNA concentrations and likewise, 150 mg of bone powder and three 50 mg bone chips (150 mg total) produced similar DNA concentrations. As expected, the 150 mg samples produced significantly more DNA than the 50 mg samples ($p < 0.001$). However, although the average DNA concentration for the

single 150 mg bone chips was significantly higher than the 50 mg bone samples ($p < 0.05$), it was significantly lower ($p < 0.001$) than the other 150 mg sets (150 mg powder and 3x50 mg chips). These data may suggest that digestion of bone chips can be an efficient alternative to powdering without compromising DNA yields, and that multiple smaller chips may digest more effectively than one large 150 mg chip.

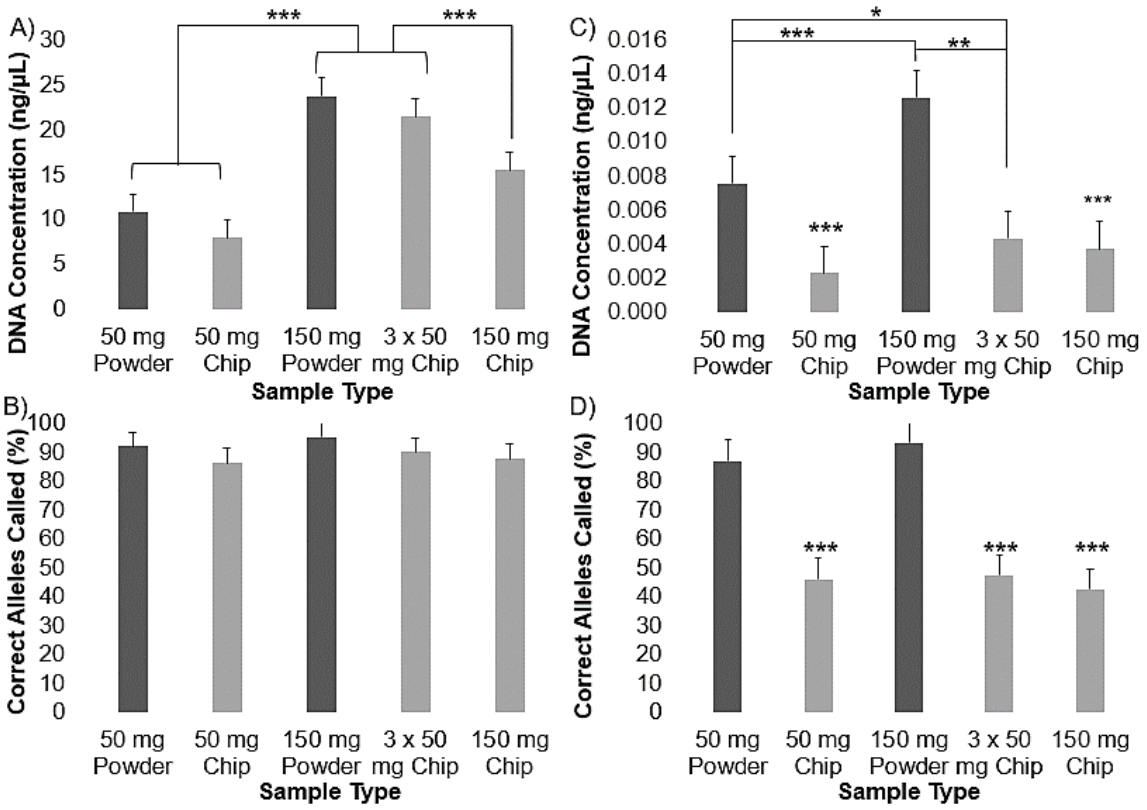

FIG. 3.2 - *Comparison of DNA concentration and STR profile results for Phase 1 based on the sample type and mass used for extraction (*n*=15 per sample type/mass). A) average DNA concentration for the high quantity samples; B) average STR profile completeness for the high quantity samples; C) average DNA concentration for the low-template samples; D) average STR profile completeness for the low-template samples. Error bars denote 0.95 confidence interval.* * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ *(NOTE: not all significant comparisons are shown)*

With the exception of the 150 mg powder set, STR data met the parametric assumption for ANOVA statistical tests as determined by a Shapiro-Wilks W test (p(normal) > 0.05)), and therefore it was deemed reasonable to proceed with the analysis. The average percent of alleles reported ranged from 86% (50 mg bone chips) to 95% (150 mg bone powder) (Fig. 3.2B). Despite the variance in DNA concentration, no significant difference in STR success was observed between any of the sample types as determined by a single factor ANOVA ($F_{4,40}$ = 1.1780, p = 0.3351). In addition, single factor ANOVAs determined that the incubation time (2, 4, or 16 hrs) had no statistically significant effect on DNA concentration ($F_{2,72}$ = 1.3629, p = 0.2624) (Appendix Fig. B2A) or reportable alleles ($F_{2,42}$ = 0.3631, p = 0.6977) (Appendix Fig. B2B) for powdered or whole bone chips (p > 0.05).

Results from the low-template sample showed some different trends to the bone samples containing more DNA. Average DNA concentrations ranged from approximately 0.002 ng/μL (50 mg bone chip) to 0.01 ng/μL (150 mg bone powder) (Fig. 3.2C). Although parametric assumptions were not met in some cases (50 mg and (3) 50 mg bone chip sets) as determined by a Shapiro-Wilks W test (p(normal) < 0.05), it was deemed reasonable to proceed with ANOVA statistical analyses. As with the previous data set, a single factor ANOVA determined that there was a statistically significant difference between the sample types ($F_{4,70}$ = 26.1719, p = 0.0000), and therefore a post hoc analysis was performed. Both sets of powdered samples (50 and 150 mg) had higher average DNA concentrations than the three bone chip sets, and 150 mg of bone powder still yielded significantly more DNA than 50 mg powder (p < 0.001). However, all bone chip sets (50 mg, 3x50 mg, 1x150 mg) performed similarly regardless of bone mass (p > 0.05). These data suggest that for bone

samples containing very little DNA, a difference in bone chip number and/or mass may not have an effect on DNA recovery, and that powdering remains the more successful method.

Unlike the high quantity sample set, allele recovery significantly differed between sample types ($F_{4,70} = 42.7478$, $p = 0.0000$). Although parametric assumptions were not met in some cases (50 mg and 150 mg bone powder sets) as determined by a Shapiro-Wilks W test (p(normal) < 0.05), it was deemed reasonable to proceed with ANOVA statistical analyses. The overall trend for STR results (Fig. 3.2D) showed that the powdered sets produced a significantly higher percentage of reportable alleles than the bone chips ($p < 0.001$). This result is not unexpected as more DNA was available for amplification with the powdered samples. However, all bone chip sets yielded comparable numbers of reported alleles with a range of approximately 42-46% ($p > 0.05$), and both powdered sets performed similarly with an average of 86-93% alleles ($p > 0.05$). As with the high quantity sample set, incubation time had no effect on DNA concentration ($F_{2,72} = 0.2369$, $p = 0.7897$) (Appendix Fig. B2C) or reportable alleles ($F_{2,72} = 0.7261$, $p = 0.4873$) (Appendix Fig. B2D) ($p > 0.05$).

Overall for both sets of samples, there was no single combination of chip weight and incubation time that was significantly better than the others for DNA concentration and/or allele recovery (Appendix Fig. B3). Therefore, as none of the variations in bone chip number/size or increase in incubation time tested in this study generated significantly better results than the manufacturer's recommendations for powdered bone (50 mg bone, and 2 hour incubation time), one 50 mg bone chip and a 2 hour incubation was selected for Phase 2.

Phase 2 - DNA Yield

Bone tissue was taken from 20 environmentally challenged bones (Table 3.1), and DNA was extracted from a single 50 mg bone chip in duplicate with the PrepFiler® BTA™ Forensic DNA Extraction Kit automated on the Automate Express™ platform (Thermo Fisher Scientific) following a 2 hour incubation. Five partial tooth fragments were also processed in this manner, but without replicates due to the availability of only a single fragment. With the exception of the tooth fragments, DNA from 50 mg of powdered bone was also extracted manually with both a total demineralization method and commercial DNA extraction method (PrepFiler® BTA™ Forensic DNA Extraction Kit) to serve as control groups for the bone samples.

Normalized DNA concentrations ranged from 0 to 35.8 ng/mg of bone for bone chips (Appendix Fig. B4) and from 0.0009 to 23.4 ng/mg of tooth fragment (data not shown) when extracted with PrepFiler® BTA™ chemistry on the AutoMate Express™. A femur sample from a body cremated in an outdoor fire pit did not yield a quantifiable amount of DNA with any of the DNA extraction methods tested, and was therefore removed from further analysis. This result was not unexpected as the sample was extremely burnt and very brittle. Overall, powdered samples extracted manually yielded slightly more DNA on average than bone chips (5.0 ± 1.6 ng/mg bone vs 3.0 ± 1.1 ng/mg bone), but this difference was not statistically significant ($p > 0.05$) (Fig. 3.3A). Additionally, no statistical difference in DNA yield was observed between the two manual methods (Fig. 3.3B), suggesting that PrepFiler® BTA™ may be just as effective at extracting DNA from 50 mg of powdered bone compared to the more time-consuming total demineralization method. These results are consistent with previous work comparing the same two traditional

powdering methods with a non-powdering method (34). As determined by the IPC ΔC_T during DNA quantitation, no inhibition was detected in any samples, and negative controls showed no signs of contamination.

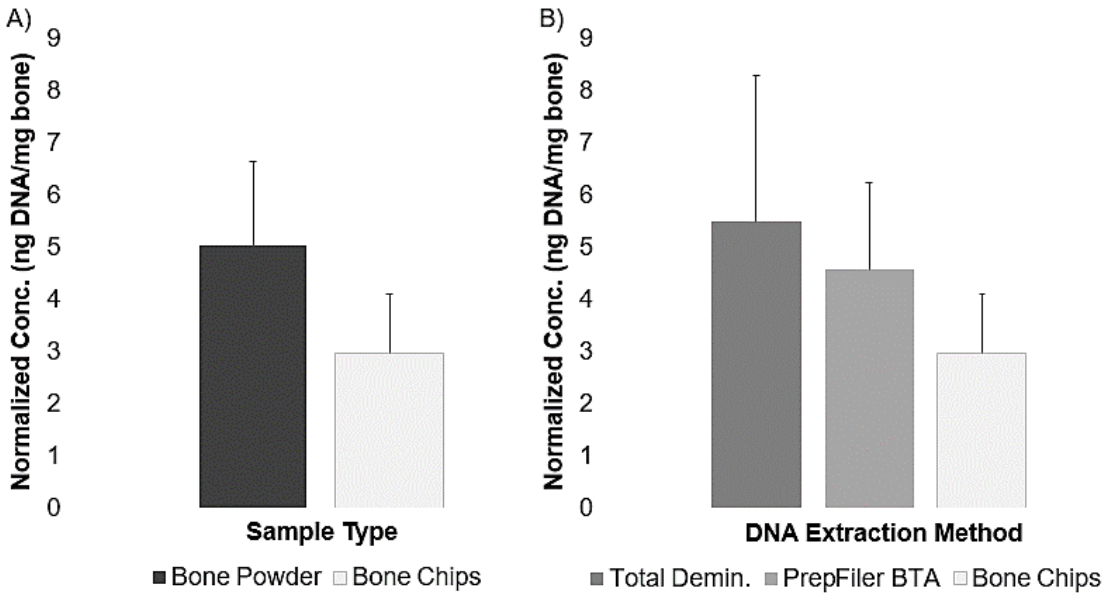

FIG. 3.3 - *Comparison of average normalized DNA concentrations for the first round of DNA extractions in Phase 2 with the powdered samples: A) combined in the sample type (*n=*20 each) and B) separated by DNA extraction method (*n=*10,* n=*10,* n=*20, respectively). Error bars denote the standard error of the mean (SEM).*

Although comparable in yield, DNA extracted from bone chips was more degraded on average compared to DNA recovered from powdered bone (7.4 ± 1.4 vs 4.2 ± 0.4; $p < 0.05$) as determined by the DI from Quantifiler® Trio. This could be due to the BTA™ buffer only having access to the surface and limited porous areas of the chip, which was subject to damaging heat during the sanding and cutting process and several aggressive cleaning steps, while the cells and crystal aggregates at the core of the chip are more protected, and then released during the powdering process (7, 35, 39). In general, samples from the embalmed and decomposed cadavers showed the highest degradation levels on average, with DIs ranging from approximately 1.9 to 17.5.

As methods to extract DNA from bones are being modified and improved, several studies have investigated the use of skeletal elements other than the more traditionally harvested femur, tibia, and humerus for the identification of human remains (6, 11, 18, 21, 22, 27, 29-31). In this study, non-traditional elements (radius, ulna, rib end, vertebral transverse process, broken tooth fragments, and hand and foot phalanges) yielded slightly higher concentrations and less degraded DNA ($p > 0.05$), and resulted in significantly more alleles reported ($p < 0.01$) compared to traditional bony elements (Fig. 3.4). The three bone chips that yielded the highest concentrations of DNA per 50 mg of bone tissue across the entire study were from a vertebral transverse process, a maxillary canine fragment, and a femur with 35, 23, and 21 ng DNA/mg bone, respectively. Except for a single replicate, all bone chips from non-traditional elements resulted in STR profiles with greater than 70% alleles called. Although results from other studies differ in their rankings of the most successful bones, non-traditional bony elements, especially those from the hands and feet, have ranked similarly or better than femurs and teeth (6, 11, 21, 22). Therefore, DNA can be effectively recovered from many different bony elements using this non-powdering method and laboratories should also consider extracting DNA from bony elements other than more traditional sources. In some cases, those weight-bearing long bones may not even be available for analysis.

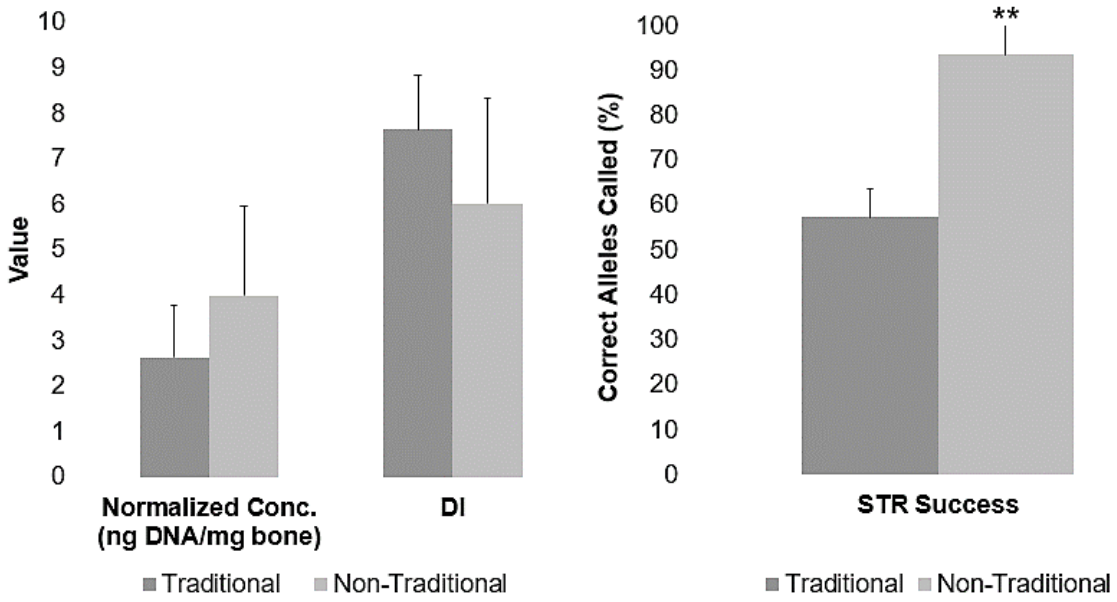

FIG. 3.4 - *Comparison of DNA extract and STR profile metrics between traditional (*n*=24) and non-traditional (*n*=21) skeletal elements in Phase 2. Traditional elements include: humerus, femur, tibia; Non-traditional elements include: teeth, radius, ulna, rib ends, hand and foot phalanges. Error bars denote the standard error of the mean (SEM).* **$p < 0.01$

Phase 2 - STR Profile Quality

In total, at least one of the replicate chips from six bone samples (Appendix Fig. B5) and three out of five tooth fragments (data not shown) resulted in full GlobalFiler® STR profiles. However, if only analyzing the 20 expanded core CODIS loci, then eight of the bone samples yielded full profiles from a single 50 mg chip. Most of these samples had high quantities of DNA and low to moderate degradation indices as determined by Quantifiler® Trio. With the exception of one sample, STR results from replicate chips varied by less than 10% reportable alleles, which shows that while there is natural variation between sequential bone chips, results are generally reproducible. One set of bone chips from a rib end from a buried cadaver failed to amplify despite showing DNA concentrations ranging from 0.016 to 0.076 ng/µL, a DI of 49.6 to 2.9, and no inhibition (IPC ΔC_T = 0.16 and -0.04, respectively). However, DNA extracted from powder of that

same sample yielded similar quantification results but near complete STR profiles. Therefore, the authors hypothesized that the soil on the bone chips was not effectively removed during the cleaning and/or DNA extraction processes and as soil is a known source of the inhibitor humic acid, inhibition may have caused the failed amplification. In an effort to rescue these failed samples, the DNA extracts from the bone chips were diluted 1:2 in an attempt to reduce the effects of inhibition, but they still failed to amplify any alleles. Unfortunately, the remaining pieces of the rib end were consumed during powdering, so the bone could not be re-sampled and was therefore removed from further analyses.

Average peak heights were comparable between the two sample types (2318.8 ± 333.3 RFUs for bone chips vs 2707.6 ± 262.1 RFUs for bone powder), but STR profiles from bone chips were less balanced than the powdered samples with average heterozygous peak heights of 49.3 ± 5.1% vs 65.7 ± 2.5% ($p < 0.01$). Average allele recovery for bone chips was also lower than the powdered samples (71.5 ± 5.3% and 93.1 ± 1.7% respectively; $p < 0.001$). An increase in the level of degradation and slightly less DNA available for amplification are likely strong factors for this difference, as 66% of alleles that dropped out for bone chips, and 86.7% for powdered samples, were at loci with larger amplicon sizes (> 200 bps). With the long target of Quantifiler® Trio being only 214 bps, the predictability of alleles amplified at these longer loci might not be as easily apparent based on small amplicon input or degradation values alone (Appendix Fig. B6). Indeed, we observed a general trend that as the amount of long amplicon DNA into PCR increases (based on the amount of short amplicon added), the percentage of alleles reported increases in a logarithmic manner (Appendix Fig. B7). The lowest amount of long amplicon DNA

in the PCR reaction that resulted in a full STR profile was 0.05 ng. This sample had a DNA input of 0.35 ng based on the short amplicon quantity and a DI of 7.

Phase 2 - Secondary Processing Strategies

With 6 of the 20 bone samples having full STR profiles and two samples being excluded from further study, the remaining 12 sets of bone chips were subjected to further testing using two approaches: A) pooling and concentrating DNA elutes of duplicate bone chips, and B) combining the replicate bone chips remaining intact after the first round of extraction and subjecting them to a second round of extraction.

As expected, when DNA extracts were pooled and concentrated, the DNA concentration increased (approx. 2.5–fold increase) (Fig. 3.5). However, an improvement in allele recovery was only observed in half of those re-worked samples, with an average increase of approximately 10% in the number of reportable alleles compared to the first extraction of the bone chip on the Automate Express™ (Fig. 3.5; Appendix Fig. B8). The six samples that showed improvement had < 100 pg in the original PCR, but the new input amount provided approximately twice the amount of DNA for amplification. Pooled eluates were concentrated to approximately 30 µL, so further concentration may have resulted in even greater allele recovery.

Combining duplicate bone chips (from the first round of extractions) into a single sample digestion tube and re-extracting manually with the PrepFiler® BTA™ kit showed no increase in the recovery of DNA or improvement in the number of reportable alleles (Fig. 3.5). As similar amounts of DNA as the first extraction were obtained, this strategy does offer the possibility to re-exact from the original bone chip if needed. An analyst also potentially could recover more DNA by performing subsequent rounds of extractions for

further pooling and concentration; however, this approach would be more time consuming and increases the risk of contamination due to multiple rounds of sample handling. When compared to the original automated extraction of bone chips, and regardless of the secondary strategy employed, the manual extraction from bone powder (control method) was still the most successful ($p < 0.01$) processing approach overall (Fig. 3.5). For samples yielding incomplete STR profiles on the first-pass, an improvement in allele recovery for all twelve reworked samples was observed, with four of those samples resulting in full STR profiles (Appendix Fig. B9).

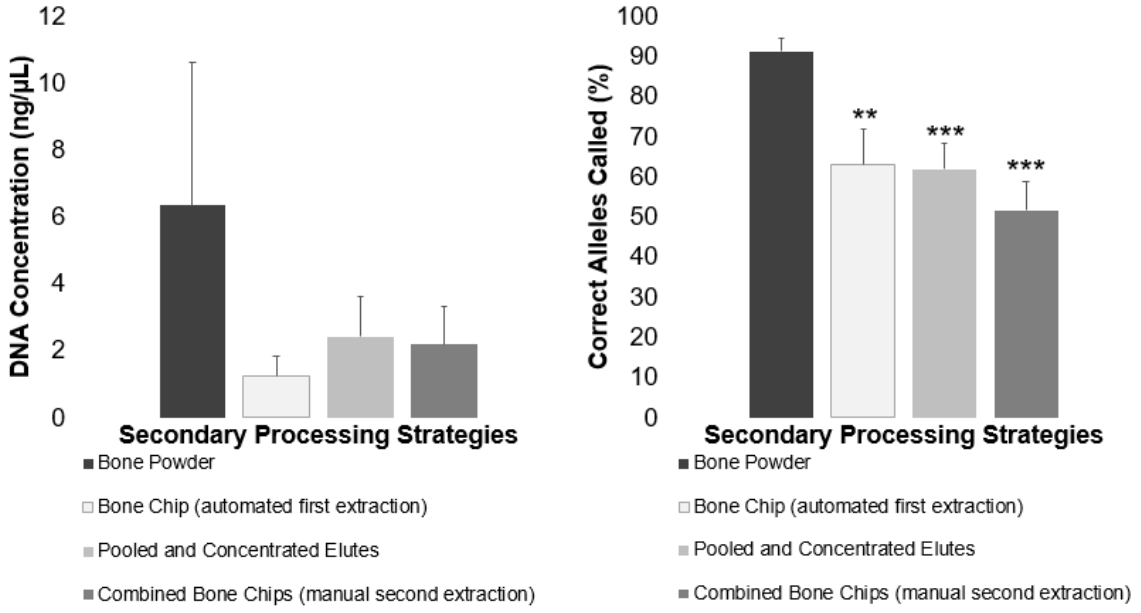

FIG. 3.5 - *Comparison of average A) DNA concentration and B) STR alleles reported for each of the processing strategies in Phase 2.* ** $p < 0.01$; *** $p < 0.001$

Conclusions

Many different protocols for processing and extracting DNA from bones and teeth are used in various laboratories across the world. These methods are almost exclusively performed manually and require bone samples to be powdered, making them time-consuming and requiring specialized equipment and technical training. Therefore, many

crime laboratories may choose to outsource these samples. However, this study has shown that the PrepFiler® BTA™ extraction kit can perform similarly to other commonly used DNA extraction protocols for bone tissue that are more time-consuming and require reagent preparation (4, 14, 23, 34, 47). The results of this study have also demonstrated that the PrepFiler® BTA™ kit can effectively extract DNA from whole bone chips without having to powder the samples. This non-powdering method using a commercial kit may make it a more attractive option for crime laboratories to process their own bone samples if they already have this chemistry integrated into their current workflow. In this study, the PrepFiler® BTA™ buffer appears to digest whole bone chips equally effectively as powdered bone for a variety of skeletal samples. While 3x50 mg bone chips produced better results in some cases, overall a single 50 mg chip yielded comparable results in DNA concentration and STR success. Neither an increase in incubation time (2, 4, or 16 hrs) nor bone chip mass (50 - 150 mg) and/or number (1 - 3 bone chips) significantly improved results compared to the current manufacturer's recommended protocol for powdered bone (50 mg powder for 2 hrs) with the standard buffer volume. Therefore, these data suggest that no further optimization of these conditions would be required in order to successfully process whole bone chips (in lieu of bone powder) using this chemistry.

In the phase 2 study, over a third of the bones sampled resulted in a full profile (all CODIS loci) in the first-pass from a single 50 mg chip processed using the PrepFiler® BTA™ chemistry on the AutoMate Express™ platform. Bone powder and chips of similar weight performed comparably in the resultant DNA concentration and STR success in most cases, but data also showed that a non-powdering extraction may not be as efficient as powdering bone prior to digestion for highly compromised or bone samples with very little

DNA. However, the bone chip approach presents a possibility for a quick triaging approach, or for additional extractions to be performed from the same bone chip to improve or supplement DNA recovery without consuming more of the original bone sample. If desired, the chip could be rinsed, powdered, and then undergo a subsequent extraction. Overall, this study has shown that full STR profiles can be quickly recovered from contemporary whole bone chips and broken tooth fragments using a commercial DNA extraction kit and an automated workflow without having to crush the sample into a fine powder. Furthermore, DNA quantity and quality from any bone sample submitted as evidence may be screened in-house before (or avoid) having to perform longer, more labor-intensive manual protocols, or outsource to specialized labs for processing.

Acknowledgements

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CHAPTER IV

The effects of extra PCR cycles when amplifying skeletal samples with the GlobalFiler® PCR Amplification Kit[1]

This dissertation follows the style and format of *Journal of Forensic Sciences*.

[1] Harrel, M., Gangitano, D. & Hughes-Stamm, S. The effects of extra PCR cycles when amplifying skeletal samples with the GlobalFiler® PCR Amplification Kit. Int J Legal Med 2019;133(3):745-750. https://doi.org/10.1007/s00414-018-1860-2

Abstract

When samples with low amounts of DNA are amplified using Short Tandem Repeats (STRs), stochastic effects such as allele and locus dropout or drop-in, allele imbalance, and increased stutter often occur making data interpretation more difficult. The most common approach to improving STR results from low template samples is to increase the number of PCR cycles. Although more alleles may be recovered, stochastic effects may be exaggerated resulting in more complicated STR profiles. This work reports the effect of additional PCR cycles (29 vs. 30, 31, and 32) on STR success from environmentally challenged bone and tooth samples using the GlobalFiler® DNA amplification kit (Thermo Fisher Scientific). In addition, we compared the efficiency of two DNA extraction kits for skeletal samples: QIAamp® DNA Investigator (QIAGEN) and PrepFiler® BTA™ Forensic DNA Extraction (Thermo Fisher Scientific) kits. Results showed that more DNA was recovered from samples using the PrepFiler® BTA™ kit, but regardless of the extraction method the number of alleles detected and the peak heights both increased with an increase in PCR cycle number. Although more alleles were reported in almost all samples, the most notable improvement was observed in samples with the DNA template <120 pg. A general increase in the number of PCR artifacts was detected in STR profiles generated using 30-32 cycles. Overall, this study provides supporting evidence that STR profile completeness and quality may be improved when low template skeletal samples are amplified with extra PCR cycles (up to 32 cycles) using the GlobalFiler® DNA amplification kit.

Keywords: low-template DNA; skeletal; low-copy number; GlobalFiler®

The effects of extra PCR cycles when amplifying skeletal samples with the GlobalFiler® PCR Amplification Kit

Introduction

Many types of forensic evidence such as blood stains and buccal swabs provide abundant amounts of nuclear DNA for forensic analysis and provide robust and reliable genotyping of individuals with high discriminatory power (1). However, some biological samples submitted to forensic laboratories prove to be more challenging. In the case of mass disasters, missing persons' cases, severe fires, and mass graves, skeletonized remains (bone and teeth) may be the only elements available for human identification (2-6). When performing STR analyses from bone samples, several difficulties may arise due to DNA damage, fragmentation, and/or very low amount of DNA available for amplification. These conditions may result in the loss of loci and increase of undesired PCR artifacts due to stochastic effects during amplification. These artifacts must be taken into consideration when genotyping difficult skeletal samples, as they can complicate data interpretation.

Several approaches have been proposed to improve the results from low template DNA (LT-DNA) bone samples (4, 5, 7-19), including optimizing DNA extraction methods for skeletal samples (2, 4, 5, 20, 21). For LT-DNA samples in general, another strategy to increase the number and confidence of the alleles reported is to generate a consensus profile. This process involves splitting a single DNA extract into multiple PCR reactions and only reporting alleles when observed more than once (4, 7, 10, 13, 19, 22-27). However, debate exists whether this splitting approach generates better results than with a single amplification (19, 23-27). Nevertheless, the most common strategy to recover more complete STR profiles from LT-DNA samples is to increase the number of cycles during

PCR amplification, a process termed high sensitivity (10, 22, 28) or low copy number (LCN) typing (5, 8, 10, 12-15, 18, 19, 28). Although varying levels of success using these LCN-typing techniques has been reported (5, 7-10, 12-15, 18, 22, 28, 29), the robustness and reliability of results from LCN typing has also been a subject of scrutiny (7, 8, 22, 28-31). PCR artifacts such as allele drop-in, heterozygous peak height imbalance or exaggerated stutter may be more severe (7-10, 14, 15, 19, 22, 28). This work documents the effect of additional PCR cycles when using the GlobalFiler® DNA amplification kit to genotype bone and tooth samples.

Materials and Methods

Sample Preparation

Bone and tooth samples (n=12) were collected from 11 cadavers exposed to various environmental insults at the Applied Anatomical Research Center (AARC) at Sam Houston State University, Huntsville, Texas, USA (Appendix Table C1). The surface (1 mm) of each bone was sanded to remove any contamination and cut into small pieces (3-5 mm^2) using a Dremel tool (Dremel, Racine, WI, USA) before being washed with a series of 5 min washes: 15% bleach, two washes of autoclaved distilled water, and 70% ethanol. The samples were dried overnight. Bone samples were crushed into a fine powder in a SPEX 6750 liquid nitrogen Freezer-mill (SPEX SamplePrep, Metuchen, NJ). An extraction blank was prepared by wetting a cotton swab with sterile water and swabbing the inside of an empty Freezer-mill tube each batch.

DNA Extraction and Quantification

Bone powder (100 mg) from twelve bone samples were extracted in duplicate using two methods; 1) QIAamp® DNA Investigator Bone and Teeth protocol (QIAGEN, Hilden,

Germany) with a 40 µL elution volume (32), and 2) PrepFiler® BTA™ Forensic DNA Extraction Kit Bone and Tooth protocol (Thermo Fisher Scientific, Carlsbad, California, USA). The PrepFiler® kit extraction was performed using two aliquots of 50 mg of powder and an 18 hr lysis incubation at room temperature as recommended (33). The two neat elutes from the PrepFiler® extractions (50 mg powder) were pooled to provide a total extract volume of 100 µL from 100 mg of bone powder. A reagent blank was run in parallel with both extraction procedures to monitor contamination. DNA reference samples were available for all cadavers.

The amount of DNA in each sample extract (n=48) was determined via quantitative real-time PCR (qPCR) using the Quantifiler® Trio DNA Quantification kit (Thermo Fisher Scientific) as per manufacturer's instructions (34). Data were accepted with an R^2 value of 0.99 or above.

STR Typing and Data Analysis

A selection of 30 DNA extracts under 0.03 ng/µL were amplified in a 25 µL reaction volume using the GlobalFiler® PCR Amplification Kit (Thermo Fisher Scientific) with 29, 30, 31, and 32 cycles on a ProFlex™ PCR System (Thermo Fisher Scientific) (35). No template controls were included to monitor contamination. Separation and detection of amplified products was performed on the ABI Prism 3500 Genetic Analyzer (Thermo Fisher Scientific). DNA profiles were generated using GeneMapper ID-X v4.1 software (Thermo Fisher Scientific) with an analytical threshold of 150 RFUs and stochastic threshold of 600 RFUs being applied. Loci with a single peak below the stochastic threshold of 600 RFUs were considered a high potential for allele drop-out, and as such only one allele was reported at that locus.

Data were tested for statistical significance by Student's *t*-test in Excel or ANOVA single factor followed by Tukey HSD *post hoc* comparisons in Statistica (36) when appropriate. $p < 0.05$ was accepted as the level of significance.

Results and Discussion

DNA Quantitation

When comparing overall DNA yields, significantly more DNA ($p = 0.018$) was recovered from 100 mg of bone and tooth samples using the PrepFiler® BTA™ Forensic DNA Extraction kit than the QIAamp® DNA Investigator kit (average yield of 1486.3 ± 551.7 ng compared to 117.9 ± 49.7 ng per 100 mg bone powder). This difference in DNA yield could potentially be due to the smaller amount of starting material for the PrepFiler® samples (2 x 50 mg powder) compared to a single batch of 100 mg of powder with the QIAamp® DNA Investigator protocol. Previous studies have shown that using less bone powder per extraction may increase the total yield of DNA due to the ability of EDTA to more efficiently break down the hydroxyapatite matrix and complex the resulting calcium ions (2, 20, 21). In addition, the use of silica spin columns during the Investigator extraction (compared to silica-coated magnetic beads in the PrepFiler® kit) may have also contributed to the loss of DNA using this method. Several studies have reported significant loss of DNA when spin columns are used for DNA purification as DNA, especially smaller fragments, may be retained on the silica membrane after elution (37-40).

An additional benefit of the PrepFiler® BTA™ kit is that the sample stays in the same tube during the entire bind, wash, and elute process which may minimize the contamination risk and sample loss due to changing tubes and increased sample manipulation. Although the overall DNA yield was significantly different between the two

methods, the extraction method did not have a significant effect on the level of DNA degradation ($p = 0.08$), as measured by the degradation index (DI) in this study. No PCR inhibition was observed in any extracts, and all negative controls/reagent blanks showed no signs of DNA contamination.

STR Typing

The DNA concentrations of the 30 samples tested in the cycling study ranged from 0.0006 to 0.03 ng/μL of DNA. The total amount of DNA amplified in each PCR reaction therefore ranged from 0.009 to 0.45 ng (up to 15 μL input). STR data is displayed in Fig. 4.1 A-D, while data normalized in relation to the average 29 cycle data can be seen in Appendix Figure C1a-d. In general, the number of reportable alleles increased when additional PCR cycles (30-32 versus 29 cycles) was used (Fig. 4.1 and Appendix Fig. C1a). For data analysis, samples were placed into one of five groups based on the amount of DNA in the PCR: <30 pg (n=4), 30-60 pg (n=5), 60-120 pg (n=6), 120-200 pg (n=6), and 200-500 pg (n=9), where 120 pg appears to denote a notable difference in STR success based on the amount of DNA amplified (Appendix Fig. C2).

A one-way ANOVA determined that there was a significant difference in the number of reportable alleles for the <30 pg ($F_{3,12} = 3.490$, $p = 0.0498$), 30-60 pg ($F_{3,16} = 3.239$, $p = 0.0002$), and 60-120 pg ($F_{3,20} = 3.098$, $p = 0.015$) groups, so a *post hoc* analysis was performed for each group. For samples with less than 30 pg of input DNA, the average number of reportable alleles significantly increased ($p < 0.05$) from 10.9 ± 3.4 % with 29 cycles to 64.1 ± 16.3 % with 32 cycles (Fig. 4.1A). For samples with 30-60 pg of input DNA, the average number of reportable alleles significantly increased ($p < 0.05$) from 28.0 ± 3.3 % with 29 cycles to 54.7 ± 9.4 % with 31 cycles and 80.3 ± 3.6 % with 32 cycles (p

< 0.001) (Fig. 4.1A). Finally, for samples with 60-120 pg of input DNA, the average number of reportable alleles significantly increased ($p < 0.05$) from 32.5 ± 6.0 % with 29 cycles to 71.3 ± 6.5 % with 32 cycles (Fig. 4.1A). Although the increase in reportable alleles was not deemed to be significant by one-way ANOVA for the 120-200 pg and 200-500 pg groups, the number of samples with full profiles more than doubled (3 to 7 samples) with the addition of a single cycle and tripled (3 to 9 samples) with the addition of three cycles. As shown in other studies (7, 12-14), the addition of even more cycles may result in an even larger increase in STR success and number of full profiles. However, the more PCR cycles that are added, the more likely stochastic effects will be exaggerated, further complicating data interpretation.

Contrary to other studies using additional PCR cycles (7, 9, 14), a significant difference ($F_{3,16} = 3.239$, $p = 0.002$) in average peak height ratio (APHR) was only observed for the 30-60 pg group rather than all samples tested (Fig. 4.1B). There was a significant improvement in APHR when amplified with 32 cycles (37.8 ± 2.6%) compared to 29, 30, and 31 cycles (10.4 ± 1.0% ($p < 0.01$), 18.1 ± 4.7% ($p < 0.05$), and 19.6 ± 6.5% ($p < 0.05$) respectively) (Fig. 4.1B and Appendix Fig. C1b). However, all samples amplified with <120 pg at 29, 30, 31 and 32 cycles generated imbalanced profiles (less than 50% APHR). When samples with >120 pg were amplified, peak balance was acceptable (55 – 67%), and additional PCR cycles did not improve amplification balance. As expected, significantly higher average peak heights (APH) were observed in all groups when amplified with 32 cycles than with 29 cycles ($p < 0.05$) (Fig. 4.1C and Appendix Fig. C1c). Increase in fluorescence ranged from an average of 29.7 ± 8.7 RFUs to 639.9 ± 251.3

RFUs for the <30 pg group to an average of 968.9 ± 180.1 RFUs to 6968.4 ± 1177.2 RFUs for the 200-500 pg group (Fig. 4.1C).

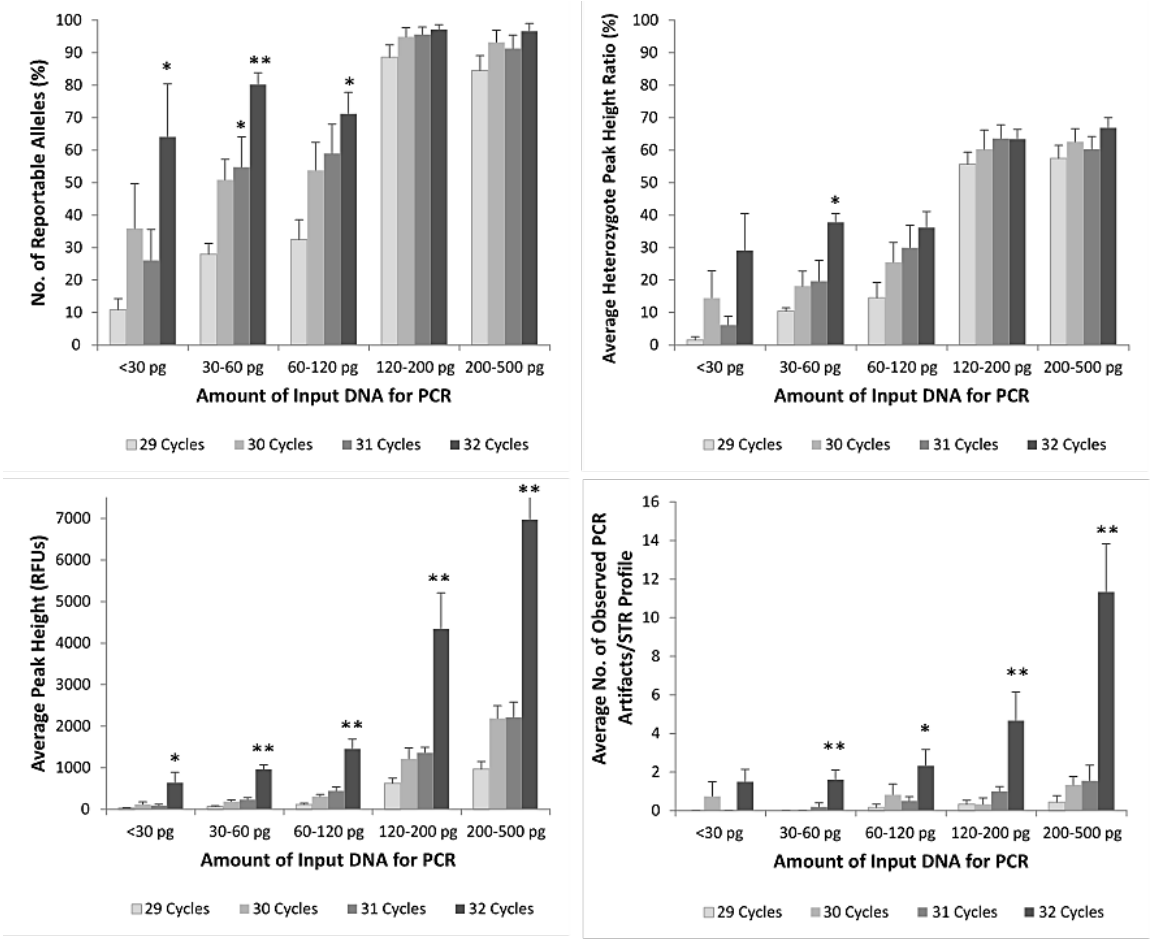

FIG. 4.1 - *Comparison of STR profile quality between the different sample groups categorized by template amount (*n=*4,* n=*5,* n=*6,* n=*6, and* n=*9, respectively) and cycle number based on (A) percentage of correct alleles reported; (B) average peak height ratio; (C) average peak height; (D) average number of observed PCR artifacts per profile (exaggerated stutter, off-ladder alleles, drop-in alleles, pull-up origins, and incomplete adenylation). Data is presented as average + standard error of the mean (SEM).* *p < *0.05,* **p < *0.01*

Consistent with other studies (7, 9, 15), we also observed an increase in the occurrence of exaggerated stutter peaks and other artifacts when samples were amplified with additional PCR cycles (Fig. 4.1D). It should be noted that most of these artifacts were observed in samples with more than 200 pg of input DNA, especially when amplified with 32 cycles (Fig. 4.1D and Appendix Fig. C1d). A summary of these artifacts can be seen in

Appendix Table C2. The most commonly observed artifacts were elevated stutter and pull-up events. For the majority of additional stutter peaks reported, the -8 stutter allele was called while the -4 stutter allele was visually present, but not reported because it fell under the stutter filter threshold. In this study, the D22, D3, D5, and SE33 markers showed the highest occurrence of elevated stutter peaks (Appendix Table C2). Additionally, two occurrences of incomplete adenylation were observed in two samples amplified with 32 PCR cycles. The STR artifacts observed in this study did complicate the data interpretation, but did not prohibit analysis due to the availability of known reference profiles. However, it is important to note that for forensic casework samples and mixtures these artifacts will likely pose a more serious complication for data interpretation.

Representative STR profiles from a sample (64 pg) amplified with 29, 30, 31, and 32 cycles can be seen in Fig. 4.2 and Appendix Figure C3. As expected, increasing the cycle number had the greatest influence on peak heights and the number of artifacts observed for samples with a larger DNA input, and greater increases in reportable alleles and peak height ratios for those with lower DNA inputs. In general, for samples with <120 pg of input DNA (n=15), the percentage of reportable alleles was significantly improved with each additional PCR cycle (30-32 cycles) while APH, APHR, and the number of artifacts were only elevated with 32 cycles. For samples with 120-500 pg of input DNA (n =15), the percentage of reportable alleles, APH, and number of artifacts were improved or elevated with 32 cycles while no difference was observed in APHR.

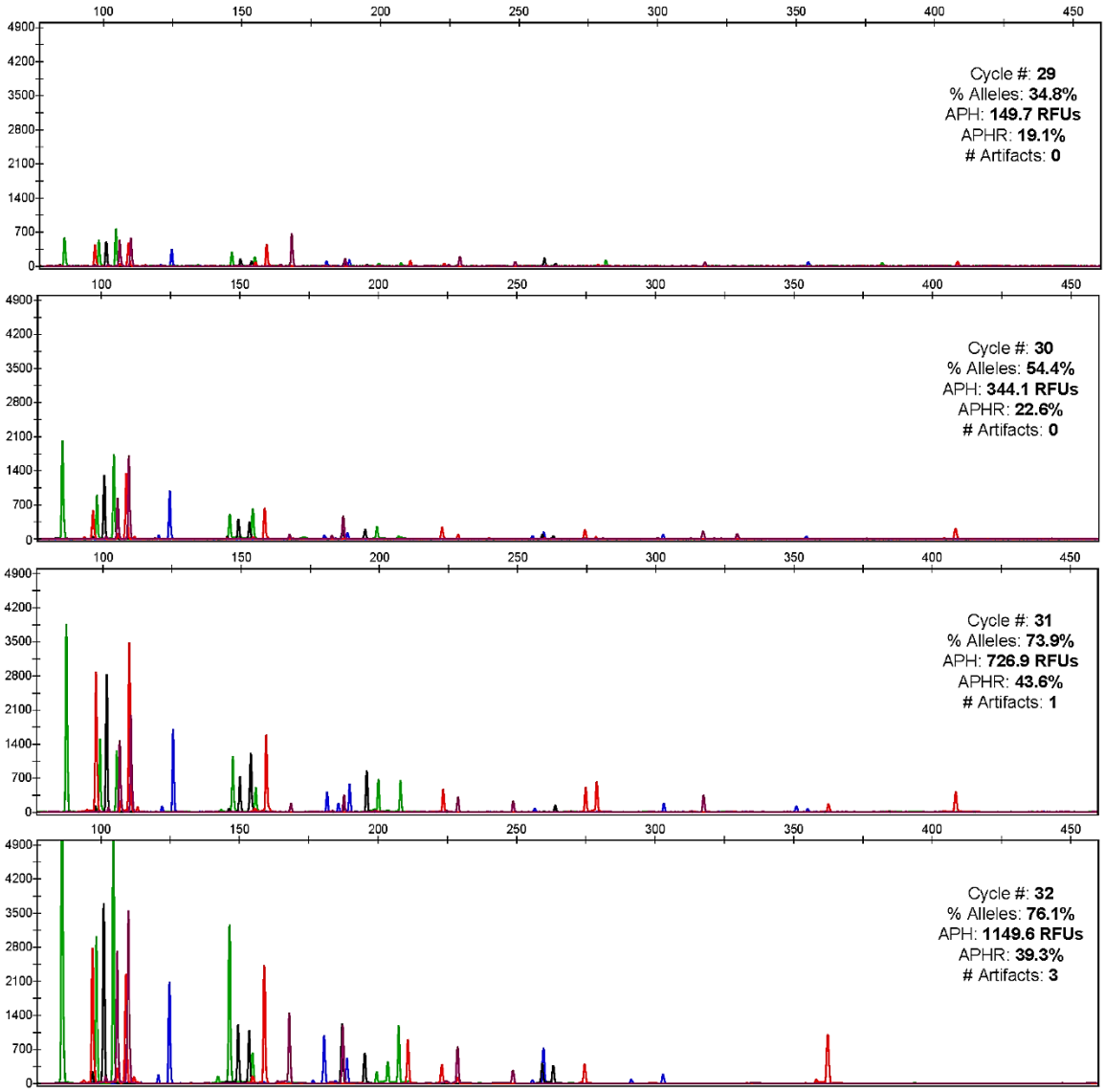

FIG. 4.2 - *Example of electropherograms and comparative STR data for a buried humerus sample (0.064 ng) amplified with 29, 30, 31, and 32 cycles using the GlobalFiler® PCR amplification kit*

Conclusions

Significantly more DNA was recovered from all bone and tooth samples using the PrepFiler® BTA™ Forensic DNA Extraction kit than the QIAamp® DNA Investigator kit. However, regardless of the extraction method, consistently more alleles were recovered from low-template skeletal samples with the addition of extra PCR cycles using the

GlobalFiler® PCR Amplification Kit with minimal adverse STR artifacts in samples amplified with up to 32 cycles. As expected, the improvement observed in STR profile quality with additional PCR cycles was most pronounced in low template samples (<120 pg DNA). At the time of this study, no publications have specifically reported the effectiveness of using additional PCR cycles to genotype low template or skeletal samples with the GlobalFiler® PCR amplification kit. Overall, this study provides supporting evidence that STR success may be increased in low template bone and tooth samples with the addition of extra PCR cycles (up to 32 cycles for less than 120 pg of input DNA and up to 31 cycles for 120-500 pg of input DNA) using the GlobalFiler® PCR amplification kit with minimal effect on data interpretation. With the inherent variability in DNA quantity and quality in skeletal samples, results may differ from laboratory to laboratory and sample to sample; therefore, a thorough internal validation and data interpretation training is necessary before applying these modifications to any type of casework samples.

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CHAPTER V

The performance of quality controls in the Investigator® Quantiplex® Pro RGQ and Investigator® 24plex STR kits with a variety of forensic samples[1]

This dissertation follows the style and format of *Journal of Forensic Sciences*.

[1] Harrel, M., Mayes, C., Houston, R., Holmes, AS., Gutierrez, R., Hughes-Stamm, S. The performance of quality controls in the Investigator® Quantiplex® Pro RGQ and Investigator® 24plex STR kits with a variety of forensic samples. Forensic Sci Int Genet (*In preparation*)

Abstract

Forensic DNA laboratories may process database reference samples on FTA® cards or buccal swabs, which commonly contain adequate amounts of quality DNA for full STR profiles and high first-pass rates. However, some reference samples and many forensic casework samples may be exposed to a variety of insults that may lead to low quantities of DNA, DNA degradation, DNA mixtures, and/or PCR inhibition, which can pose challenges to downstream genotyping success. The inclusion of multiple targets and internal PCR controls (IPCs) in DNA quantification kits and quality sensors within STR amplification kits can aid in the accurate interpretation of sample/profile quality, and guide more efficient rework strategies when needed.

In order to assess the effectiveness of these quality systems we subjected database-like samples (buccal swabs and blood or saliva on FTA® cards), mock casework samples (low-template, degraded, inhibited, DNA mixtures), and authentic post-coital samples to various challenging conditions. Concordance between the quality flags in the Investigator® Quantiplex® Pro RGQ kit (QIAGEN), the QS markers in QIAGEN's Investigator® 24plex QS kit, and overall STR profile quality was evaluated for all casework-type samples. To assess the value of the QS markers in the Investigator® 24plex QS and GO! STR kits, samples with partial or failed STR profiles were reworked based on the quality of the electropherogram 1) with the QS markers redacted, and 2) in conjunction with the QS markers. Results from each of the rework approaches were compared to determine which strategy, if any, improved the STR profile quality and the number of reportable alleles.

Overall, the processing of challenged database and mock casework samples resulted in a wide range of quantification data (when applicable) and STR success

depending on the type of sample. The QS markers in the 24plex STR kits correctly confirmed sample quality in 99.9% of databasing samples and 98.4% of mock casework samples. Quality flags during DNA quantification were concordant with the STR profiles for the majority (77%) of the mock casework samples. Additionally, when samples with partial STR profiles were reworked, more complete profiles were obtained for 80% of the samples regardless of the rework strategy used. However, the most notable improvement in STR completeness was observed in inhibited samples that were reworked based on the information provided by the STR quality sensors, with an average increase of 56% reportable alleles.

Keywords: forensic science; DNA; challenging samples; Investigator Quantiplex Pro RGQ; Investigator 24plex QS; Investigator 24plex GO!; QS markers; rework strategies

The performance of quality controls in the Investigator® Quantiplex® Pro RGQ and Investigator® 24plex STR kits with a variety of forensic samples

Introduction

While many samples submitted for analysis have ample amounts of DNA available for STR typing and produce complete STR profiles, forensic analysts still encounter reference and casework samples that fail or result in incomplete profiles. Common challenges include PCR inhibition (1-3), DNA degradation (4-6), DNA mixtures (7-9), and low amounts of DNA (10-13). Sources of PCR inhibition can be from the collection/preservation method, the substrate, environment, or the sample itself. Endogenous hematin in blood (1-3, 14) and EDTA (3) from blood collection tubes and in FTA® cards are common sources of PCR inhibitors in blood samples submitted to forensic agencies for DNA analysis. With buccal swabs, poor collection techniques may result in inadequate amounts of cells being collected and transferred to the swab and/or paper, and exogenous inhibitors from food and beverages (3) can interfere with STR typing.

Forensic DNA quantification and STR kits are designed to be both sensitive to low amounts of DNA and tolerant enough to overcome many common inhibitions for a wide range of forensic samples. However, when they do fail, samples often need to be reworked in order to generate the most probative DNA result possible. To avoid multiple reworks, as much information about the sample quality and quantity should be known in order to make the most informed decision and apply the most appropriate strategy. For casework and some databasing samples that are processed with the traditional workflow of DNA extraction, quantification, amplification, and capillary electrophoresis (CE), sample quality and quantity information is provided during the quantification step. However, with

databasing samples that are processed using direct amplification without quantification, no quality metrics are known prior to STR typing (14-17). This lack of information can hinder profile interpretation when a sample fails to produce a complete or high quality STR profile, and can make rework determination difficult, especially with ambiguous, low-level, partial and/or failed profiles.

The Investigator® 24plex GO! and Investigator® 24plex QS kits are designed to amplify database and casework samples, respectively (18). In addition to the expanded 20 CODIS core loci and three additional loci (Amelogenin, SE33, DY391), these STR kits also include two quality sensor targets within the purple dye channel (QS1 - 74 bp and QS2 - 435 bp) (19, 20) that serve as an internal PCR control (IPC), similar to those included in many DNA quantification kits. The signal of the QS2 ('S') marker tends to markedly decrease in height (or drop out) with PCR inhibition, compared to the QS1 ('Q') (19, 20), except in the presence of extreme inhibitor concentrations when both markers fail to amplify. The presence/absence of one or both QS markers can often assist in differentiating STR profiles that are of poor quality due to PCR inhibition, DNA degradation, failed amplification or absence of DNA (18-23). Overall, the behavior of these quality sensors may simplify the interpretation of STR profiles and could direct analysts towards the most appropriate rework strategies (21-23).

Investigator® Quantiplex® Pro RGQ is a DNA quantification kit that has been designed to run on a Rotor-Gene Q system (24, 25) and pair with downstream genotyping kits such as the Investigator® 24plex QS kit (25). It is a unique expansion of the Investigator® Quantiplex® Pro kit in that it includes a fifth target; an additional male target that indicates degradation of any male DNA in the reaction. This allows for the

simultaneous detection of human autosomal and Y-chromosomal degradation (24-27) while maintaining high sensitivity, especially for male DNA in the presence of high amounts of female DNA (e.g. post-coital vaginal swabs) (26), and the ability to monitor inhibition with its Internal Control (IC) (24-26). According to the manufacturer (28), the sensitivity of the Investigator® Quantiplex® Pro RGQ IC in the presence of PCR inhibitors mirrors that of the QS markers in the Investigator® 24plex QS kit. Therefore, the IC can be used to accurately predict the presence and influence of PCR inhibitors within a sample. Together, the quality flags in the RGQ kit and the QS markers in the 24plex STR kits should be consistent with each other and accurately reflect the quality of the sample and resultant STR profile.

In this study, the performance of the Investigator® 24plex GO! and Investigator® 24plex QS kits and their QS markers were tested against a wide range of high quality and challenged databasing and casework-type samples. The informativeness of the Investigator® Quantiplex® Pro RGQ kit quality controls were also assessed for a range of casework-like samples (low template, degraded, and inhibited single-source DNA extracts, mock and authentic DNA mixtures). Finally, we investigated any potential benefit in applying the information provided by the STR quality sensors when determining any subsequent rework strategy, to the interpretation of electropherograms (EPGs) with reduced profile completeness. Two approaches were taken: the first was to remove the QS marker information and have external analysts determine a rework strategy based solely on the STR profile quality, and the second was to use the QS markers in conjunction with the overall EPG quality to guide the rework strategy. The STR results for each sample when

these two rework strategies were followed and compared (if different) to see if any improvement in allele recovery or profile quality was achieved.

Materials and Methods

Sample Collection and Preparation

A summary of sample information and chemistries used is listed in Table 5.1. All live-donor samples used in this study were collected from informed and consenting participants pursuant to approved Sam Houston State University IRB 2018-05-40949.

Databasing Samples

Saliva samples were collected using sterile cotton tipped applicators (Puritan, Guilford, Maine, USA), Bode buccal DNA collectors (Bode Cellmark Forensics, Lorton, Virginia, USA), and Whatman® FTA® cards with Easicollect™ devices using manufacturers recommended protocols; hereafter referred to as cotton swabs, Bode swabs, and FTA® saliva samples, respectively. For cotton and Bode swabs, samples were also collected with poor and dirty collection protocols to mimic poor quality samples that may be submitted to laboratories. The poor collection method was a single swipe from one cheek with cotton swabs (*n*=20) and Bode swabs (*n*=30). Dirty collections for cotton (*n*=7) and Bode swabs (*n*=7) were performed using the manufacturer's protocol immediately after subjects had chewed gum or drank coffee.

Whole blood (100 µL) collected from donors (*n*=10) in purple topped 3.0 mL K2E BD Vacutainer blood collection tubes (Beckton Dickinson) was deposited onto Whatman® FTA® cards and allowed to dry overnight. To recreate low blood volume samples, approximately 125 µL - 700 µL of blood (~0.25 - 1 cm in height from bottom of tube) from the original donor collection tube was transferred via BD Vacutainer Safety-Lok blood

collection set needles into unopened purple top tubes. The low blood volume tubes were vortexed to mix with the EDTA before transferring 100 µL onto FTA® cards.

Mock Case Samples

Aged biological stains were simulated by depositing 500 µL of saliva (*n*=5) and blood (*n*=5) on polyester and cotton blend bed sheets. The stains were air-dried overnight before exposure to simulated environmental insults, described in section "Simulated Environmental Insults on Samples".

DNA extracts identified as low template, inhibited, or degraded were also used in this study. Low template samples were sourced from touch DNA collected from handled rifle magazines using cotton CEP® Swabs (*n*=12) (FITZCO, Spring Park, Minnesota) and nylon FLOQSwabs™ (*n*=12) from Copan (Murrieta, California, USA). Low template and/or degraded DNA was also recovered from skeletal samples (*n*=20).

Inhibited (*n*=20) and degraded (*n*=10) DNA samples were sourced from muscle tissue and swabs used to collect DNA from decomposing human bodies, including unpreserved nylon swabs and muscle tissue preserved in Tent buffer (10mM Tris, 10mM EDTA, 1M NaCl, 2% Tween 20; 100 mL, pH 8.0). In addition, samples (*n*=12) spiked with the inhibitors hematin (*n*=3, 500-1500 ng/µL), melanin (*n*=3, 25-60 ng/µL), humic acid (*n*=3, 100-400 µM), and 70% ethanol (*n*=3, 1-3 µL/PCR reaction) were made using neat inhibitor stocks and TaqMan® control genomic DNA (Applied Biosystems).

Mock sexual assault samples were created by adding semen to female vaginal swabs. Male donors were provided a specimen container and asked to collect one emission of semen in the privacy of their own home. Female donors were provided cotton swabs and asked to collect vaginal material by swabbing for 20-30 seconds in the privacy of their own

home. Neat semen from two male donors was diluted with laboratory grade water to 1:25, 1:50, 1:100, 1:250, 1:500, and 1:1000. From these dilutions, 20 µL of solution was added onto half of a vaginal swab. The dilution series was used with 4 different sets of female vaginal swabs for a total of 24 samples. Additionally, post-coital vaginal swabs (*n* =4/donor) were also collected from two female donors with collections ranging from 9 hours to 7 days post-coitus.

Simulated Environmental Insults on Samples

Samples stored in hot and humid conditions were placed in an oven at 37 °C with open sources of water to create high levels of humidity. The cotton swabs (*n*=20), Bode swabs (*n*=20), and FTA® blood and saliva cards (*n*=10 each) were sampled from hot and humid conditions at various timepoints between 10 and 32 weeks to screen for degradation. Mock casework saliva- and blood-stained cloths were stored for 16 weeks in this same hot and humid environment.

A set of Bode (*n*=20) and saliva FTA® saliva (*n*=10) swab samples were placed uncovered in a UVP CL-1000 ultraviolet crosslinker (AnalytikJena, Upland, California, USA). Bode swabs were exposed to UV radiation for 2.5, 5, 10, and 15 mins (*n*=5 donor swabs per timepoint; 1 punch per swab) and FTA® saliva samples (*n*=10) exposed to UV radiation were sampled after 1, 2.5, 5, 10, and 15 mins of exposure (*n*=2 donor cards per timepoint; 10 replicate punches per card). Cotton swabs (*n*=20) were degraded similarly under a built-in UV lamp in an Optimizer PCR Workstation (C.B.S, San Diego California, United States) with exposure times of 6, 12, 18, and 24 hours (*n*=5 donor swabs per timepoint; full swab consumed).

TABLE 5.1 – *Summary of sample information and chemistries used in this study*.

SAMPLE	DETAILS	NO. SAMPLES	EXTRACT.	QUANT.	PCR AMP.
COTTON SWAB	Room Temp	20	N/A	N/A	24plex GO!
	Hot and Humid; 37°C, 13-27 weeks	20	N/A	N/A	24plex GO!
	Poor Collection	20	N/A	N/A	24plex GO!
	Dirty Collection; Gum, coffee	7	N/A	N/A	24plex GO!
	UV Exposure; 6-24 hours	30	N/A	N/A	24plex GO!
BODE BUCCAL	Room Temp	20	N/A	N/A	24plex GO!
	Hot and Humid; 37°C, 9-12.5 weeks	20	N/A	N/A	24plex GO!
	Poor Collection	30	N/A	N/A	24plex GO!
	Dirty Collection; Gum, coffee	7	N/A	N/A	24plex GO!
	UV Exposure; 2.5-15 mins	20	N/A	N/A	24plex GO!
FTA® SALIVA	Room Temp	150	N/A	N/A	24plex GO!
	Hot and Humid; 37°C, 13-27 weeks	100	N/A	N/A	24plex GO!
	UV Exposure; 1-15 mins	100	N/A	N/A	24plex GO!
FTA® BLOOD	Room Temp	150	N/A	N/A	24plex GO!
	Hot and Humid; 37°C, 13-27 weeks	100	N/A	N/A	24plex GO!
	Low Blood Volume (LBV); 0.25-1 cm	100	N/A	N/A	24plex GO!
	LBV Direct Amp; 0.25 cm no lysis buffer	20	N/A	N/A	24plex GO!
STAINED CLOTH	Blood; 37°C, 16 weeks	5	EZ1 Investigator	Investigator Pro RGQ	24plex QS
	Saliva; 37°C, 16 weeks	5	EZ1 Investigator	Investigator Pro RGQ	24plex QS
INHIBITED	Identified	20	Varied	Investigator Pro RGQ	24plex QS
	Spiked	12	N/A	Investigator Pro RGQ	24plex QS
TOUCH	Nylon Swab	12	QIAamp Investigator	Investigator Pro RGQ	24plex QS
	FloQ Swab	12	QIAamp Investigator	Investigator Pro RGQ	24plex QS
SKELETAL		20	Total Demin.	Investigator Pro RGQ	24plex QS
DECOMP.		10	Varied	Investigator Pro RGQ	24plex QS
MOCK SEXUAL ASSAULT	Cotton Swab; 1:25- 1:1000	24	EZ1 Investigator	Investigator Pro RGQ	24plex QS
POST COITAL	Cotton Swab; 9 hrs-7 days	8	EZ1 Investigator	Investigator Pro RGQ	24plex QS

DNA Extraction

Mock Case Samples

From the biologically stained cloths, 0.5 cm^2 cuttings were added to 490 µL of ATE buffer and 10 µL proteinase K (QIAGEN) and incubated at 56 °C for 15 mins. The resulting lysate was purified using the Large Volume Protocol on an EZ1 Advanced XL automated extraction platform (QIAGEN) with a 40 µL elution volume.

Mock sexual assault samples and post coital samples underwent manual differential separations with the sperm and epithelial fractions extracted separately using automated protocols on the EZ1 XL. Briefly, 480 µL of G2 solution and 20 µL of proteinase K (QIAGEN) were added to a half-swab cutting. The cuttings were then lysed at 56 °C and 900 RPM for 1.5 hours on a thermal shaker. After removing the cutting from the lysis tube, the lysate was centrifuged at 15,000 RPM to pellet the sperm fraction. The epithelial fraction (~460 µL) was then aspirated and added to a 2 mL sample tube containing 400 µL of MTL buffer (QIAGEN) before performing DNA purification on an EZ1 XL using the Large Volume Protocol with an elution volume of 40 µL in TE. The sperm fraction was processed according to the Pretreatment for Epithelial Cells Mixed with Sperm Cells protocol of the EZ1 DNA Investigator® Handbook (29) and purified with the EZ1XL Trace Sample Protocol with resulting DNA eluted in 40 µL of TE buffer.

DNA Quantification and Amplification

A QIAGEN QIAgility® liquid handling platform was used to prepare all samples for quantification and amplification. All mock case samples were quantified using the Investigator® Quantiplex® Pro RGQ kit on a QIAGEN Rotor-Gene® Q platform with Q-

rex software as per manufacturer's guidelines. Results were analyzed with the QIAGEN Data Handling Tool and data were accepted with an R^2 value > 0.99.

Databasing samples were amplified with no quantification using the QIAGEN Investigator® 24plex GO! chemistry. An overview of workflows for cotton and Bode swabs, as well as saliva and blood FTA® cards, can be seen in Appendix Figures D1 and D2, respectively. Both cotton and Bode buccal swabs were amplified according to the manufacturer's guidelines in the Investigator® 24plex GO! Handbook with the cycling number modified to total 26 PCR cycles. Full swab heads were lysed and consumed for each cotton swab and a single 1.2 mm punch was taken from each Bode swab.

For both saliva and blood FTA® cards, a single 1.2 mm punch was deposited in a 0.2 mL 96- well plate with 10 punch replicates per sample. Using the QIAgility, 20 µL of STR GO! Lysis Buffer was directly added to the punches. This was then centrifuged and incubated at 95 °C for 5 mins. The QIAgility was then used to add 2 µL of the appropriate crude lysate to 20 µL of 24plex GO! Master Mix for FTA® samples. Additionally, a subset of 10 low blood volume FTA® samples were directly amplified in duplicate without use of the GO! Lysis buffer. All FTA® blood and saliva samples were amplified with a total number of 27 PCR cycles.

All mock case work samples targeted a final input DNA per reaction of 0.8 ng. Samples were amplified with the QIAGEN Investigator® 24plex QS kit according to manufacturer's protocol on either a ProFlex (Thermo Fisher, Waltham, Massachusetts, USA) or Veriti (Applied Biosystems, Foster City, California, USA) thermal cyclers.

Capillary Electrophoresis and Data Analysis

Amplified fragments were separated and detected on an Applied Biosystems 3500 Genetic Analyzer (Thermo Fisher Scientific) according to manufacturers recommended guidelines. Data analysis was completed using GeneMapper IDX v1.4 (Thermo Fisher Scientific) with tertiary analysis being accomplished with in house excel workbooks. Stochastic and analytical thresholds were set at 200 RFU and 100 RFU, respectively.

Quality Metrics Concordance Study

Following STR data analysis, any discrepancies between information provided by the qPCR quality control flags, QS markers, and the overall STR profile quality were identified for all mock case samples. The categories of incorrect flags or information mismatches relative to the qPCR quality controls were as follows: unidentified degradation, inflated DI, unidentified inhibition, false inhibition flag, unidentified mixture, false mixture flag, and more than one incorrect. “Unidentified” categories were defined as having an issue observed in the STR profile, either based on the QS markers or visual inspection, but were not flagged during qPCR. Conversely, when an issue was flagged during qPCR, but not observed in the STR profile it was labeled as a “false” flag. To note, any sample that did not have a DNA concentration at one or both of the male targets was automatically flagged as having “possible male degradation” by the Data Handling Tool. This meant that all female samples had this quality flag, but this was not considered as an occurrence of an “incorrect flag” for the purpose of this study.

Sample Reworks

A sub-set of single-source database and mock casework samples with <90% reportable alleles (n=79) after amplification were further evaluated to test the applicability

and benefits (if any) of the information provided by the QS markers. This was achieved by comparing the first-pass results to those of secondary amplifications performed based on rework strategies that were determined either with or without the quality sensor information. First, electropherograms with the QS markers redacted were provided to external forensic DNA analysts to remove the potential for bias. Basic information necessary for the determination of the appropriate rework strategy, such as sample group (databasing vs casework) and type (blood vs buccal vs DNA extract), substrate (cotton/Bode swab vs FTA® card), and quantification data (if applicable), was also provided. Using their experience, the analysts were asked to identify what they thought was causing any problems observed in the STR profile, and using their judgement and laboratory's SOPs, what their approach to additional analysis (if any) would be based on the overall quality of the STR profile. This rework strategy was then compared to how other analysts chose to reanalyze the same samples when electropherograms were assessed with the QS markers included.

Samples were categorized as being either inhibited, low template and/or degraded, no DNA, or a failed amplification. When assessing EPGs with the QS markers included, inhibition was suspected and/or confirmed when one or more of the following occurred: possible inhibition was flagged during qPCR (IPC shift of 1+ cycles); the S/Q ratio fell between 20-70% with some visual indicators of inhibition throughout the EPG; the S/Q ratio fell below 20%; or one or both QS markers failed to amplify. Low template/degraded samples were classified when consistently low peak heights (average peak heights of approximately <750 RFUs) and/or a ski slope effect throughout the EPG were observed

with balanced QS markers. Finally, failed amplifications due to no DNA template had no alleles present throughout the EPG except for balanced QS markers.

After determining an approach for each sample, the two rework strategies were compared and performed on all samples (if different). Databasing samples labeled as low template/degraded were reworked with an increased template by either adding an additional microliter of lysate (total of 3 μL) if available, or two 1.2 mm punches in the amplification reaction. However, for casework samples requiring additional template in the PCR, if the maximum input had already been used, no rework was performed. Inhibited samples were either diluted 1:3, or a punch was washed with GO! lysis buffer before reamplifying. For a failed amplification reaction, samples were processed in the same manner as before either from the original lysate/extract or a new punch. No PCR cycling or CE parameters were changed for any rework strategy.

Results and Discussion

First-Pass Results

Databasing Samples

A summary of first-pass results for all databasing samples is shown in Figure 5.1. The control group consisted of swabs or FTA® cards collected and then stored at room temperature. All other samples were intentionally exposed to challenging conditions to create a range of compromised samples in order to test the benefits of the quality sensors. All room temperature controls for cotton and Bode buccal swabs generated full STR profiles on the first amplification (Fig. 5.1). All but a single replicate from one FTA® card resulted in full STR profiles for punches taken from the center of FTA® blood cards stored at room temperature (Fig. 5.1). However, direct amplification from saliva on FTA® saliva

cards resulted in a lower first-pass (83.3% of samples) (Fig. 5.1) with an average of 93.8% reportable alleles. The observance of reduced or variable STR success for saliva samples stored on FTA cards compared to that of blood has been previously reported as likely due to inhibitors within the saliva (30), low amounts of collected cells (31), or cells clumping in different areas of the substrate (32). A majority of the sample replicates that failed to generate full STR profiles were attributed to two of the ten donors, which could be due to a natural variation in shedding propensity between donors and/or the collection process itself as swabs were self-collected by the donors. QS markers did not indicate inhibition (all S/Q ratios >20%) for any room temperature sample (Appendix Fig. D3). Overall, with the exception of some FTA® saliva samples, STR profiles for room temperature controls were well balanced (average PHR >80%), with average peak heights greater than 1000 RFUs.

First-pass rates for swabs and FTA® cards stored in a hot and humid environment ranged from 65-85% of samples generating complete profiles (Fig. 5.1). Sub-sampling from two of the FTA® saliva donor cards after 13 weeks in hot and humid conditions yielded notably different results from the others. While the rest of the donor cards averaged over 95% reportable alleles, one donor card had an average of 63.4% reportable alleles and the other had no reportable alleles for any of the replicate punches. The Q/S ratios did not indicate PCR inhibition; therefore, it was likely that little or no DNA was present on the cards. It was not possible to re-collect those samples from the donors, and therefore, these data from those donor cards were excluded from the remainder of the study. However, this observation was a perfect example of when the QS markers were able to inform the analyst

that no DNA was present (as opposed to severe inhibition) and therefore avoided unnecessary reamplification of those samples.

For the remaining FTA® cards, overall profile quality declined with prolonged exposure to heat and humidity, but saliva samples consistently generated complete (or near complete) and balanced profiles on average (>90% alleles; >70% average peak height ratios), regardless of the collection substrate, for up to 32 weeks (Appendix Fig. D4-5). However, blood preserved on FTA® cards showed to be the most susceptible to hot and humid conditions as the average number of reported alleles dropped from nearly 100% after 10-14 weeks of storage, to less than 75% after 24-27 weeks of storage (Appendix Fig. D4) and profiles became less balanced with an average PHR of 55% (Appendix Fig. D5).

For samples subjected to UV damage (cotton, Bode, and FTA® saliva/buccal swabs), first-pass success rates ranged from 0-50% (Fig. 5.1), with cotton swabs consistently yielding more reportable alleles on average compared to Bode and FTA® samples. Cotton swabs also required much longer exposure to UV light than the Bode swabs and FTA® cards before allele dropout was observed (several hours vs 2.5 mins). These results may be explained by fact that the surface area and composition of cotton swabs likely allow for more protection against UV damage compared to Bode and FTA® cards, where DNA is trapped on a flat and relatively exposed surface. As expected, the overall quality of STR profiles for each sample type decreased with increasing UV exposure (Appendix Fig. D6-7). For Bode swabs and FTA® samples, no full profiles were observed after 2.5 mins of exposure to UV light (Appendix Fig. D6). However, full profiles were obtained with cotton swabs through 24 hours of exposure (Appendix Fig. D6).

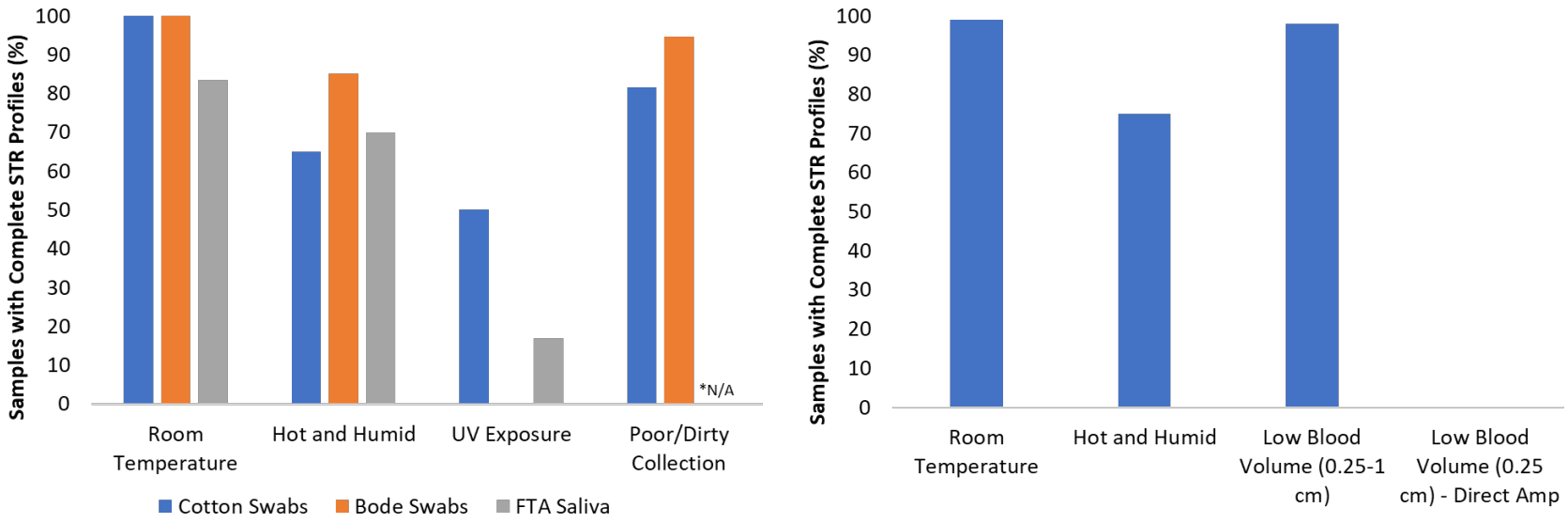

FIG. 5.1 - *First-pass success rates for environmentally challenged databasing samples: buccal swabs (left) and blood on FTA® cards (right). First-pass success rate was defined as the percentage of samples that generated complete STR profiles using the Investigator® 24plex GO! Kit (QIAGEN). *N/A indicates that the insult category was not tested for that substrate. (Cotton* - n = *20,* n = *20,* n = *20,* n = *27,; Bode* - n = *20,* n = *20,* n = *20,* n = *37; FTA saliva* - n = *150,* n = *100,* n = *100; FTA blood* - n = *150,* n = *100,* n = *100,* n = *20, respectively).*

While these conditions may not accurately reflect real-life UV exposure, they demonstrate the differences in susceptibility to UV damage between the substrate types and the performance of the QS markers. The QS markers did not indicate the possibility of inhibition for any hot and humid samples, but did accurately confirm that allele dropout was likely due to DNA degradation in all but one sample. The single UV degraded swab that flagged possible inhibition had an S/Q ratio of 19%, which is just below the 20% threshold, but still resulted in a complete reportable profile. When the EPG was interrogated further, there appeared to be potential chromatography issues, so the sample was simply reinjected instead of fully reworked. As a result, the overall profile quality was resolved and the S/Q ratio was restored to 86% (Appendix Fig. D8). This case demonstrates that rather than using the 20% S/Q ratio as a strict threshold for rework determination, the QS information can support the analysts' best judgement and experience for the most appropriate strategy moving forward to avoid any unnecessary reamplification.

The first pass rate for FTA® blood cards spotted with blood from EDTA tubes containing 0.25 - 1 cm of blood was 98% when processed with the GO! Lysis Buffer prior to amplification. However, when the 0.25 cm blood collection cards were directly amplified without a pre-lysis step, no full STR profiles were obtained, with the percentage of reportable alleles ranging from 0 - 78% (Appendix Table D1). According to the Investigator® 24plex GO! Handbook (20), an S/Q ratio of <20% is indicative of inhibition. Although not explicitly stated, a high degree of PCR inhibition could also be assumed with the complete dropout of the larger quality sensor ('S'), and inhibition must also be considered when dropout of both quality sensors occur (severe inhibition that stalls PCR completely). The direct amplification sample group was the only set of databasing samples

that had one or both quality sensors drop out, which occurred for both duplicate punches (Appendix Table D1).

We did observe one instance of an S/Q ratio <20% for a lysed 0.25 cm low blood volume sample, as well as a previously mentioned UV-exposed cotton swab (with 19% Q/S ratio). However, for both of these samples 100% alleles were reported with high average peak heights (>3300 RFUs), and balanced heterozygous peak height ratios (>85%), which demonstrates how the information provided by the quality sensors is not an absolute determination and should be considered in conjunction with the STR profile as a whole during the data interpretation process.

When swabs were used to mimic sub-optimal collection techniques (a single swipe of one cheek or when the subject had chewed gum or drank coffee immediately before collection), full profiles were generated from all but four cotton and two Bode buccal swabs (Fig. 5.1). The poor collection swabs that did not yield full profiles had notably less amplified DNA (average peak heights <250 RFUs), but nevertheless resulted in over 80% reportable alleles. These results may be expected due to the small number of buccal cells (and saliva) that were likely collected. The sample that yielded the worst result was a cotton buccal swab collected after an individual chewed cinnamon gum. Only five alleles (11.4%) were reportable, but the S/Q ratio was 70%, confirming that a low amount of DNA was likely collected or released during lysis, and that PCR inhibition was not likely a contributing factor.

Mock Case Samples – Single Source

A range of low template and/or degraded samples were represented by a set of previously extracted skeletal (*n*=20) and touch (*n*=24) items that resulted in first-pass rates

of 60% and 20.8% samples generating full profiles, respectively (Fig. 5.2). For the skeletal samples, one DNA extract had a concentration of 2.97 ng/µL while the rest ranged from 0.013 – 0.457 ng/µL. All of the touch samples had DNA concentrations of less than 0.031 ng/µL. In addition to having higher DNA concentrations, DNA from the skeletal samples was more degraded than the touch DNA, with degradation indices (DIs) ranging from 2.7 – 168.2 versus 1.0 - 7.8. However, it should be noted that the larger DIs (DI > 50) for the skeletal samples may be artificially inflated due to also being in very low amounts of DNA (0.01-0.03 ng/µL) and stochastic effects during amplification. Additionally, four low template samples had a DNA concentration of either 0.0 or 'unknown' for the Human Degradation target (long autosomal target) and therefore a DI could not be calculated. No inhibition was detected during DNA quantification, as determined by the Investigator® Quantiplex® Pro RGQ inhibition index (all IPC ΔCT shifts were <1 cycle). As there was more DNA template available for amplification in the skeletal extracts, it was not unexpected that more alleles on average were reported for skeletal samples (90%) compared to touch samples (60%). Nevertheless, all samples generated at least a partial profile (>10% alleles) and full STR profiles were obtained with as little as 0.05 ng DNA. Finally, with the exception of a single skeletal DNA extract, no PCR inhibition was indicated by the STR Quality Sensors, which was concordant with the information provided by the IPCs during DNA quantification. In all other low template samples, the QS markers also confirmed that the observed allelic dropout was due to low amounts of DNA and/or degradation rather than inhibition.

Degraded samples were extracted from decomposed human muscle tissue (n=10) and blood or saliva stains on cloth (n=10) that were artificially degraded in a hot (37 °C)

and humid oven for 16 weeks. DNA concentrations averaged 14.2 ng/µL and 0.8 ng/µL for decomposed tissues and degraded stains, respectively. With the high amount of DNA available, the target amount of DNA for PCR (0.8 ng) amplified for all but one sample, which was the only sample to generate a profile with less than 97% reportable alleles. In fact, this one sample failed to amplify any alleles despite having a DNA input of 0.3 ng and no indicator of inhibition during DNA quantification or when the QS peaks were examined (Q/S ratio of 130%). Therefore, this sample was selected to be further evaluated during the Sample Reworks phase of the study. Although full STR profiles were generated for most samples and the DIs were not high enough to flag degradation (all DIs were < 10) during DNA quantification, the characteristic "ski-slope" effect was still observed throughout the EPGs. No PCR inhibition was indicated during DNA quantification or STR amplification as determined by the IPCs or QS markers respectively.

A variety of common PCR inhibitors (melanin, hematin, humic acid, and ethanol) in a range of concentrations, and DNA extracts that had been previously identified as inhibited were also examined. These samples produced two of the lowest first-pass rates for all of the mock casework samples with only 5% of soft tissue DNA extracts (n=20) and 50% of spiked DNA samples (n=12) resulting in full STR profiles (Fig. 5.2). During DNA quantification, inhibition was flagged for all of the tissue extracts and the samples with high and moderate concentrations of hematin and humic acid. Due to the high levels of inhibition, the longer ("degradation") human and male targets failed to amplify (with the exception of the moderate concentration hematin sample), which resulted in DIs not being able to be calculated. Although these samples would normally be diluted and then re-quantified to determine a more accurate DNA concentration, for this study we wanted to

assess the full effects inhibition would have on the QS markers in the STR kit. Therefore, samples that flagged as inhibited during qPCR were not pre-diluted unless it was required to bring the DNA concentration to the 0.8 ng target for STR amplification.

The Investigator® 24plex QS kit was highly tolerant to the low concentrations of ethanol, hematin, and humic acid as well as all three concentrations of melanin used in this study (Appendix Fig. D9). However, despite a full profile being generated for the high melanin concentration, the S marker dropped to 12.7% of the Q marker (falling below the 20% threshold for detecting PCR inhibition), and visible signs of inhibition (inter- and intra-locus allele imbalance) were apparent. Partial inhibition was observed for the moderate and high concentrations of humic acid and moderate concentration of hematin, with samples resulting in 10-99% reportable alleles. The moderate and high concentrations of ethanol, and high concentration of hematin, resulted in near (<10% alleles) or complete (0% alleles) inhibition (Appendix Fig. D9). Finally, only two of the tissue extracts resulted in any alleles being amplified (>97% alleles) as these samples were the only extracts that required dilution prior to amplification; the rest were completely inhibited and failed to amplify any alleles. As anticipated, a wide range of QS values and STR profile completeness were observed with these samples containing various levels of common PCR inhibitors (Fig. 5.3). When full STR profiles were obtained, both of the QS markers were present, as expected. However, in samples with sufficient DNA template in the PCR, dropout of the S marker mirrored the amplification failure of the larger loci, while the Q marker did not drop out until severe inhibition was observed across all loci (<10% alleles). This behavior of the QS markers in the presence of increasing concentrations of inhibitors has also been previously reported (18, 33, 34).

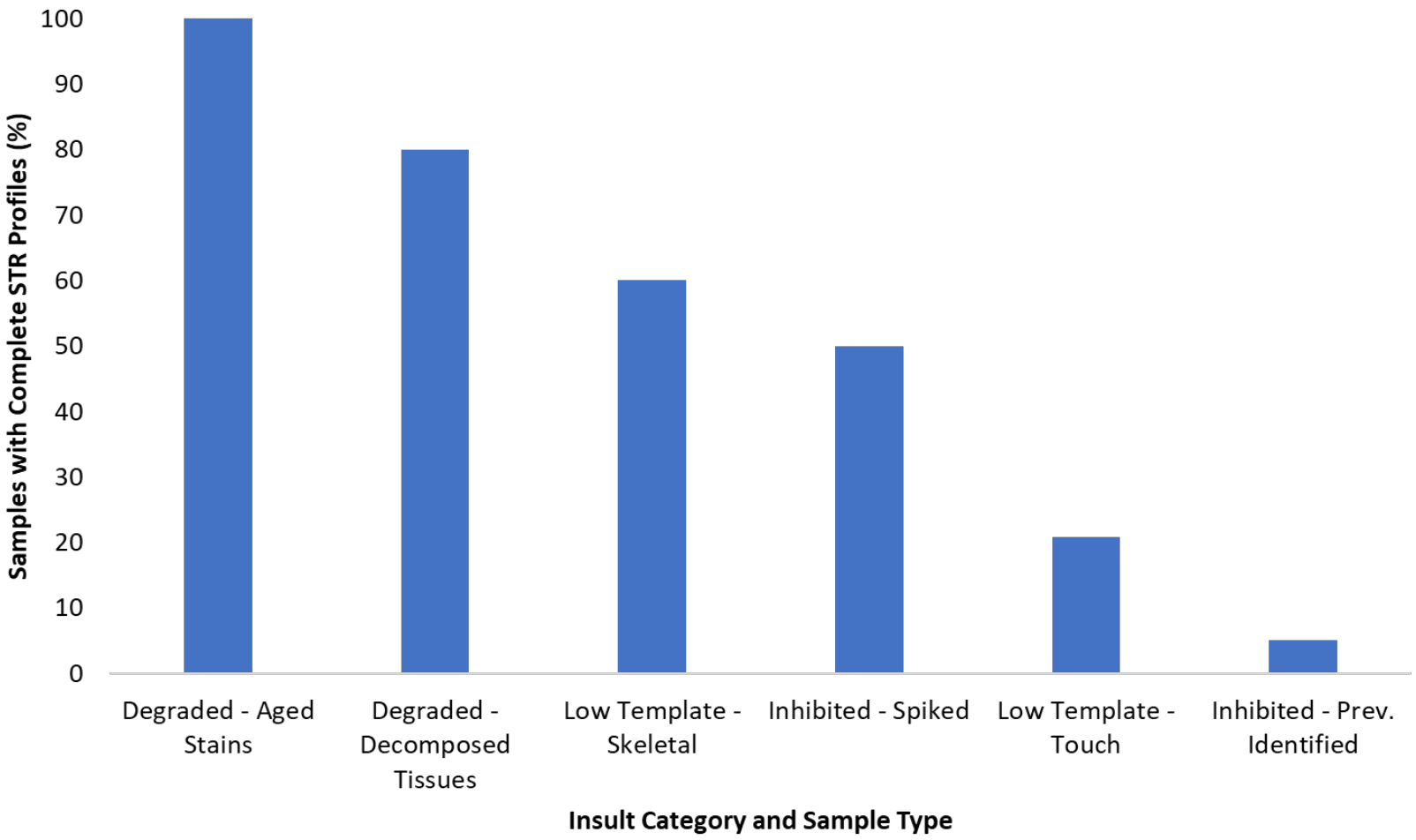

FIG. 5.2 - *First-pass success rates for single-source mock casework samples based on insult category and sample type. First-pass success rate was defined as the percentage of samples that generated complete STR profiles using the Investigator® 24plex QS Kit (QIAGEN).* (n = *10,* n = *10,* n = *20,* n = *12,* n = *24,* n = *20, respectively).*

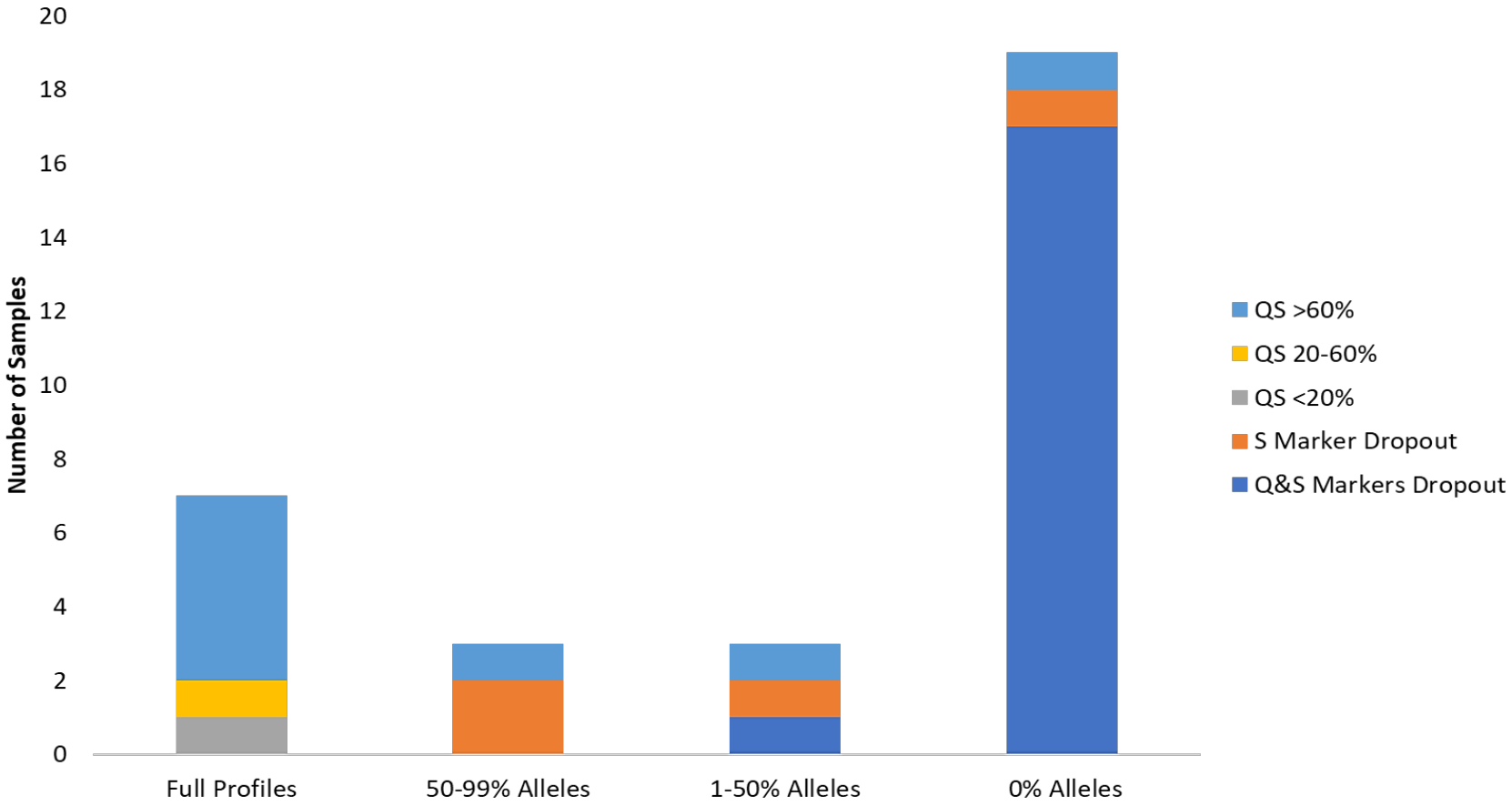

FIG. 5.3 - *Distribution of QS values for inhibited mock casework samples (*n*=32) based on STR completeness.*

Mock Case Samples – Mixtures

As expected, the amount of male DNA recovered in the sperm fractions decreased as the serial dilution of neat semen (Appendix Fig. D10) and the time post-coitus (Appendix Fig. D11) increased. The female epithelial fractions were not STR-typed for this study. Despite the multiple wash steps for the sperm fractions, there was still a high proportion of female carry-over (>5:1 ratio) for nearly half of the mixture samples, with a consistently low recovery of male DNA (<0.06 ng/μL on average). This negatively affected the overall recovery of male alleles as some of the extracts then required dilution prior to amplification, further reducing the amount of male DNA available for amplification. Although not tested in this study, the use of Y-STRs may have led to more probative information in these cases as a dilution would not be necessary, allowing for maximal male DNA for amplification. Previous studies have shown the benefits of Y-STR typing for low-level male:female mixtures, with successful detections of male alleles from samples with mixture ratios as high as 1:4000 (35-38). Additionally, Y-STRs might also provide an opportunity to better observe a correlation between any male degradation flagged during quantification and any degradation present in the profile (26, 27) for mixed samples such as these.

The number of shared alleles between the male and female donors in the dilution series group ranged from 10-16 alleles and sperm fraction human to male ratios ranged from 0.76:1 to 45.9:1. Obligate male alleles were able to be detected in STR profiles with as little as 15 pg of male input DNA and a human to male ratio of 24:1 for a semen dilution sample of 1:1000. However, across the dilution series for sperm fractions with a human to male ratio greater than 9:1, no more than two obligate male alleles were detected. Of the

24 sperm fractions, four full male profiles were obtained for 1:25 and 1:50 semen dilutions, no male alleles were detected in the STR profiles for two of the 1:1000 semen dilutions, and the rest had partial male profiles ranging from 2% to 91% of obligate male alleles detected (Fig. 5.4).

For the authentic post-coital samples, male DNA was detected as late as 5 days post-coitus (0.003 ng/μL); however, more than a single male allele was only amplified only until day 3 post-coitus, and a full male profile only being obtained after 9 hours post-coitus (Fig. 5.5). Major and minor contributor alleles were distinguishable for three of the sperm fractions at 9 hrs, 12 hrs, and 3 days post-coitus. This is consistent with other studies that show that 2-3 days post-coitus is typically the limit for obtaining male autosomal STR alleles (7, 9, 39). However, the use of Y-STRs provide more complete male profiles (7-9) and may be able to extend the detection window as far as 7-9 days (8, 9).

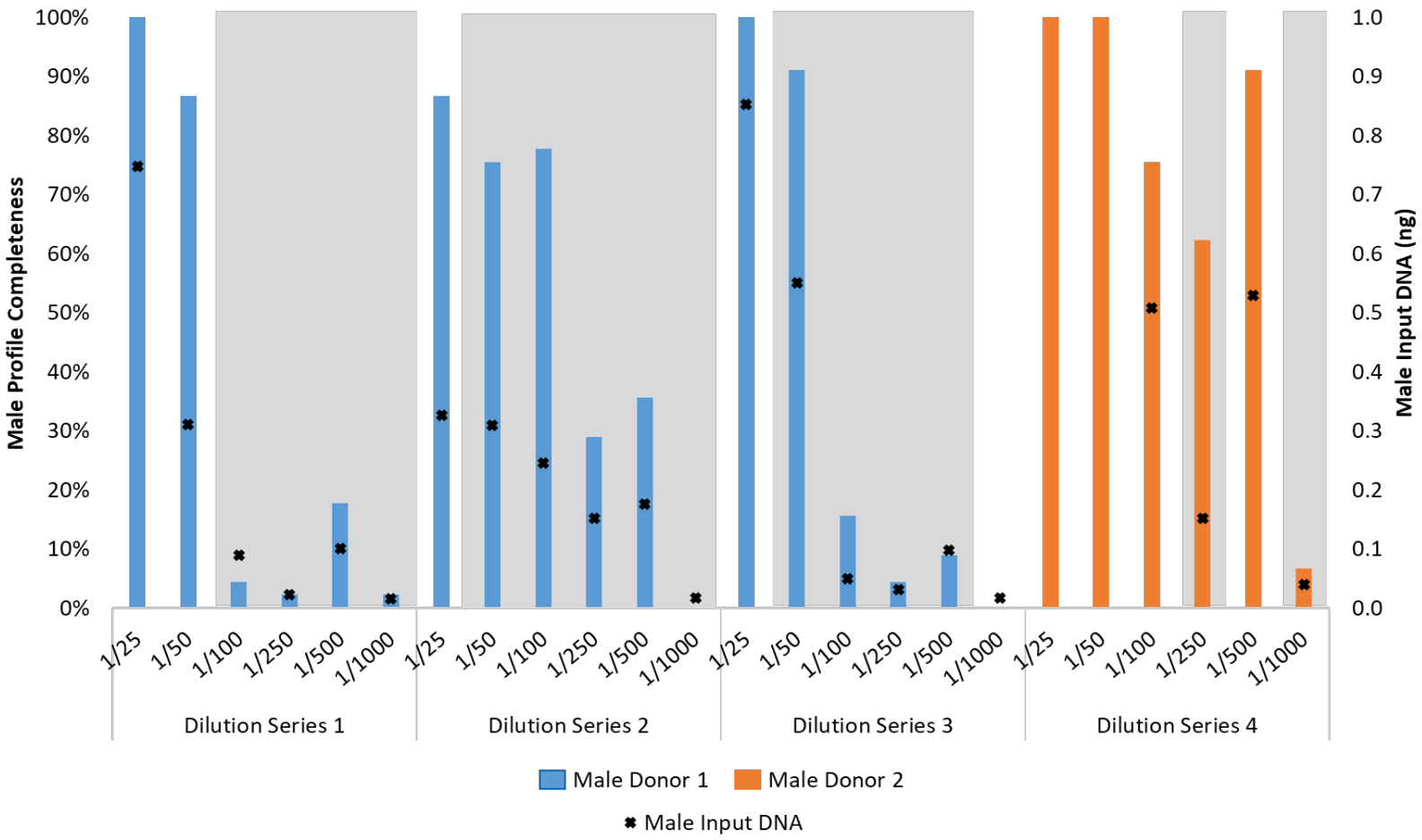

FIG. 5.4 - *Male profile completeness observed in the sperm fractions (*n*=1 each) of mock sexual assault samples based on the dilution of semen, and amount of male DNA amplified. Semen from two male donors were used for the dilution series. Bars with a shaded background denote samples that were flagged as mixtures during DNA quantification.*

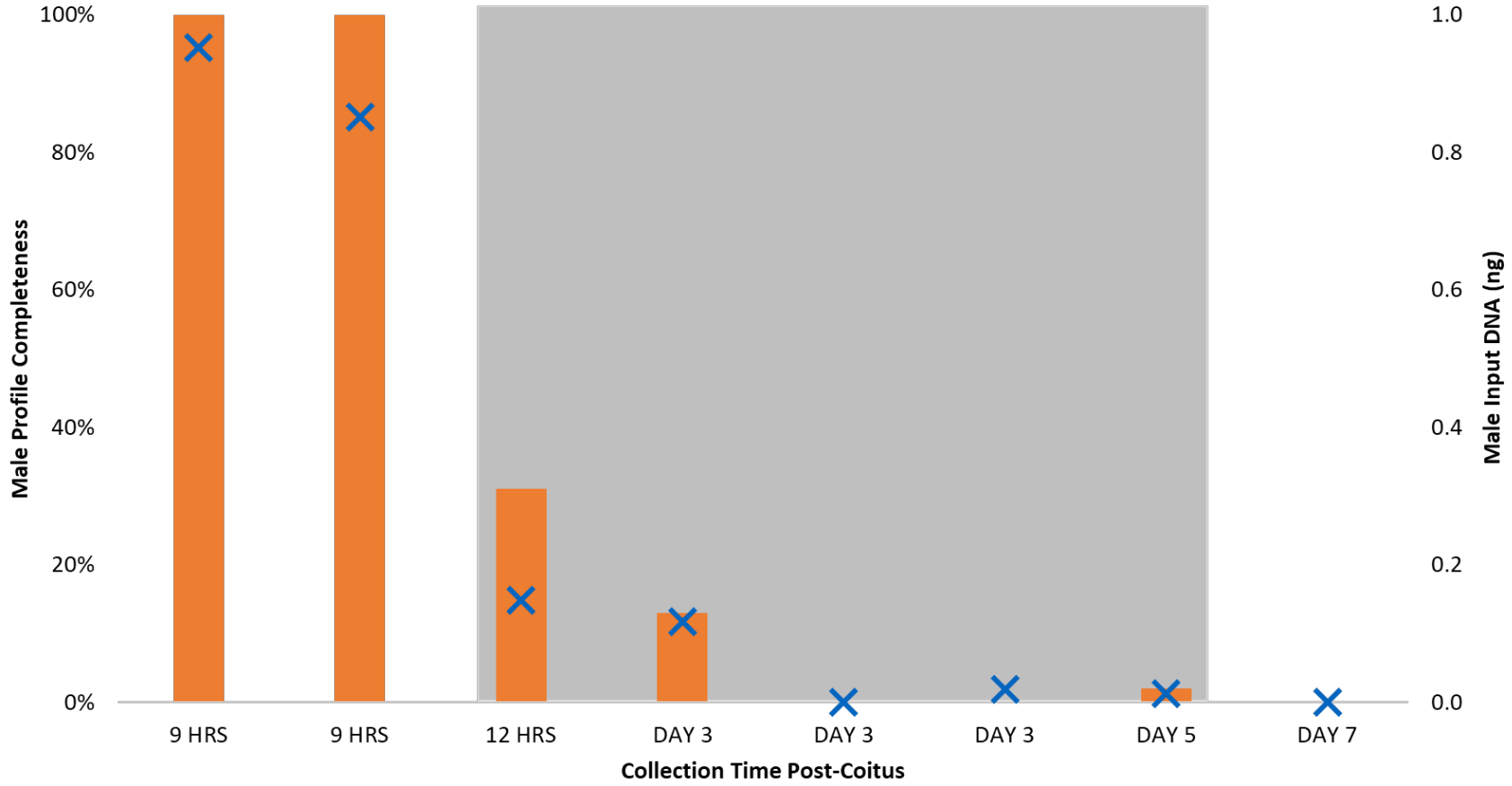

FIG. 5.5 - *Male profile completeness observed in the sperm fractions of authentic post-coital samples (*n*=1 each) after (9 hrs, 12 hrs, 3, 5, and 7 days), and amount of male DNA amplified. Bars with a shaded background denote samples that were flagged as mixtures during DNA quantification.*

Concordance Study

Concordance between quality flags during qPCR in the RGQ kit, the STR quality sensor information, and the overall profile quality was evaluated. Of the 133 mock casework samples in this study (single-source and mixtures), 103 showed full concordance between the two internal quality control metrics and the overall STR profile (Fig. 5.6). As the Investigator® Quantiplex® Pro RGQ kit is relatively new to the market, limited research has been published regarding the predictability of the qPCR data for STR typing. However, the few studies that have published these kinds of data report a high concordance between the two sets of metrics (25, 26, 40). In this study, the most common mismatch observed was when degradation in the STR profile was not detected during quantitation (6% of samples), followed by false inhibition flags (4.5% of samples flagged as inhibited during qPCR but not seen in STR profiles) and unidentified mixtures (mixture seen in profile but not detected during qPCR) (Fig. 5.6). Other, less frequent discrepancies, included inhibition not detected during qPCR (2.3%), two mismatched flags (e.g. false inhibition plus unidentified mixture), and a single instance of a false mixture being flagged for a known single-source male sample with no extra alleles in the EPG.

The samples with unidentified degradation (not flagged during quantification) showed a visible ski-slope effect throughout the profile, and although no allele dropout had occurred, the allele peak heights at the larger loci were reduced to less than half of those at the smaller loci. The default settings in the Data Handling Tool flag degradation at a DI of >10 during quantification, and even though signs of DNA degradation were consistently visible in STR profiles at DI values of 2.5 or greater, the default threshold of 10 was sufficient for predicting when allele dropout started to occur in this study (Fig. 5.7).

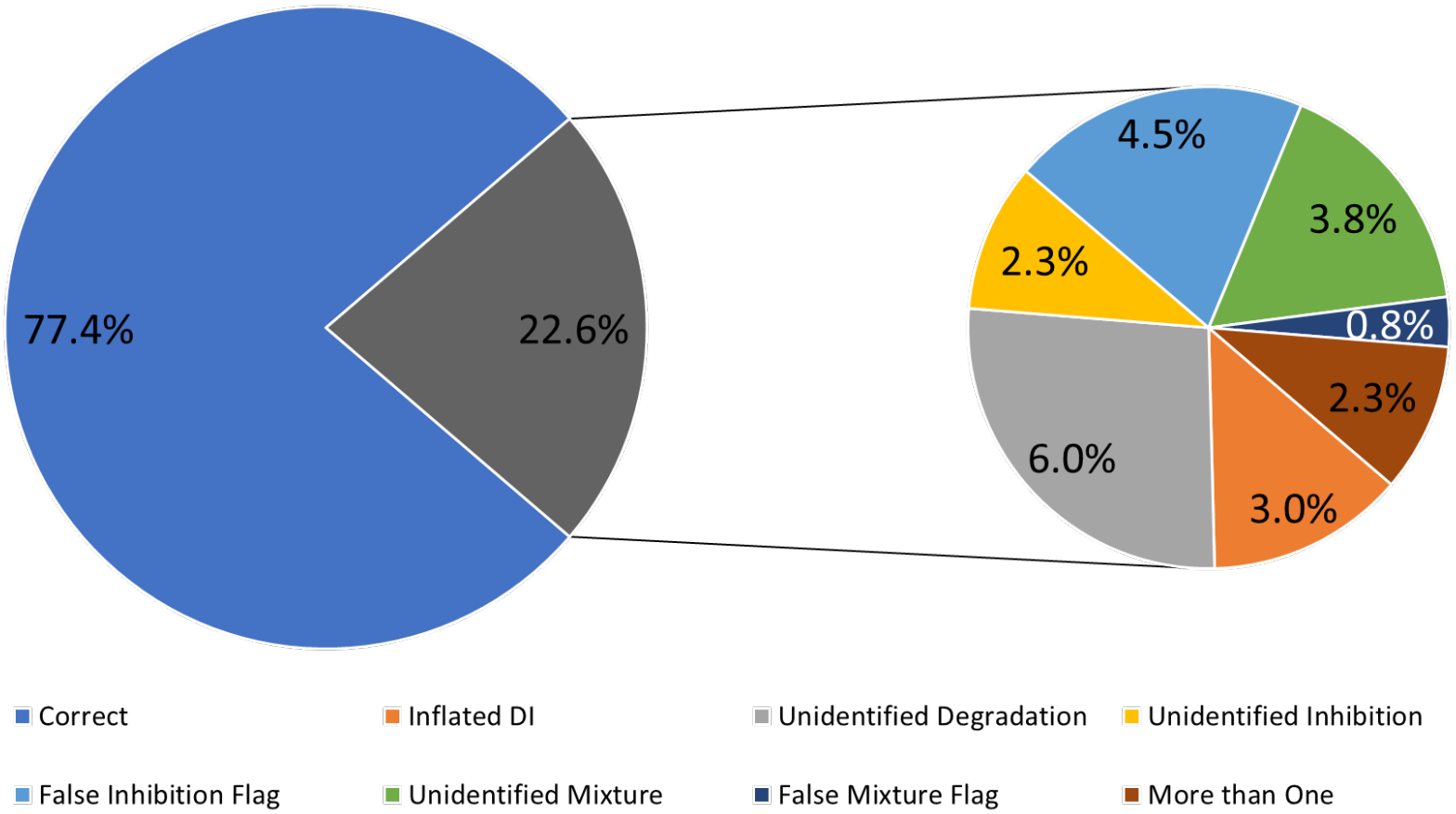

FIG. 5.6 - *Percentage of mock casework samples (*n*=133) that showed concordance between quality flags in the Investigator® Quantiplex® Pro RGQ kit during DNA quantification and the Quality Sensors and profile quality in the Investigator® 24plex QS kit (left). Disconcordance (right) was categorized as follows: inflated degradation index (DI), unidentified degradation, unidentified inhibition, false inhibition flag, unidentified mixture, false mixture flag, and more than one category.*

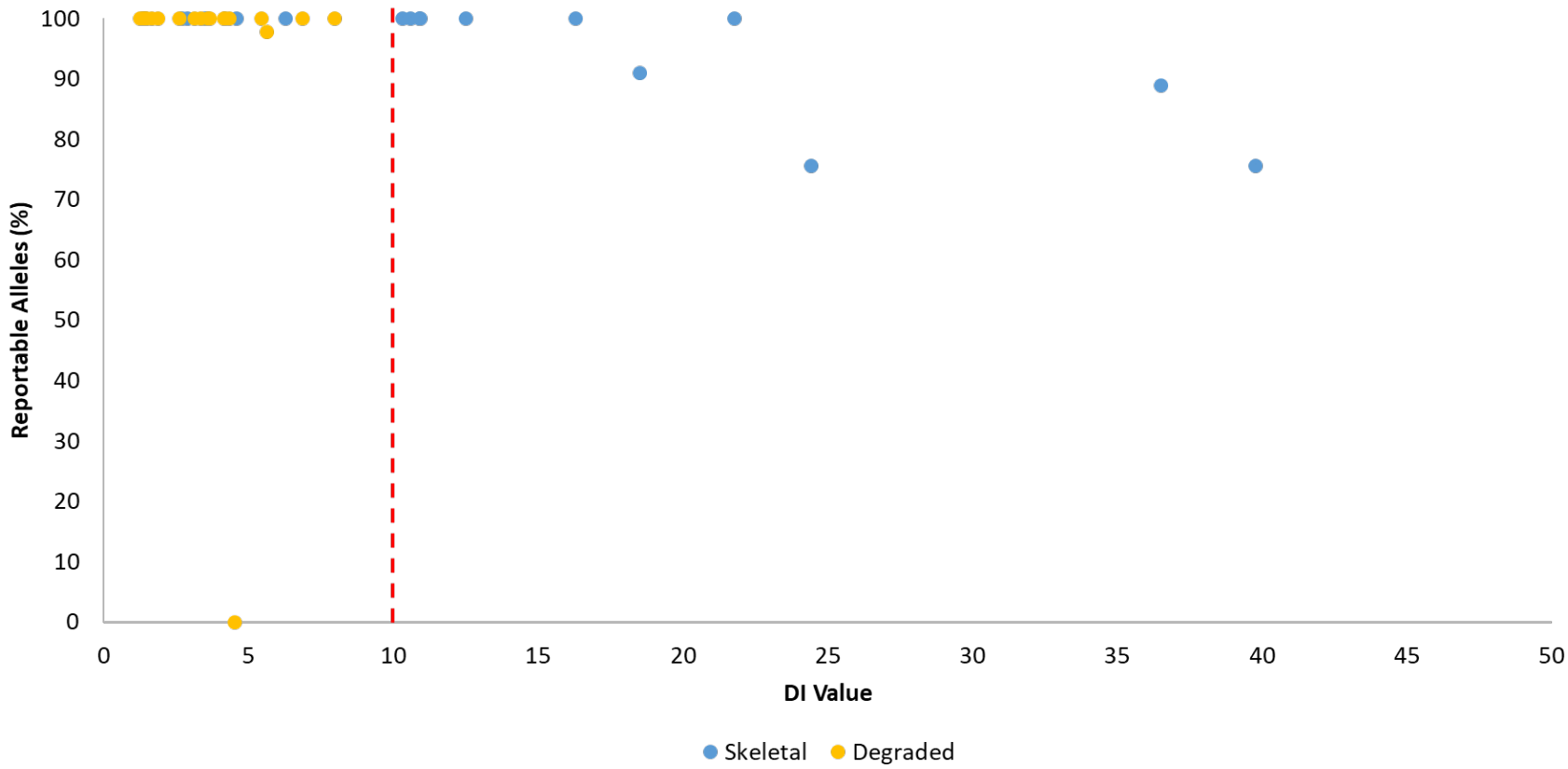

FIG. 5.7 - *The relationship between the level of degradation (DI value) and the resultant percentage of reportable alleles from skeletal (*n*=20) and other degraded tissue (*n*=20) samples. The dashed line represents the default degradation analysis threshold (DI of 10), when degradation is automatically flagged by the Data Analysis tool during DNA quantification. Three skeletal samples with DIs over 50 were not included in this graph, but have the following values: 1) DI 74.8, 55.6% alleles; 2) DI 113.3, 82.2% alleles; 3) DI 168.2, 64.4% alleles.*

All but one of the false inhibition flags were from samples that had an IPC shift just over the 1-cycle threshold (e.g. -1.069), but the EPGs showed balanced QS markers with no visible effects of inhibition in the STR profile. This threshold of a 1-cycle shift is the default setting in the Data Handling Tool, but can be modified as needed based on internal laboratory validation. Additionally, the Investigator® 24plex QS kit has been shown to be highly tolerant to common inhibitors than other commercial STR kits (33, 41), and although the Investigator® Quantiplex® Pro RGQ kit was designed to reflect the same level of tolerance, the two kits may not always behave exactly the same due to the different sample input amounts. The only other sample that was flagged as inhibited during qPCR, but did not exhibit signs of inhibition upon STR typing, had a high DNA concentration and was therefore diluted prior to PCR, which therefore also diluted out the inhibitor. Lastly, the unidentified mixture samples had flagged mixture indices ranging from 0.94:1 to 1.9:1, which again fell just under the default threshold of 2:1. This was an interesting phenomenon as EPGs with nearly full male and female profiles present were generated from samples that had similar DNA concentrations for the human and male targets as determined by the Quantiplex® Pro RGQ kit (Example – Appendix Fig. D12), when the human target should theoretically have double the male target DNA concentration for a 1:1 female/male mixture, as previously demonstrated with this kit (40). With observance of some discordance between human and male targets for single-source samples, it is hypothesized that either the human DNA concentration was under-estimated and/or the male DNA concentration was over-estimated, causing the true mixture ratio to be misrepresented.

Sample Reworks

In addition to the Investigator® 24plex QS and GO! kits, several commercial STR kits that include internal PCR controls, such as Thermo Fisher Scientific's NGM Detect® (42) and VeriFiler® Plus (43) kits and QIAGEN's Investigator® ESSplex SE QS (44) and Investigator® 26plex QS (45) kits, have been designed with the goal of assisting analysts in the interpretation of challenging profiles. The most commonly reported benefit of these quality control systems is being able to differentiate between inhibited and degraded samples (21-23, 42-48), and using that information to determine the workflow for further analyses.

Although a few published studies have reported that the QS markers in the Investigator® 24plex QS and GO! kits have the ability to better guide rework strategies, most of these studies were performed by the manufacturer and included minimal rework data (21-23). At the time of this study, our work was the most extensive comparison of reworks using the QS markers, for both mock casework and database-type samples. A subset of samples (n=79) with incomplete STR results (<90% alleles reported) during the first-pass analyses were selected for potential reworking. Samples were reworked according to the strategy determined by DNA analysts when they reviewed 1) the STR profile alone (no QS markers), and 2) the STR profile with the QS markers included.

After the rework strategies had been performed, there was an increase in reportable alleles for 63 samples, regardless of the approach used to define the rework strategy. The 16 samples that showed no improvement after reworking either had very little or no DNA and/or were highly degraded, or in the case of one sample, was both low template and severely inhibited. When the two rework strategies were the same (n=54), the average

number of reportable alleles increased from 13% to 48.7% (Fig. 5.8), overall profile quality improved (Fig. 5.8) and full profiles were able to be generated for 14 additional samples.

For almost one third of the samples (*n*=25) the analysts determined a rework strategy based on the STR profile alone that was different from the rework approach to be taken when the profile was examined with the QS markers included. An average increase in alleles (from 29.3% to 41.9% profile completeness) and overall profile quality was observed (Fig. 5.9), as well as generation of 8 full profiles with the non-QS information approach. However, even greater improvement was observed when samples were reworked based on the QS markers. Allelic balance improved, STR completeness increased to 76.2% reportable alleles (Fig. 5.9), and 17/25 samples produced full profiles when reworked based on the QS information (Fig. 5.10).

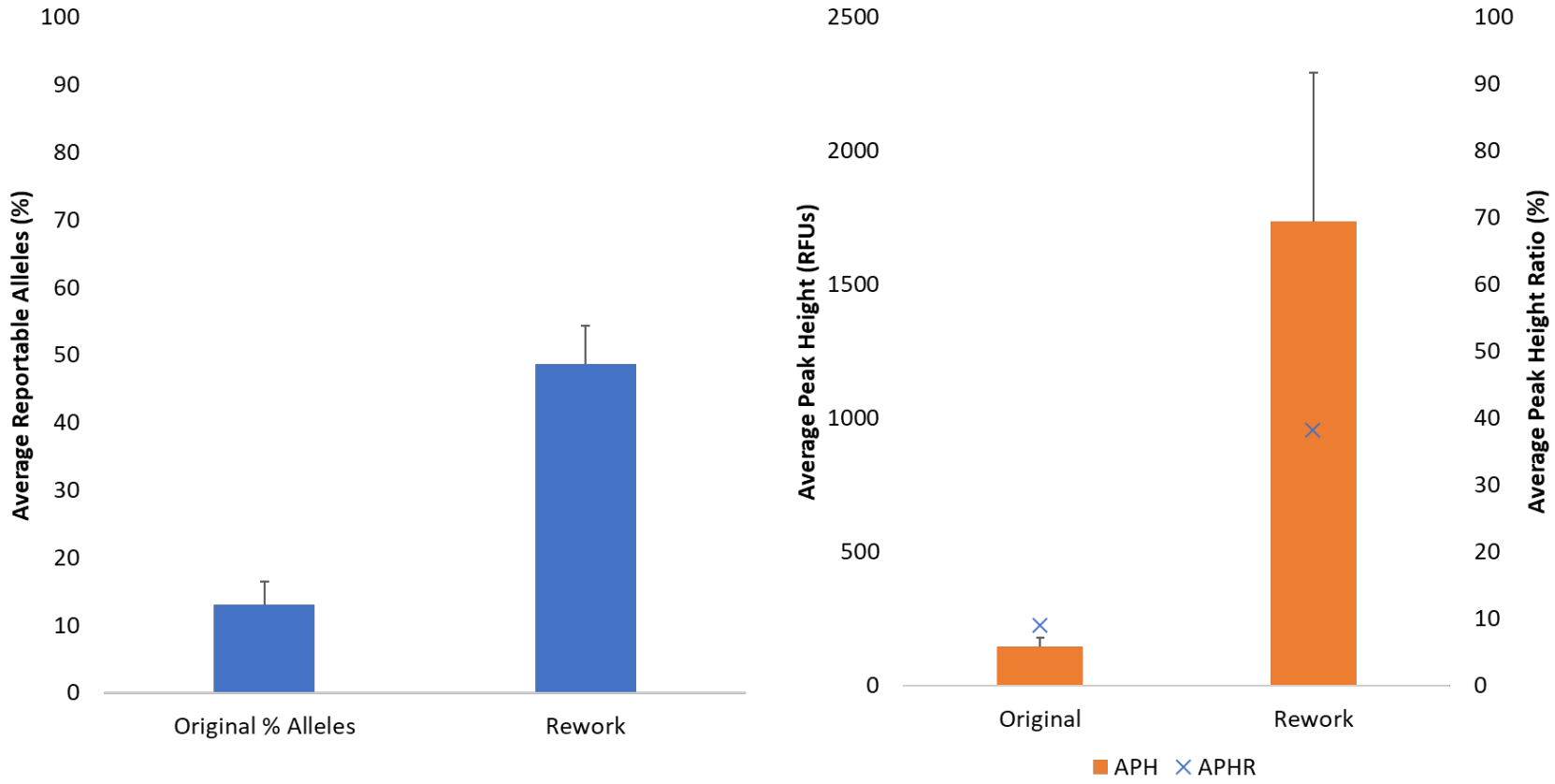

FIG. 5.8 - *Comparison of STR profile quality metrics between the original amplification and the secondary rework amplification for samples (*n*=54) that were reworked using the same strategy regardless of whether the QS marker information was taken into consideration. Quality metrics included average: reportable alleles (left), peak heights and peak height ratios (right). Error bars denote the standard error of the mean (SEM).*

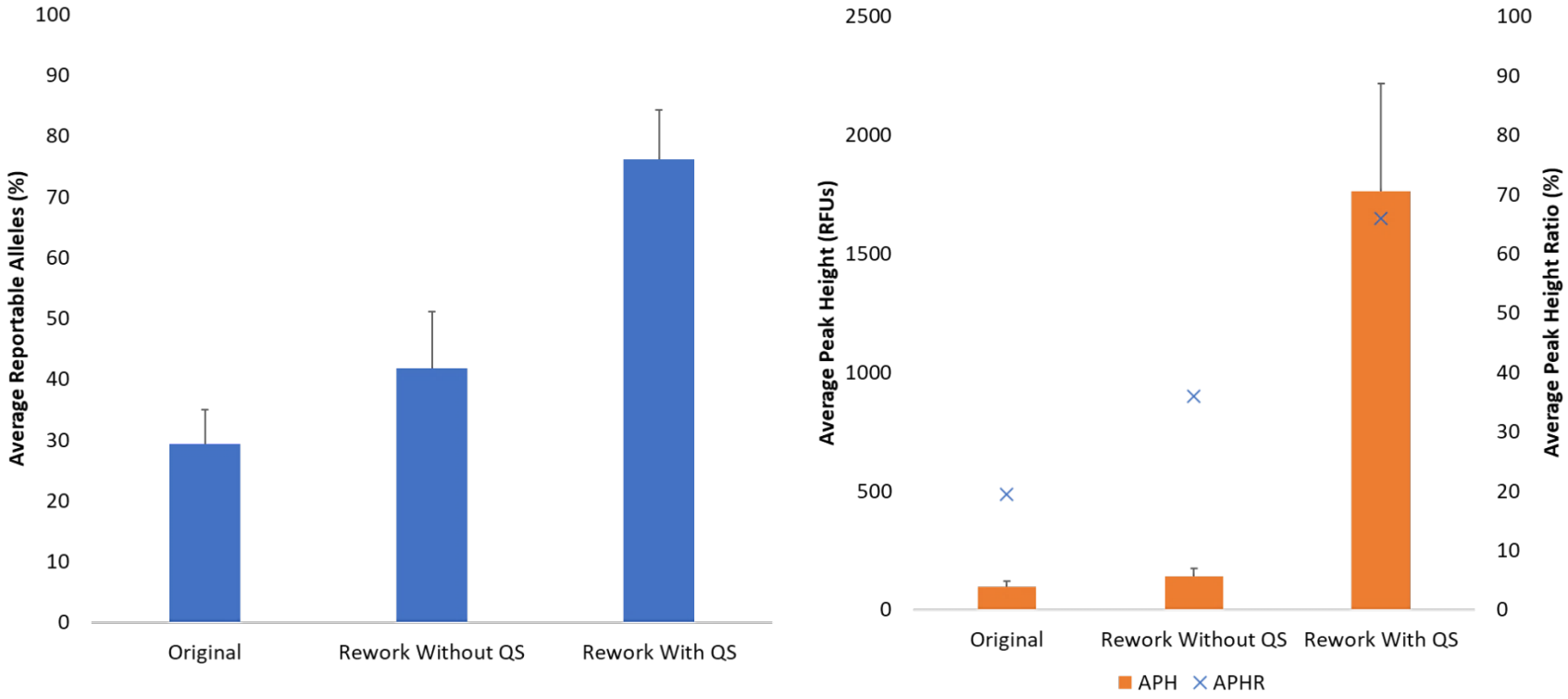

FIG. 5.9 - *Comparison of STR profile quality metrics between the original amplification and the secondary rework amplification for samples (*n*=25) that had different rework strategies with and without the QS marker information being considered. Quality metrics included average: reportable alleles (left), peak heights and peak height ratios (right). Error bars denote the standard error of the mean (SEM).*

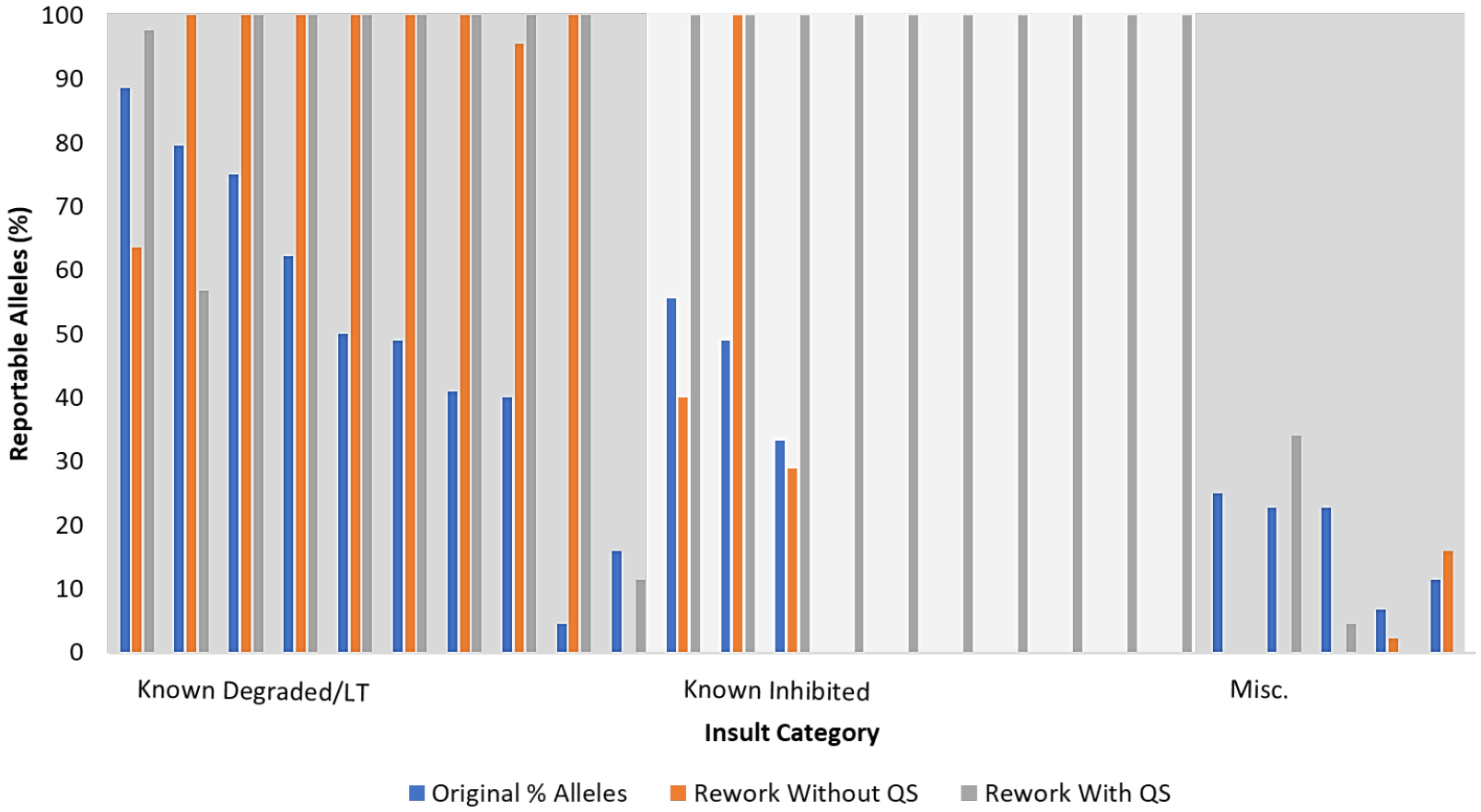

FIG. 5.10 - *Comparison of amplification success between the original and secondary amplifications (reworks determined with and without the QS marker information) for each sample that was reworked with different approaches (*n=*25). Samples were classified three into general insult categories; degraded (*n = *10), inhibited, (*n=*10), and miscellaneous (*n=*5).*

The most notable improvement was observed for severely inhibited samples that originally failed amplification (Fig. 5.10-11). Without the QS markers, the analyst assumed no DNA was present in that particular sample and therefore the strategy was to fully reprocess the sample (e.g. process a new FTA® punch). As inhibition was the reason for failure, the same result as before, or worse, was achieved. However, for these samples, one or both of the QS markers dropped out which is an immediate indicator of severe inhibition and therefore, the appropriate strategy would be to perform a dilution or water wash prior to re-amplification. This strategy resulted in full profiles for all 10 inhibited samples (Fig. 5.10).

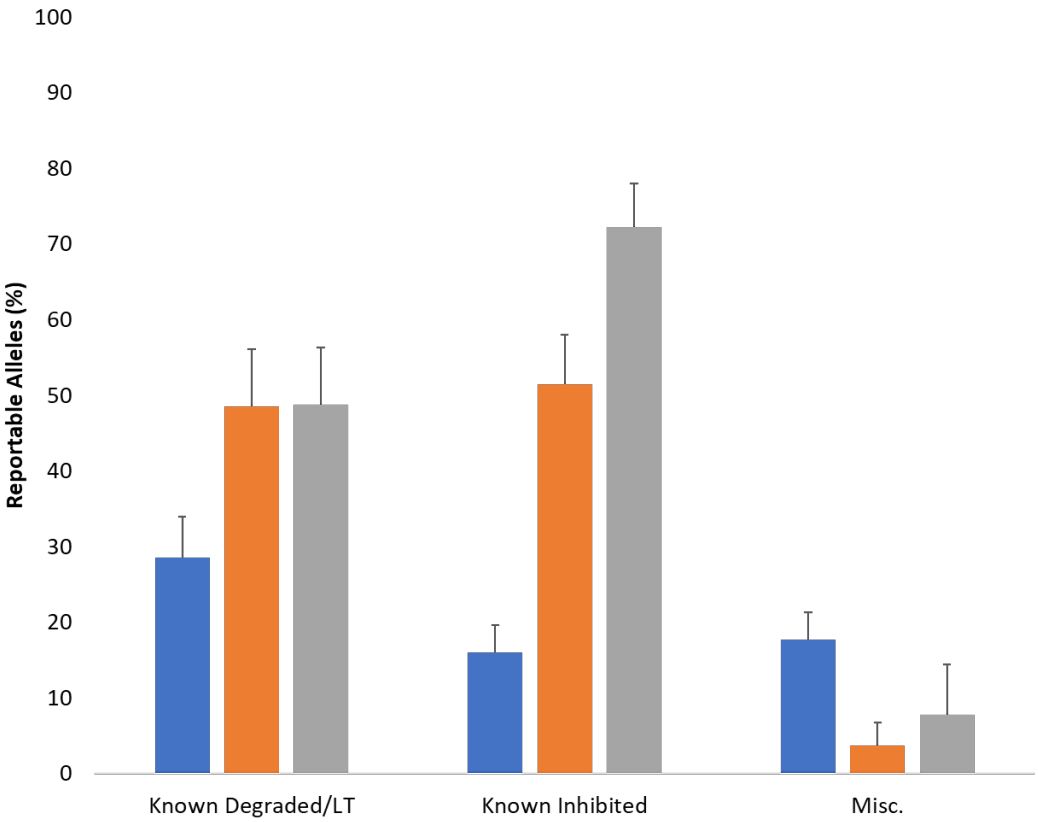

FIG. 5.11 - *Comparison of the average amplification success between the original and secondary amplifications (reworks determined with and without the QS marker information) for all reworked samples (*n*=79). Samples were classified three into general insult categories; degraded (*n*=34), inhibited (*n*=40), and miscellaneous (*n*=5). Error bars denote the standard error of the mean (SEM).*

There were two instances where the internal analysts' rework strategy determination was uncertain based on their interpretation of the overall profile quality and the QS marker information. Obvious signs of DNA degradation were observed throughout both profiles, but there were also some indications of possible inhibition coupled with the S marker height reduced to 55-70% of the Q marker. Therefore, a dilution was performed to see if inhibition was having an adverse effect during PCR. The diluted samples produced less alleles (11-23% less). However, when the samples were reamplified with more template, more alleles (4-21% improvement from original) were recovered. While the manufacturer's recommendation of a 20% S/Q ratio can be adjusted and interpreted as necessary based on laboratories' internal validations, throughout this study it was a reliable indicator for determining the presence of inhibition that would negatively affect downstream STR typing results to the point when allelic dropout occurred. Finally, there were four samples that showed no improvement with either strategy (Fig. 5.10). These were severely degraded and/or had very low amounts of DNA.

Overall, these results suggest that although experienced analysts may be proficient at identifying the reason for sub-optimal results solely based on the STR profile and apply the appropriate rework strategy for a wide variety of samples, the information provided by the QS markers can provide additional support for interpreting more unclear STR data. The QS markers were able to more frequently and accurately resolve ambiguous low-quality or failed STR profiles (particularly severely inhibited samples) (Fig. 5.12), leading to the most appropriate rework strategy.

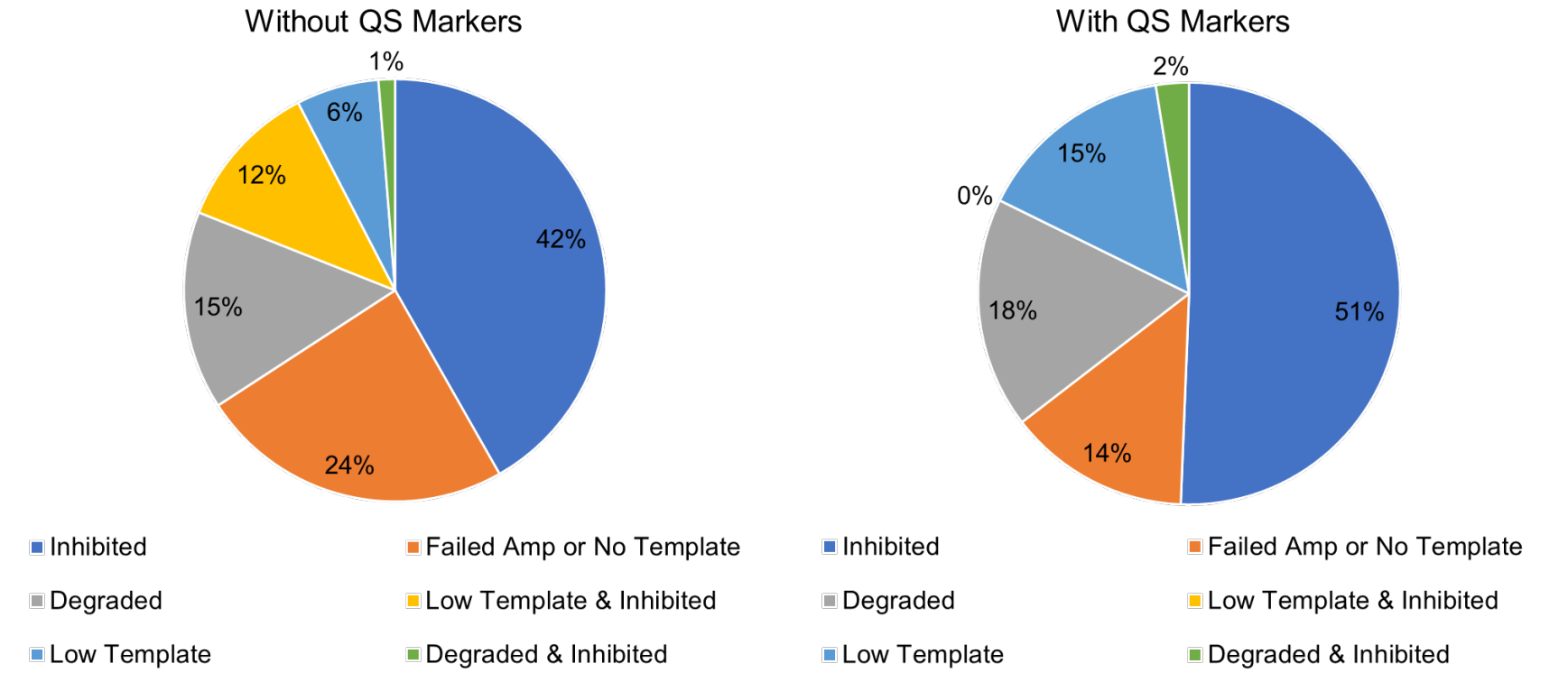

FIG. 5.12 - *Identified reasons for suboptimal STR results for all samples with <90% alleles which were selected for reworking (*n=*79). Classifications were determined based on the overall EPG quality: without (left) and with (right) the QS marker information. Samples were designated as being: inhibited, degraded, low template, a failed amplification/no template, low template and inhibited, or degraded and inhibited.*

Conclusion

The Investigator® Quantiplex® Pro RGQ and Investigator® 24plex QS and GO! kits performed well for a wide variety of challenging database and casework-type samples showing consistency between the sample quality metrics in both quality systems and as a HID workflow as a whole. Both casework chemistries were also sensitive and robust enough for a wide range of female:male mixtures. Following a manual differential separation and automated DNA extraction, male DNA was able to successfully be detected and amplified in sperm fractions from vaginal swabs collected as late as 5 days post-coitus (0.003 ng/µL; single obligate male allele at DYS391) and with spiked semen dilutions as low as 1:1000 (0.001-0.003 ng/µL; 1-2 obligate male alleles). As expected, the control samples collected and stored room temperature generated high first-pass rates, while a much higher percentage of samples with partial profiles were obtained from extremely degraded, low template, and/or inhibited samples.

The QS markers correctly confirmed the quality of samples and STR profiles for 99.9% of databasing samples and 98.4% of mock casework samples. Regardless of the approach used to determine the rework strategy, a notable improvement in allele recovery was achieved for 80% of samples that were reworked with an average increase of 21 additional alleles, and an additional 32 samples generated full profiles after reamplification. However, the greatest improvement in STR quality and completeness was attained for samples when the EPG was analyzed in conjunction with the QS marker information. In particular, the quality marker systems were most beneficial in confirming PCR inhibition, which could guide the analyst to use the most effective rework strategy, and thereby avoid any unnecessary sample processing.

In summary, the quality sensors in the Investigator® 24plex QS and GO! kits allowed for a reliable and simplified interpretation of ambiguous and failed STR profiles to confirm sample quality and inform the best strategy for reworking samples. Additionally, quality flags in the Investigator® Pro RGQ kit accurately predicted sample quality in a majority (77.4%) of the mock casework samples tested in this study and were consistent with the QS markers in the Investigator® 24plex QS and GO! kits and the STR profiles as a whole. The combination of these quality control systems within a DNA workflow can help analysts determine the best way to efficiently triage and process challenging samples to reduce the amount of reworks required, ultimately saving both time and resources. However, as this study has demonstrated, these systems are not 100% predictive and have been shown to occasionally provide conflicting information. Therefore, the data provided by the sample quality flags and quality sensors should be used to complement an analyst's experience. In addition, all data analyses involving the quality sensors and how they could be used to triage samples or determine rework strategies should be guided by comprehensive internal validation studies.

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CHAPTER VI

Conclusions

The overall results of this research have shown that the use of alternative DNA extraction and STR profiling methods can provide solutions for commonly encountered forensic issues, such as DNA degradation, low amounts of DNA, and PCR inhibition, to improve overall genotyping success for a wide range of challenging samples. The results of Phase 1 show that powdering of bone tissue may be avoided without significantly reducing DNA yield and STR success. Although the complete demineralization method seemed to extract the most DNA from bone samples and generate the most complete STR profiles, no statistical difference was observed compared to the other two manual methods tested in this study (PrepFiler® BTA, and TBone kit coupled with the PrepFiler® BTA kit). Data suggest that manual DNA extraction from whole bone chips (using the TBone kit) is comparable to traditional complete demineralization and commercial kit-based methods that require powdering of the bone tissue prior to extraction. Avoiding the bone powdering steps during sample preparation can reduce the potential for contamination and the overall sample processing time. Additionally, it can also eliminate the possibility of sample loss during crushing (e.g. tube breaking, metal shavings from magnetic bar), provides the option for re-extraction from the same bone fragment rather than having to cut more bone, and most importantly, this method may also allow more forensic laboratories to perform extractions from skeletonized remains that otherwise would not have been able to.

Although automation reduces processing time and the potential for human error, there are also some disadvantages. Compared to performing a manual DNA extraction, a significant decrease in DNA yield was observed when bone samples were extracted using

an automated platform. Sample loss can be detrimental, especially in forensic or ancient cases that have a very limited amount of bone available; therefore, most laboratories prefer to perform DNA extractions manually. For skeletonized remains, automated techniques could be used for screening samples and determining the most appropriate DNA workflow. Samples that have adequate amounts of starting material and/or are less degraded could be genotyped using standard laboratory protocols. If the DNA yield is insufficient for routine STR typing, then a more vigorous manual DNA extraction method, such as total demineralization, may be performed. As a last resort, mitochondrial DNA typing has traditionally been relied upon for identifying samples that have low amounts of DNA. However, with the emerging technology of next generation sequencing, highly degraded and low template samples have been successfully genotyped with STRs and/or the combination of other alternate markers, such as SNPs and INDELs.

For Phase 2, a powder-free workflow for skeletonized remains was optimized and applied to a variety of environmentally challenged skeletal elements. A single 50 mg chip yielded comparable results in DNA concentration and STR success to 50 mg of bone powder, and an increase in incubation time and chip size/number yielded no significant improvement over samples processed using the manufacturer's recommendations. Over a third of the bones sampled resulted in a full CODIS-eligible profile from a single 50 mg chip with an automated workflow. Although a non-powdering extraction may not have been as efficient for highly compromised or low template bone samples, this method could be applied in a laboratory setting as a screening tool. The ability to process a smaller amount of bone tissue compared to many other traditional skeletal DNA extraction methods allows for a wider variety of skeletal elements to be tested. Interestingly, many

smaller bony elements frequently out-performed traditionally harvested bones for HID purposes regardless of the processing method used.

In Phase 3, significantly more DNA was recovered from all bone and tooth samples using the PrepFiler® BTA Forensic DNA Extraction kit than the QIAamp® DNA Investigator® kit. However, regardless of the extraction method, consistently more alleles were recovered from skeletal samples with the addition of extra PCR cycles using the GlobalFiler® PCR Amplification Kit with minimal adverse STR artifacts. As expected, the improvement in STR profile quality observed with an additional PCR cycle was most pronounced in low template samples (<120 pg DNA). At the time of this study, no publications had reported the effectiveness of additional PCR cycles using the GlobalFiler® kit to genotype low template and/or skeletal samples.

For the final phase of this research, sample quality flags in the Investigator® Quantiplex® Pro RGQ kit during DNA quantification accurately predicted STR quality in the majority of samples (75%) tested. The QS markers in the Investigator® 24plex QS & GO! kits correctly confirmed sample and/or overall STR quality in 99.9% of reference samples (buccal swabs and FTA® cards) and 98.4% of mock casework samples. More complete STR profiles were consistently obtained when samples were reworked based on the QS markers in conjunction with overall STR quality compared to when the EPG alone was used to determine sample quality and the most effective rework strategy.

Overall, this research has demonstrated that the digestion of whole bone fragments may be an efficient and attractive alternative to powdering bone for DNA extraction and STR success may be increased in low template samples with the addition of extra PCR cycles using the GlobalFiler® DNA amplification kit. Additionally, this research has

demonstrated that Quality Sensors can aid analysts in more accurately assessing sample quality, and triage samples for more efficient rework strategies to improve STR success and avoid unnecessary reworks for ambiguous failed/low-quality STR profiles.

Products of this work have been used as the scientific basis for changes in the manufacturer's recommended protocols for STR typing of low template samples and influenced software updates for the analysis of STR profiles with internal quality sensors. Additionally, the forensic community will further benefit from the reporting of data exploring alternative methods for performing in-house processing, extracting and triaging of bone samples which will ultimately have the potential to eliminate/reduce outsourcing, decrease sample processing time, reduce contamination risks, and increase sample throughput. The various DNA workflow strategies explored throughout this research may facilitate an improvement in overall laboratory efficiency, leading to faster and more complete identifications of skeletonized human remains and other challenging forensic samples.

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APPENDIX A

Consent for Participation in Research

Investigation into the benefits of the quality controls in QIAGEN's Quantiplex and 24plex kits as part of the STR profiling DNA workflow

Why am I being asked?

You are being asked to be a participant in a research study about the forensic identification of forensically relevant biological samples conducted by Dr. Sheree Hughes-Stamm, the Department of Forensic at Sam Houston State University. You have been asked to participate in the research because you are eligible to participate. We ask that you read this form and ask any questions you may have before agreeing to be in the research.

Your participation in this research is voluntary. Your decision whether or not to participate will not affect your current or future relations with Sam Houston State University or the Forensic Science program. If you decide to participate, you are free to withdraw at any time without affecting that relationship.

Why is this research being done?

The biological samples provided in this study will serve as mock casework evidence and database samples. These materials will be used for research, and will provide researchers with the appropriate human samples for forensic analysis investigating the utility of QIAGEN's products as part of a DNA profiling workflow.

Biological samples from living humans are required in order to simulate mock crime scene samples such as small amounts of blood, semen, epithelial cells, and saliva on items of evidence in addition to post-coital samples. The ability to recover and get a "match" from

the minute amounts of human DNA from these types of samples is vital for forensic analysis.

What is the purpose of this research?

The purpose of this project is to evaluate the utility of these DNA quantification and amplification products to identify the quantity and quality of biological source of evidence (saliva, semen, vaginal material, epithelial cells, and blood) in conjunction with traditional DNA profiling methods used for human identification.

What procedures are involved?

If you agree to be in this research, we may ask you to do one or more of the following things:

• Wipe the inside of your cheek with two swabs for approximately 30 seconds

• Collect saliva in a tube (approximately 1 mL).

• Have your venous blood collected by a qualified phlebotomist (approximately 15 mL)

• Touch various items to deposit cells from hands onto 'touched' evidence

• Collect a sample of vaginal material using a cotton swab (before or after coitus) in the privacy in your own home.

• Collect one emission of semen in a container in the privacy in your own home.

Any remaining samples will be destroyed after a period of 2 years after the completion of the project.

Samples will be disposed of via the standard pathological waste collection service.

Approximately 10-*20 subjects* may be involved in this research at Sam Houston State University.

What are the potential risks and discomforts?

There is minimal risk for the participants. These may include:

• Mild irritation and bruising whilst getting blood drawn (arm).

There are no significant physical or psychological, legal or reputational risks to participation.

If you feel uncomfortable at any time during the study, please notify Dr. Sheree Hughes-Stamm on 936 294 4359.

All collection procedures will be done at the same time and no future participation is required.

What other options are there?

There are no other options. Animal samples cannot be used for forensic human identification work.

What about privacy and confidentiality?

The only person who will know that you are a research participant is the principle investigator (PI) of this research project. No information about you, or provided by you during the research will be disclosed to others without your written permission, except:

• -if necessary to protect your rights or welfare (for example, if you are injured and need emergency care or when the SHSU Protection of Human Subjects monitors the research or consent process); or

• -if required by law.

When the results of the research are published or discussed in conferences, no information will be included that would reveal your identity. If photographs, videos, or audiotape recordings of you will be used for educational purposes, your identity will be protected or

disguised. Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law.

All samples are treated as anonymous, and are assigned a numbered code upon collection. No personal information is collected except the sex, ancestry, hair color, eye color and skin color of the participant. All personal information will only be accessed by the PI.

What if I am injured as a result of my participation?

In the event of injury related to this research study, you should contact your physician or the University Health Center. However, you or your third party payer, if any, will be responsible for payment of this treatment. There is no compensation and/or payment for medical treatment from Sam Houston State University for any injury you have from participating in this research, except as may by required of the University by law. If you feel you have been injured, you may contact the researcher, Dr. Sheree Hughes-Stamm at 936 294 4359.

What are the costs for participating in this research?

There are no research costs for which the subject is responsible.

Will I be reimbursed for any of my expenses or paid for my participation in this research?

The subject will not receive payment, remuneration or reimbursement for participation in this study.

Can I withdraw or be removed from the study?

You can choose whether to be in this study or not. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind. You may also refuse to

answer any questions you don't want to answer and still remain in the study. The investigator may withdraw you from this research if circumstances arise which warrant doing so.

The participant can withdraw from this study at any time for any reason.

Who should I contact if I have questions?

The researcher conducting this study is Dr. Sheree Hughes-Stamm. You may ask any questions you have now. If you have questions later, you may contact the researchers at: Phone: 936 294 4359.

What are my rights as a research subject?

If you feel you have not been treated according to the descriptions in this form, or you have any questions about your rights as a research participant, you may call the Office of Research and Sponsored Programs –Sharla Miles at 936-294-4875 or e-mail ORSP at sharla_miles@shsu.edu.

You may choose not to participate or to stop your participation in this research at any time. Your decision whether or not to participate will not affect your current or future relations with the University. Non-participation in this study will not result in any sanction.

Participation is completely voluntary and you are under no obligation to participate. If you are a student, this will not affect your class standing or grades at SHSU. The investigator may also end your participation in the research. If this happens, your class standing or grades will not be affected.

If you are a staff person at SHSU, your participation in this research is in no way a part of your university duties, and your refusal to participate will not in any way affect your

employment with the university, or the benefits, privileges, or opportunities associated with your employment at SHSU.

You will not be offered or receive any special consideration if you participate in this research.

Agreement to Participate

I have read (*or someone has read to me)* the above information. I have been given an opportunity to ask questions and my questions have been answered to my satisfaction. I agree to participate in this research.

Consent: I have read and understand the above information, and I willingly consent to participate in this study. I understand that if I should have any questions about my rights as a research subject, I can contact Dr. Sheree Hughes-Stamm at 936 294 4359 or by email at shereehs@shsu.edu. I have received a copy of this consent form.

Your name (printed): _____

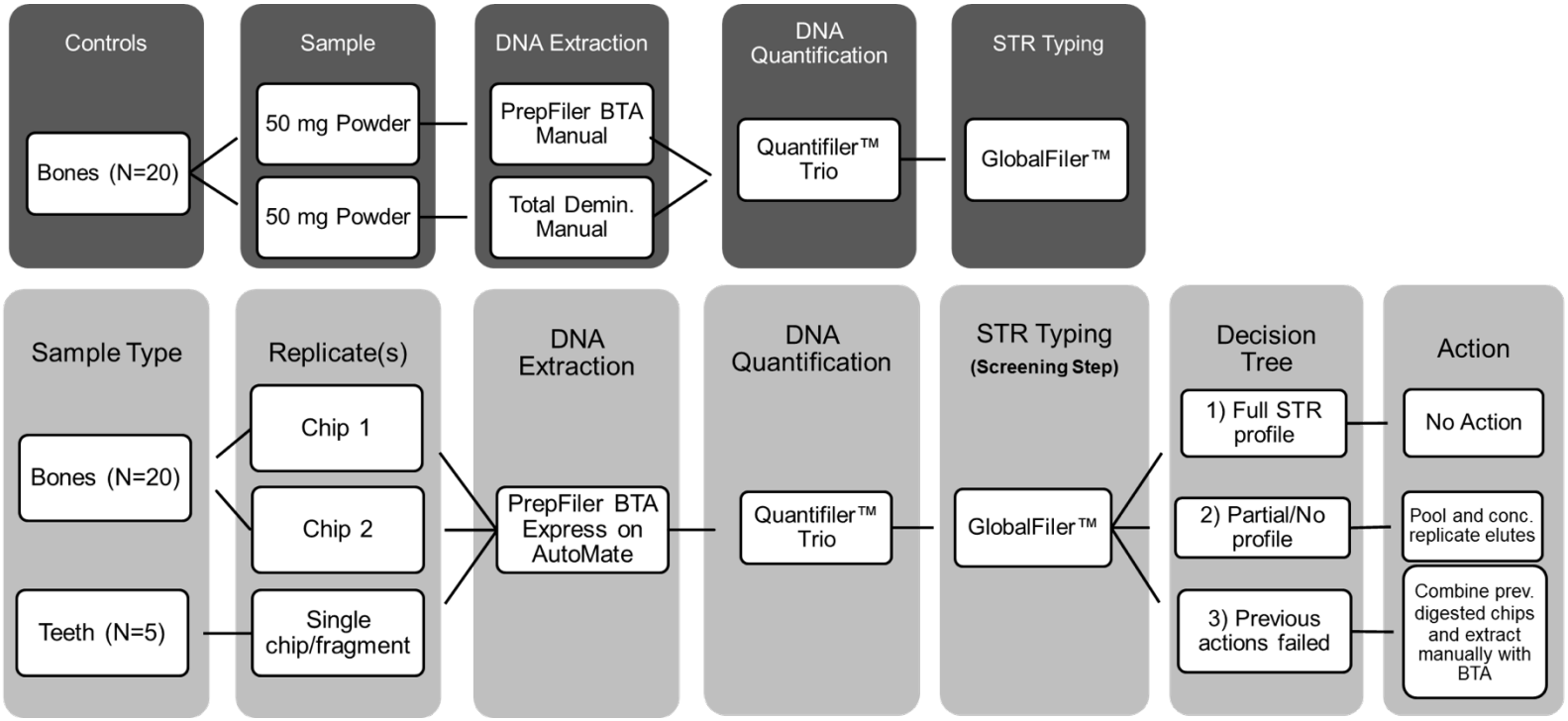
Signature: _____

Date: _____

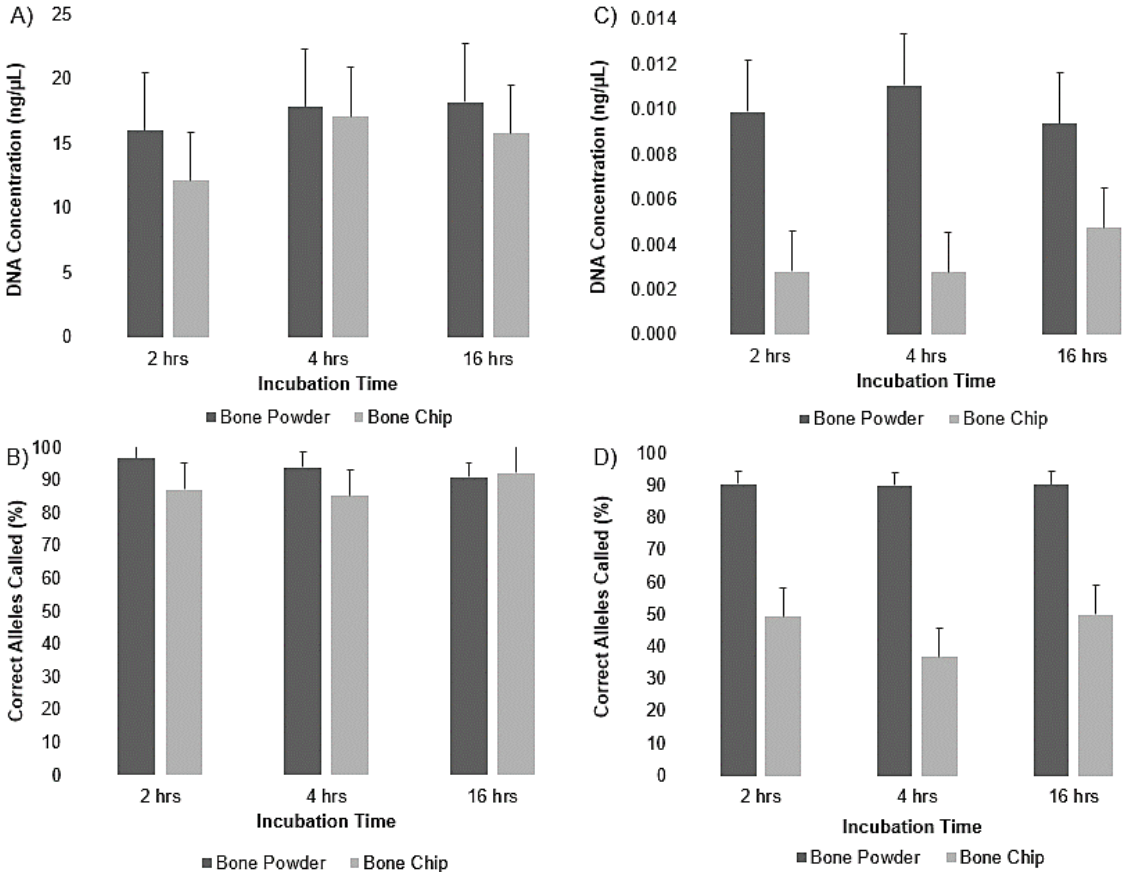
Assigned number:

APPENDIX B

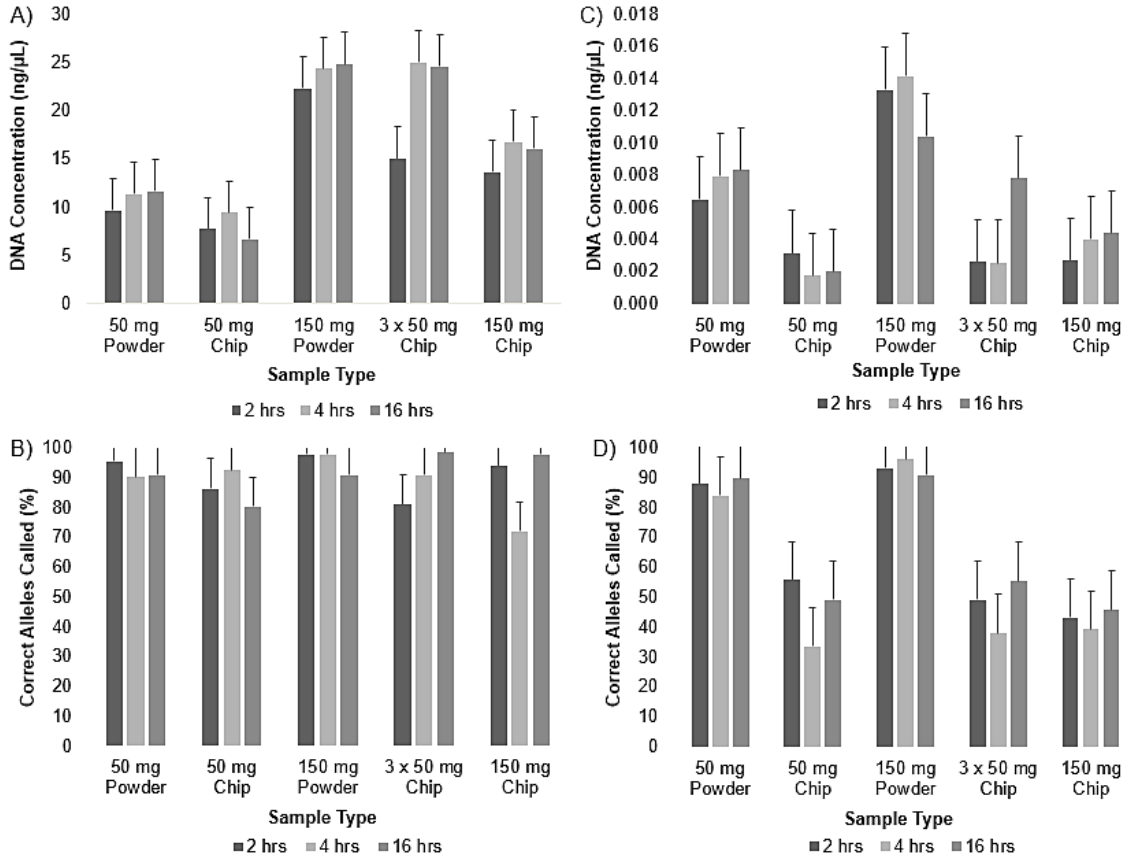
Chapter 3 Supplemental Tables and Figures



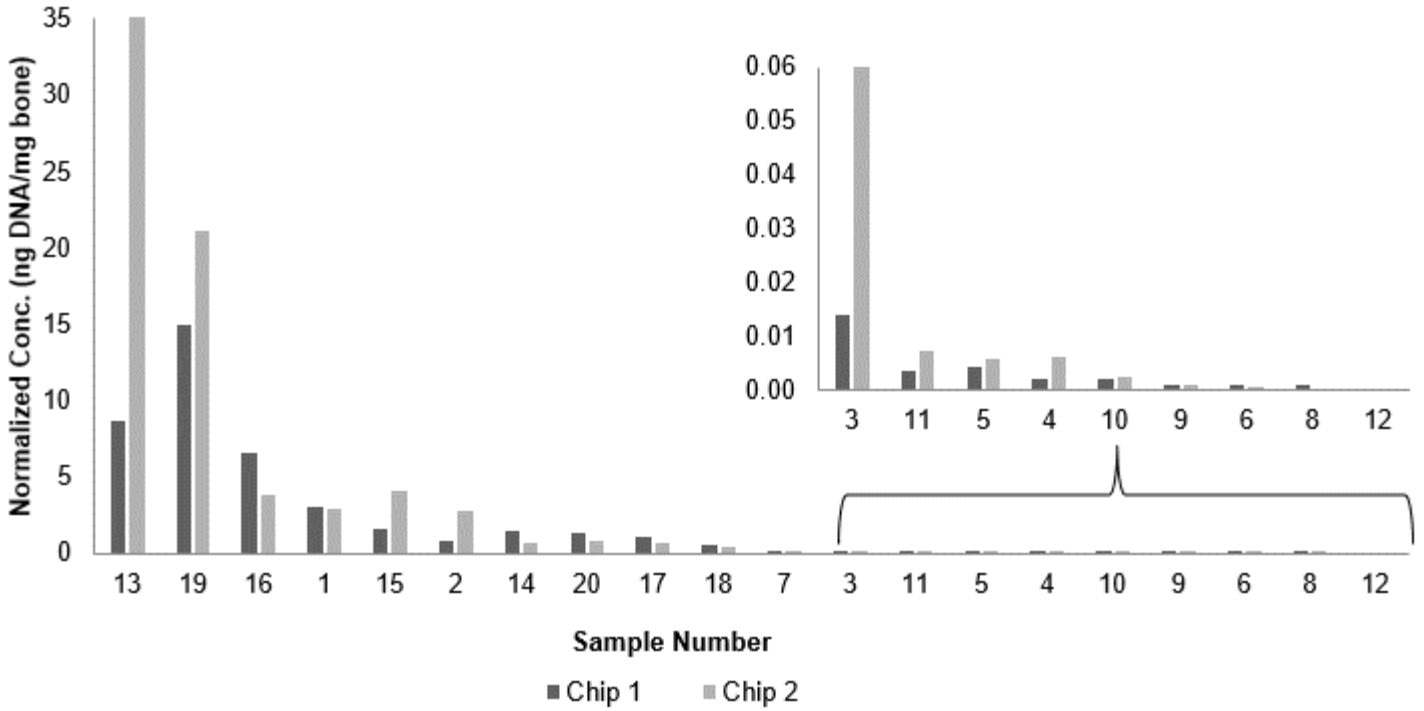
Appendix Figure B1 - Phase 2 methodology flowchart for powdered (top) and chipped bone and tooth (bottom) samples.



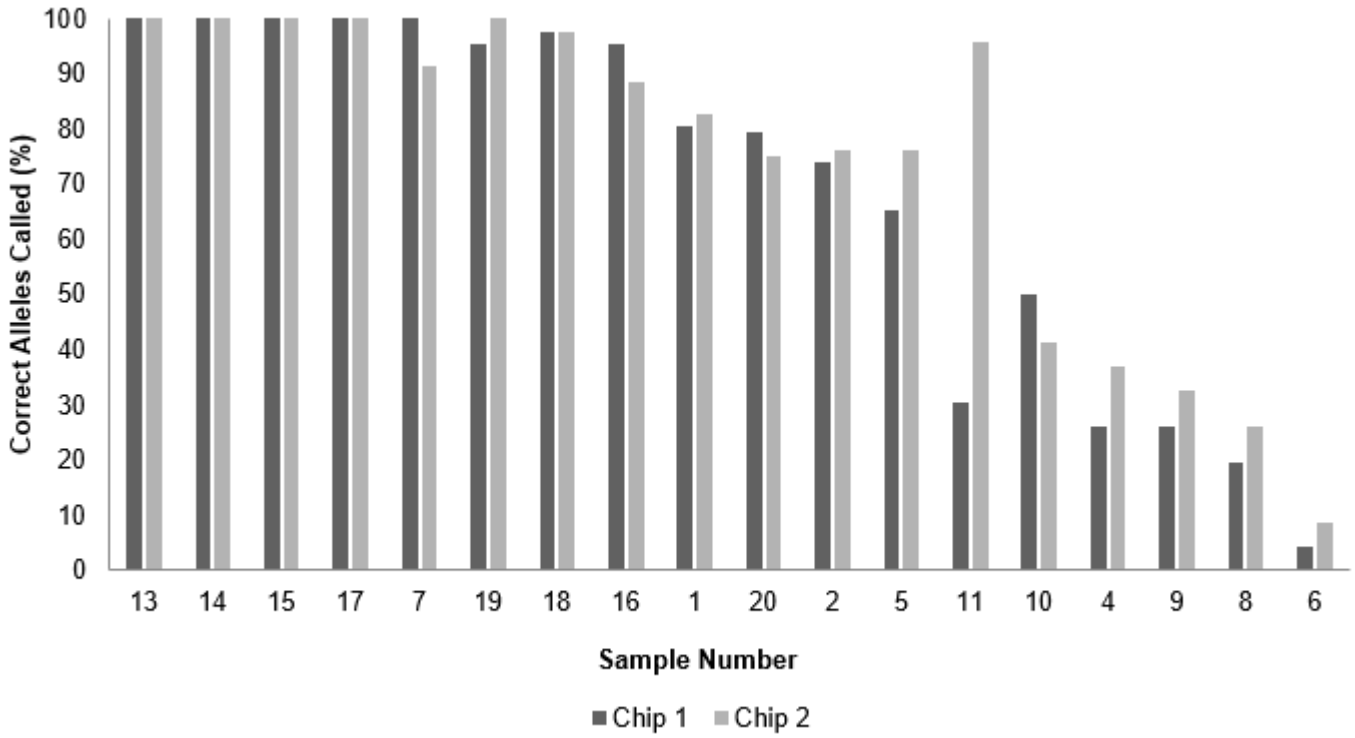
Appendix Figure B2 - Comparison of DNA concentration and STR profile results for Phase 1 based on incubation time for powder (*n*=10) and chip (*n*=15) samples. A) average DNA concentration for the high quantity samples; B) average STR profile completeness for the high quantity samples; C) average DNA concentration for the low-template samples; D) average STR profile completeness for the low-template samples. Error bars denote 0.95 confidence interval.



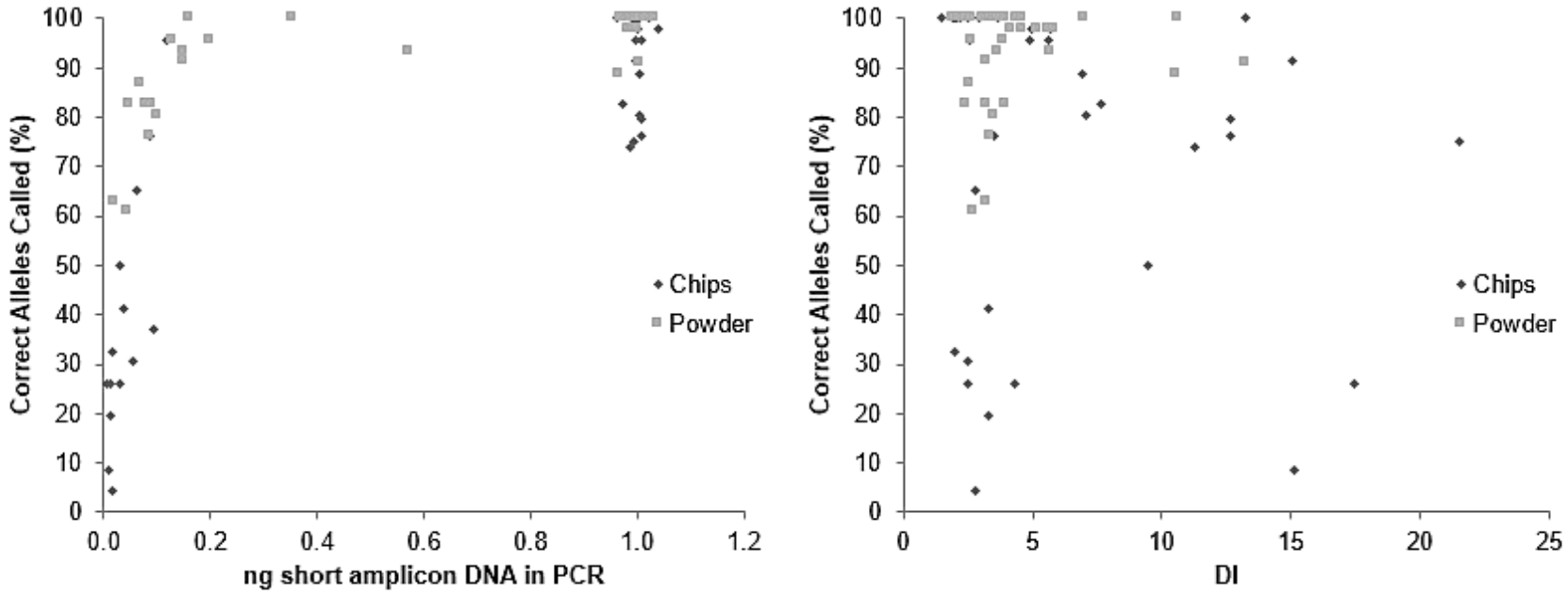
Appendix Figure B3 - Comparison of DNA concentration and STR profile results for Phase 1 based on sample type/mass and incubation time (*n*=5). A) average DNA concentration for the high quantity samples; B) average STR profile completeness for the high quantity samples; C) average DNA concentration for the low-template samples; D) average STR profile completeness for the low-template samples. Error bars denote 0.95 confidence interval.



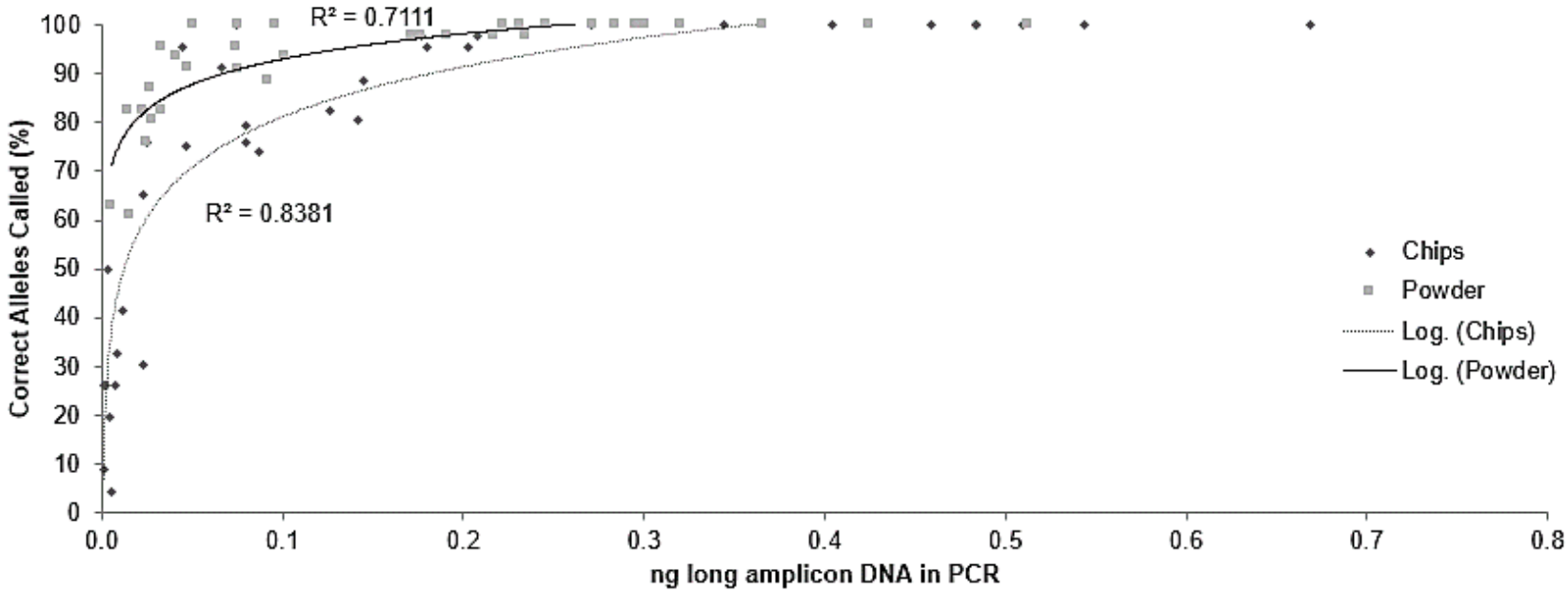
Appendix Figure B4 - DNA concentration for 20 bone chips processed in duplicate with an automated extraction in Phase 2. Values were normalized to the weight of the bone chip for accurate comparisons and samples are sorted by decreasing average DNA concentration between the replicate bone chips.



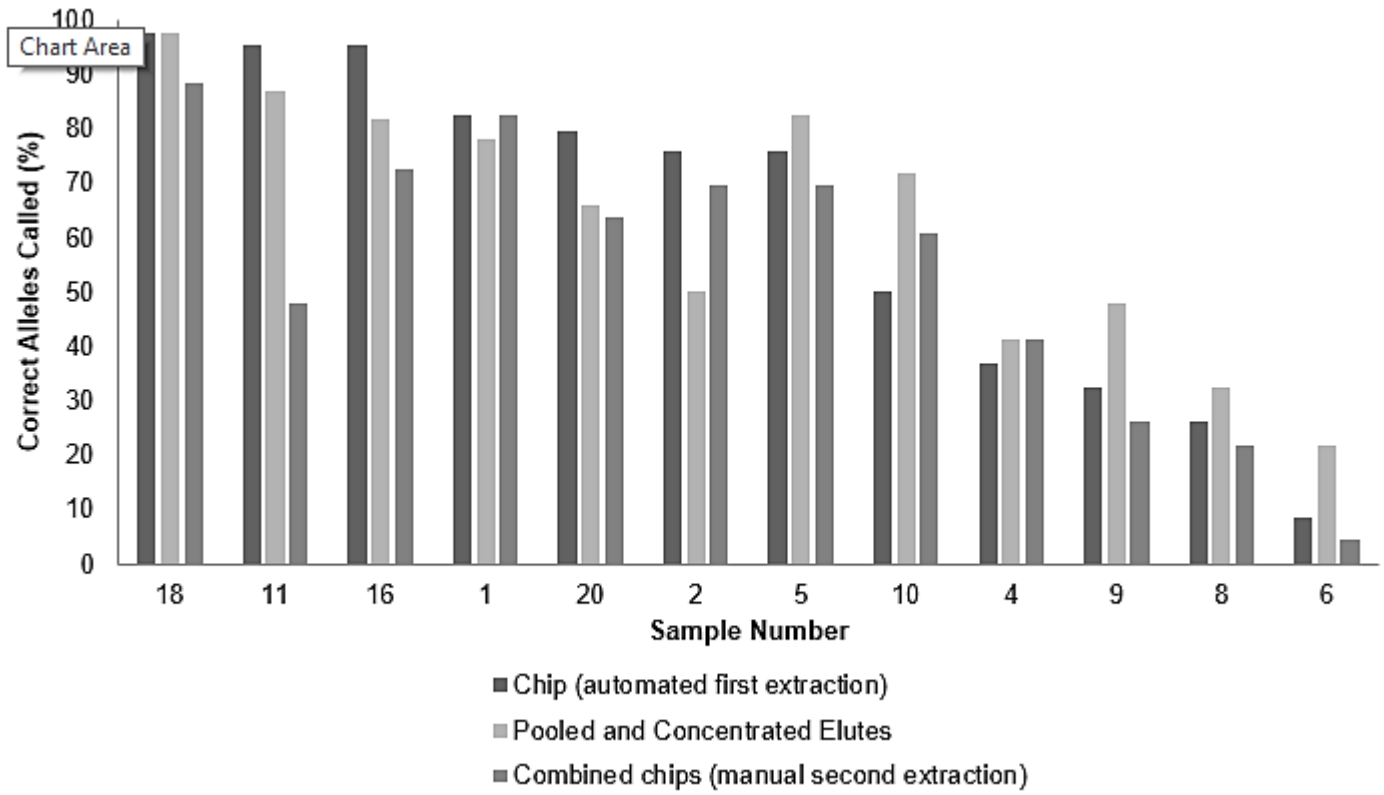
Appendix Figure B5 - Percentage of correct alleles called for 20 bone chips processed in duplicate with an automated extraction in Phase 2. Samples were sorted by decreasing average alleles called between the replicate bone chips.



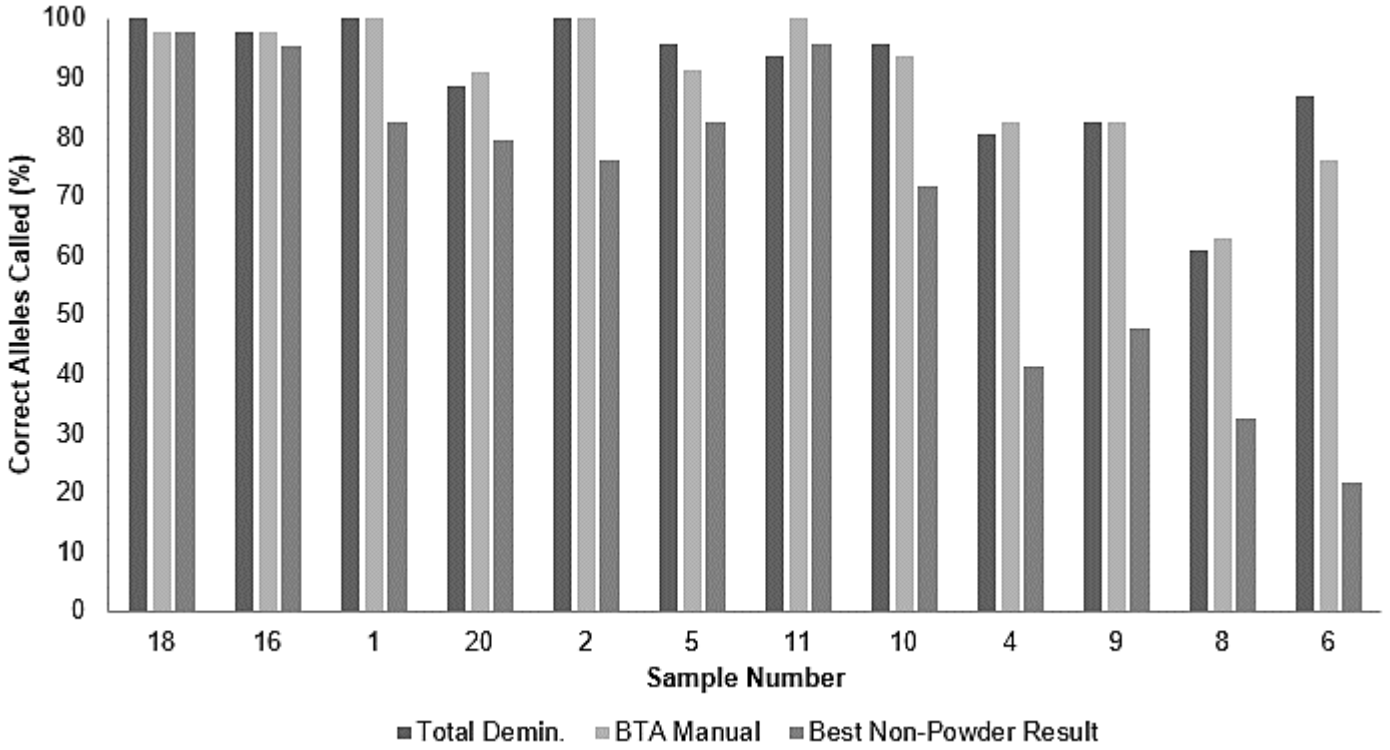
Appendix Figure B6 - Correlation plots for the predictability of STR profile completeness based on short amplicon DNA input into PCR (left) or sample degradation (DI; right) in Phase 2.



Appendix Figure B7 - Correlation plot for the predictability of STR profile completeness based on the amount of long amplicon DNA input into PCR in Phase 2.



Appendix Figure B8 - Comparison of secondary processing strategy STR results to the original automated extraction for each of the 12 reworked bone samples in Phase 2. Sample numbers retained the same order as Append. Fig. B5 for ease of comparisons.



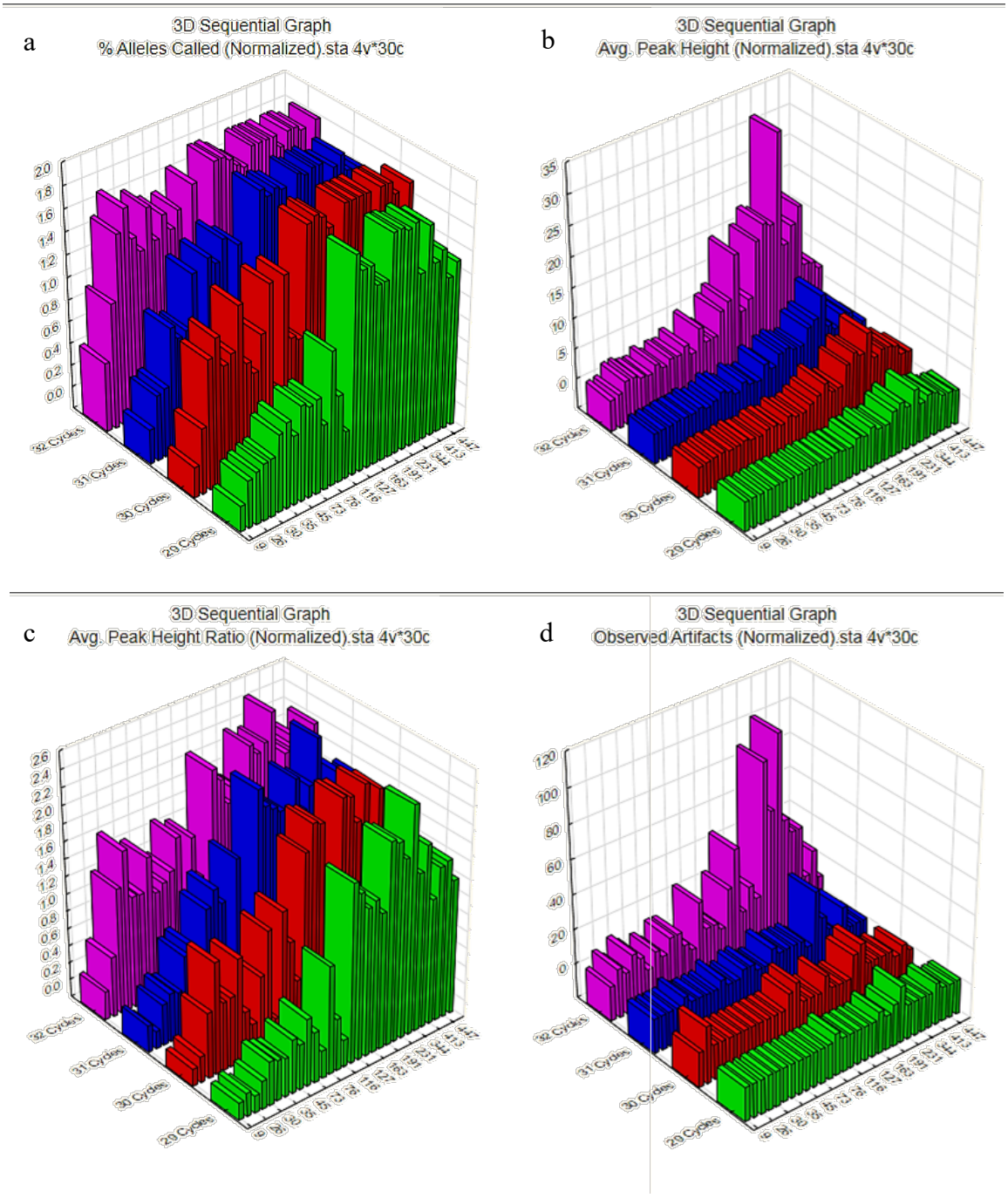
Appendix Figure B9 - Comparison of the best non-powder bone STR results to the traditional powdered bone extraction methods for each of the 12 reworked bone samples in Phase 2. Sample numbers retained the same order as Append. Fig. B5 for ease of comparisons.

APPENDIX C

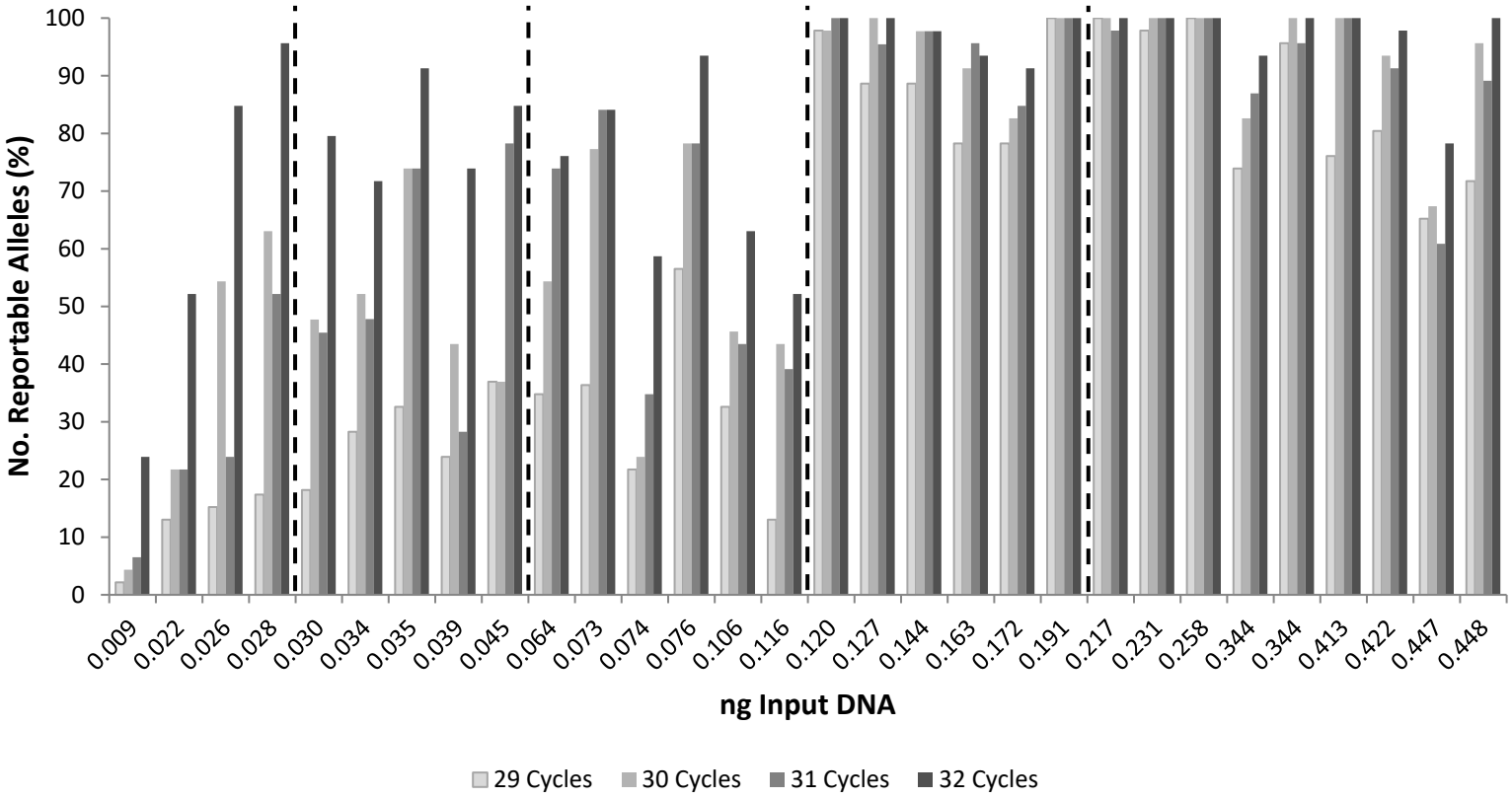
Chapter 4 Supplemental Tables and Figures

Appendix Table C1 - Information regarding the skeletal samples used in this study.

Sample #	Skeletal Element	Insult
1	Top Vertebral Arch	Cremated
2	Bottom Vertebral Arch	Cremated
3	Canine/Teeth	Embalmed
4	Humerus	Embalmed
5	Femur	Embalmed
6	Tibia	Decomposed body (3 weeks)
7	Humerus	Buried (24 months)
8	Femur	Buried (24 months)
9	Femur	Burned
10	Molar	Artificially degraded - boiled (4 hrs.)
11	Molar	Artificially degraded - boiled (6 hrs.)
12	Molar	Artificially degraded - boiled (8 hrs.)



Appendix Figure C1 - 3-Dimensional comparison of change in STR profile quality based on amount of input DNA (pg) (z-axis) and cycle number (x-axis) for (a) percentage of correct alleles reported; (b) average peak height; (c) average peak height ratio; (d) number of observed PCR artifacts (exaggerated stutter, off-ladder alleles, drop-in alleles, pull-up origins, and incomplete adenylation). Data is normalized to each mean value from 29 cycles.



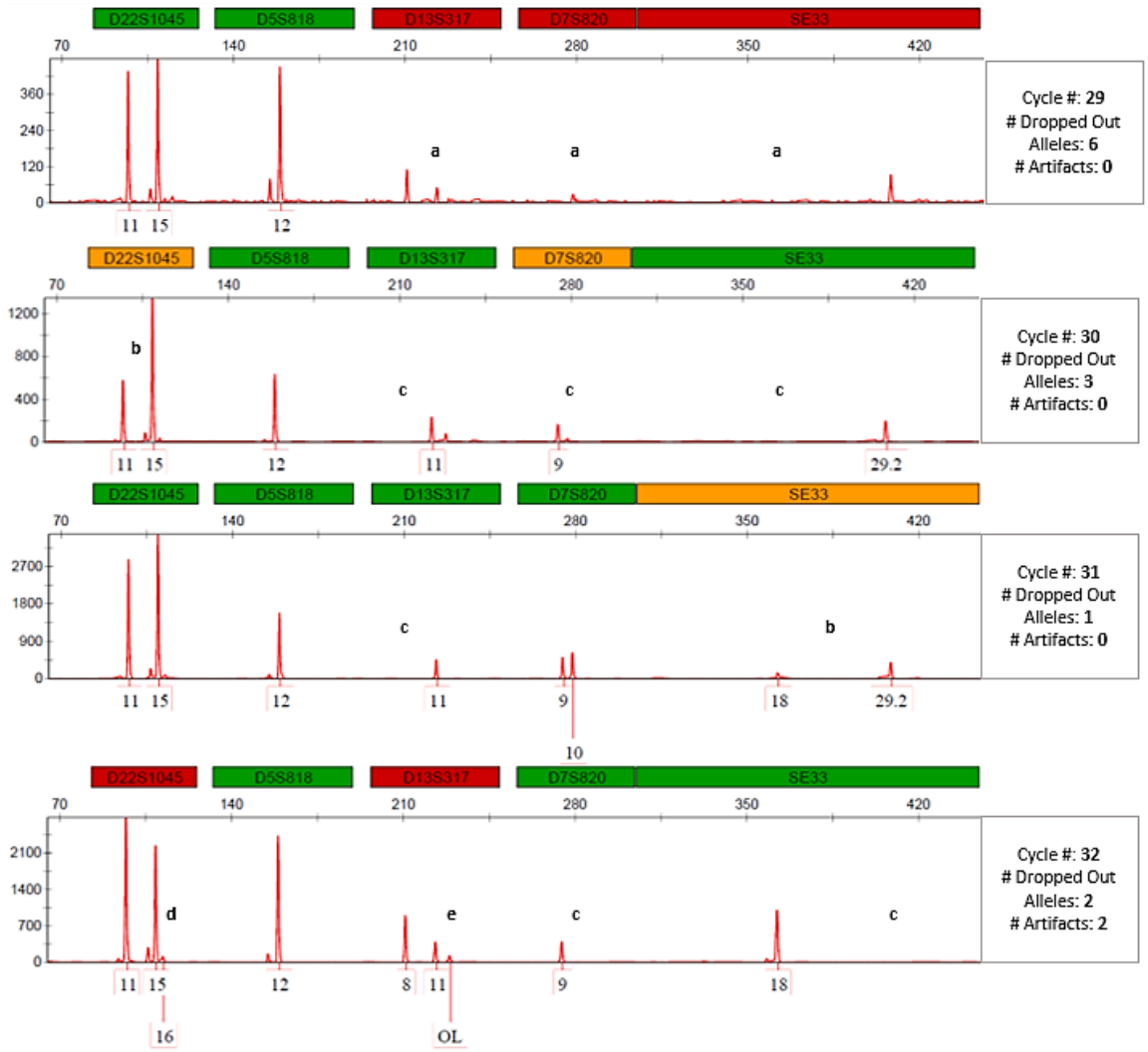
Appendix Figure C2 - Comparison of STR success for all 30 samples when amplified using 29, 30, 31, and 32 PCR cycles. Dotted lines indicate the group samples are sorted into based on DNA template in PCR for data analyses. The table below (next page) indicates which of the 12 donors (Table C1) each amplified sample originates.

(Figure C2 cont.)

Sample (numbered left to right)	Input DNA (ng)	Biological Source Sample
1	0.009	2
2	0.022	1
3	0.026	5
4	0.028	6
5	0.030	9
6	0.034	1
7	0.035	6
8	0.039	5
9	0.045	5
10	0.064	7
11	0.073	9
12	0.074	4
13	0.076	7
14	0.106	2
15	0.116	4
16	0.120	11
17	0.127	10
18	0.144	12
19	0.163	1
20	0.172	8
21	0.191	11
22	0.217	4
23	0.231	5
24	0.258	12
25	0.344	3
26	0.344	4
27	0.413	5
28	0.422	5
29	0.447	2
30	0.448	1

Appendix Table C2 - Expanded summary of all artifacts observed in STR profiles based on PCR cycle number and locus (in order of increasing amplicon size). *n*=30.

	29 Cycles				30 Cycles				31 Cycles				32 Cycles					TOTAL
	Stutter	Off Ladder	Pull-up origin	Drop-in	Stutter	Off Ladder	Pull-up origin	Drop - in	Stutter	Off Ladder	Pull-up origin	Drop - in	Stutter	Off Ladder	Pull-up origin	Drop - in	Incom. Aden.	
Y INDEL						1	1								4			6
D2S441										1			3	5				9
AMEL															5			5
D22S1045	1				1				3				8	1	6	1		21
D10S1248					2			1	3				3		10	2		21
D3S1358	1				4				3				10		2			20
D8S1179													7		2	3		12
D19S433										1				1	1			3
D5S818	1				1	1	1						10	5	7			26
D1S1656					1				1				7					9
vWA					1				3				2		3			9
TH01													2		2			4
D21S11						1							4					5
D13S317		1				1				1		1	2	2	1			9
D12S391	1												1		2			4
D16S539	1				1								1					3
D7S820					1								1		2		1	5
FGA													1				1	2
CSF1PO													1					1
D18S51					1				1				6			1		9
D2S1338					1				1				5		1			8
TPOX																		0
SE33								1	3				8	2		1		15
DYS391									1				2					3
<120 pg	0	1	0	0	2	3	1	2	3	1	0	0	19	6	1	1	1	**41**
120-500 pg	5	0	0	0	12	1	1	0	16	2	0	1	65	10	47	7	1	**168**
TOTAL	**5**	**1**	**0**	**0**	**14**	**4**	**2**	**2**	**19**	**3**	**0**	**1**	**84**	**16**	**48**	**8**	**2**	**209**



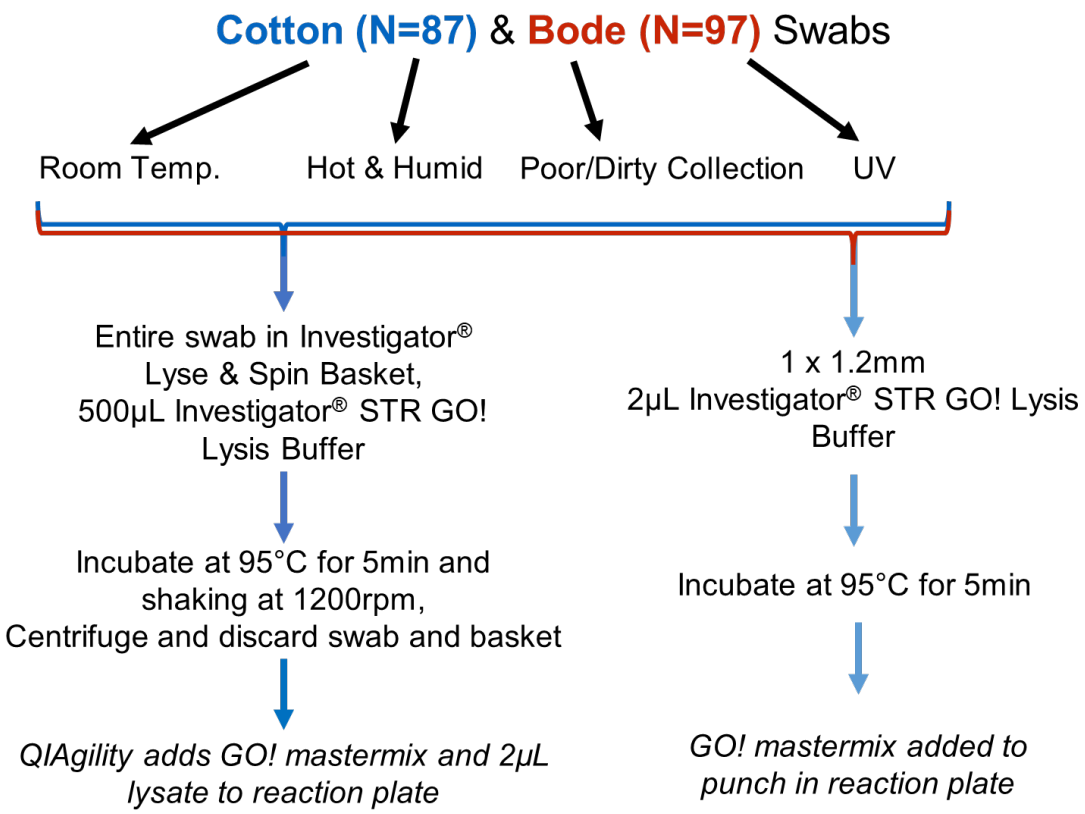
Appendix Figure C3 - A representative electropherogram (red channel only) for one buried Humerus sample (0.064 ng) amplified with 29, 30, 31, and 32 cycles using the GlobalFiler® PCR amplification kit. Stochastic effects and other artifacts are labeled as follows: a) locus dropout, b) peak height imbalance, c) allele dropout, d) exaggerated stutter, e) off-ladder peak.

APPENDIX D

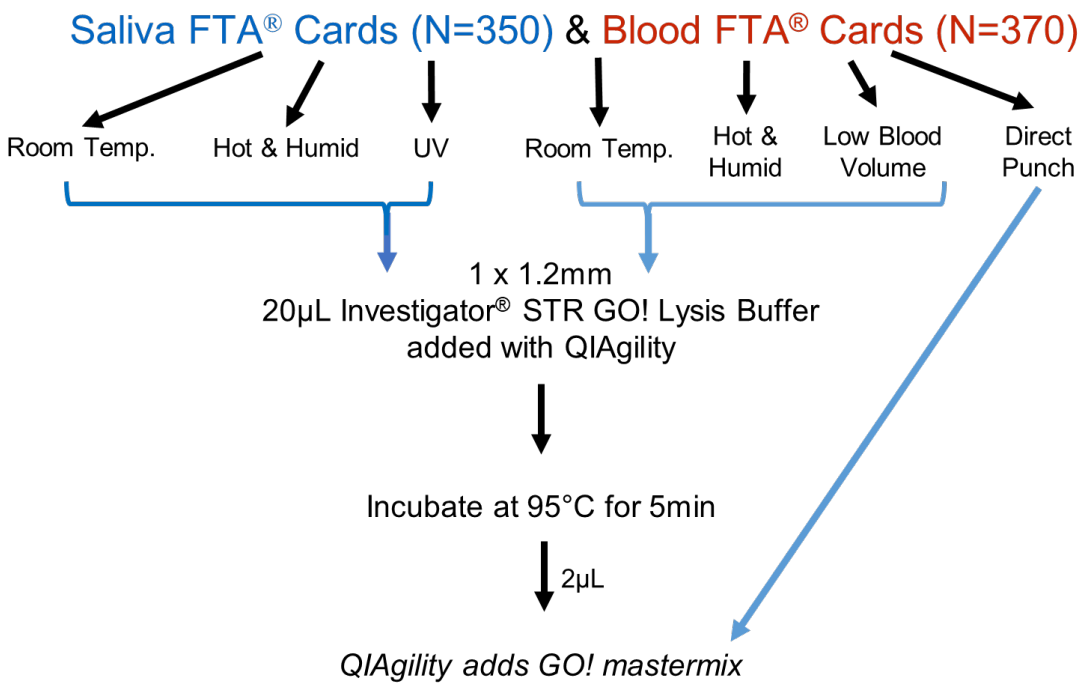
Chapter 5 Supplemental Tables and Figures

Appendix Table D1 - STR profile metrics for direct amplification low blood volume samples.

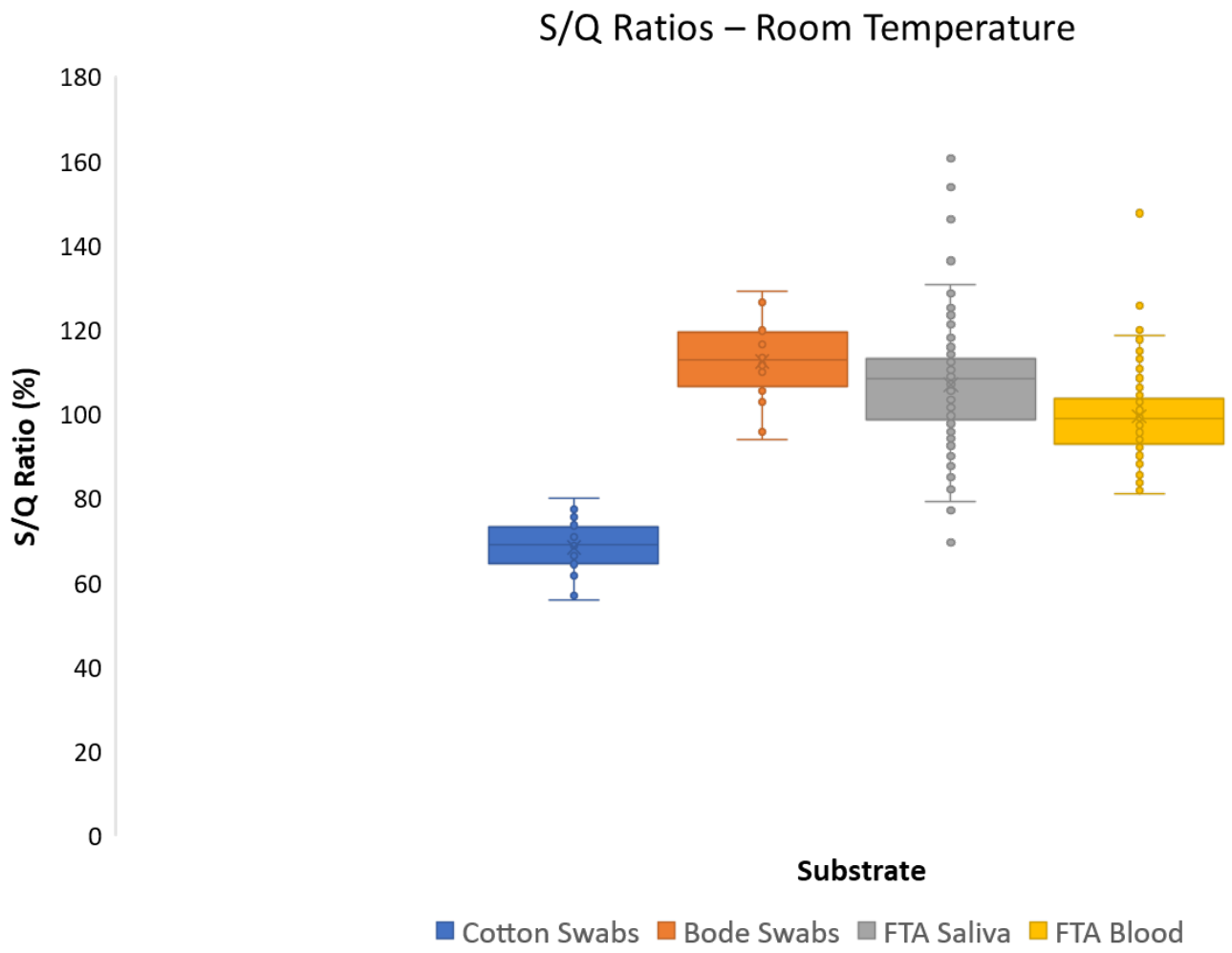
Donor #	Replicate #	QS1	QS2	S/Q*100 (%)	Reportable Alleles (%)	APH (RFUs)	APHR (%)
1	01	-	-	-	0.00	0.00	0.00
	02	-	-	-	0.00	0.00	0.00
2	01	-	-	-	0.00	0.00	0.00
	02	Q	-	-	0.00	0.00	0.00
3	01	Q	-	-	48.89	539.11	36.49
	02	Q	-	-	33.33	161.62	24.77
4	01	Q	-	-	0.00	0.00	0.00
	02	-	-	-	0.00	0.00	0.00
5	01	Q	-	-	55.56	186.22	48.41
	02	Q	-	-	28.89	67.87	25.76
6	01	Q	-	-	71.11	372.02	57.68
	02	Q	-	-	77.78	716.62	62.07
7	01	Q	-	-	31.82	701.36	22.56
	02	Q	-	-	29.55	140.55	16.80
8	01	Q	-	-	27.27	225.14	13.34
	02	Q	-	-	31.82	1173.43	15.25
9	01	Q	-	-	4.55	13.77	0.00
	02	Q	-	-	2.27	2.45	0.00
10	01	Q	-	-	15.56	31.71	7.58
	02	Q	-	-	31.11	237.16	19.40
				AVG	**24.47**	**228.45**	**17.51**
				SEM	**5.48**	**71.84**	**4.48**



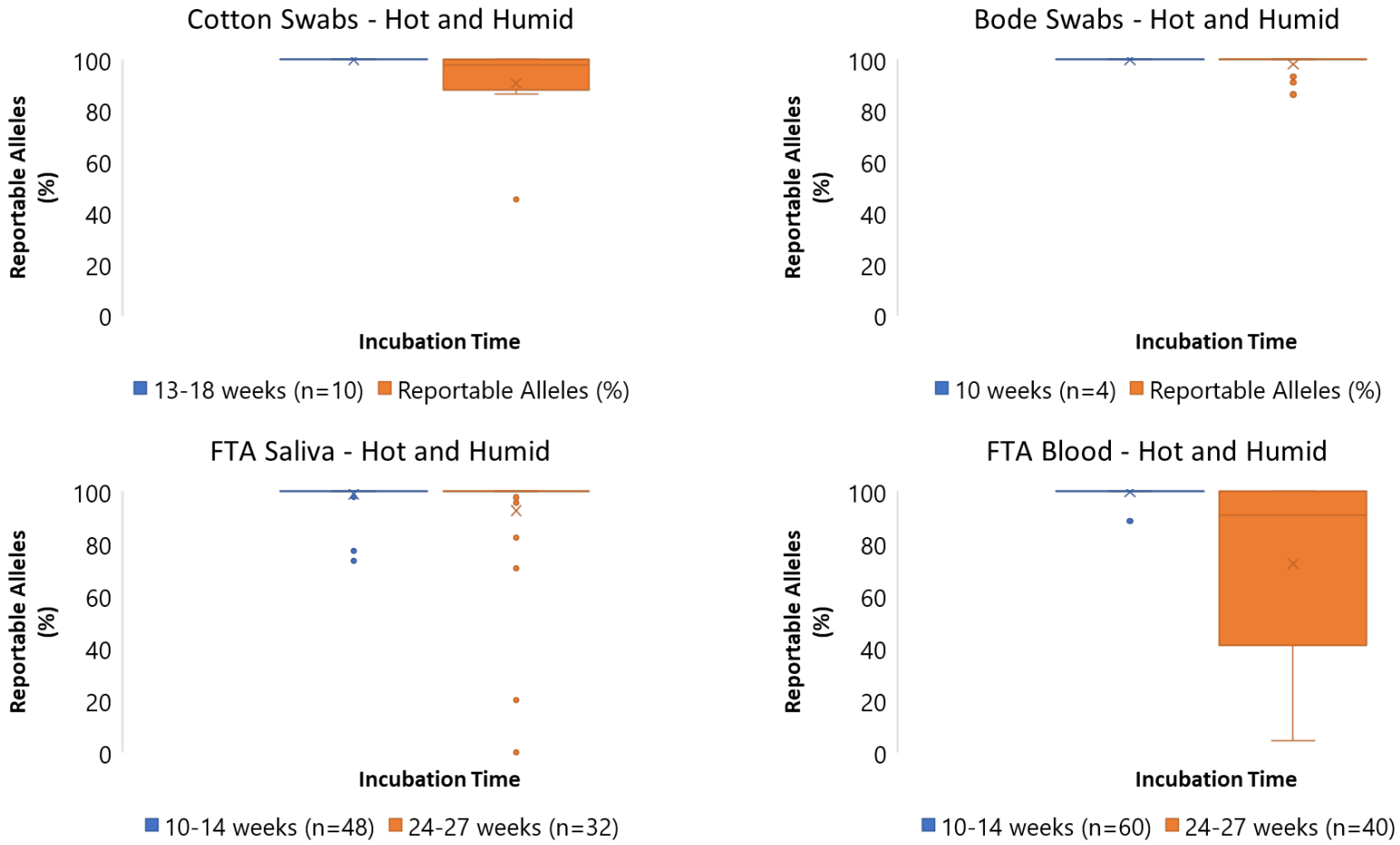
Appendix Figure D1 – Direct amplification workflow used in this study for processing cotton and Bode buccal swabs. Swabs stored at room temperature were used as controls and insult categories included: storage in hot and humid conditions, poor or dirty collection, and UV-exposure.



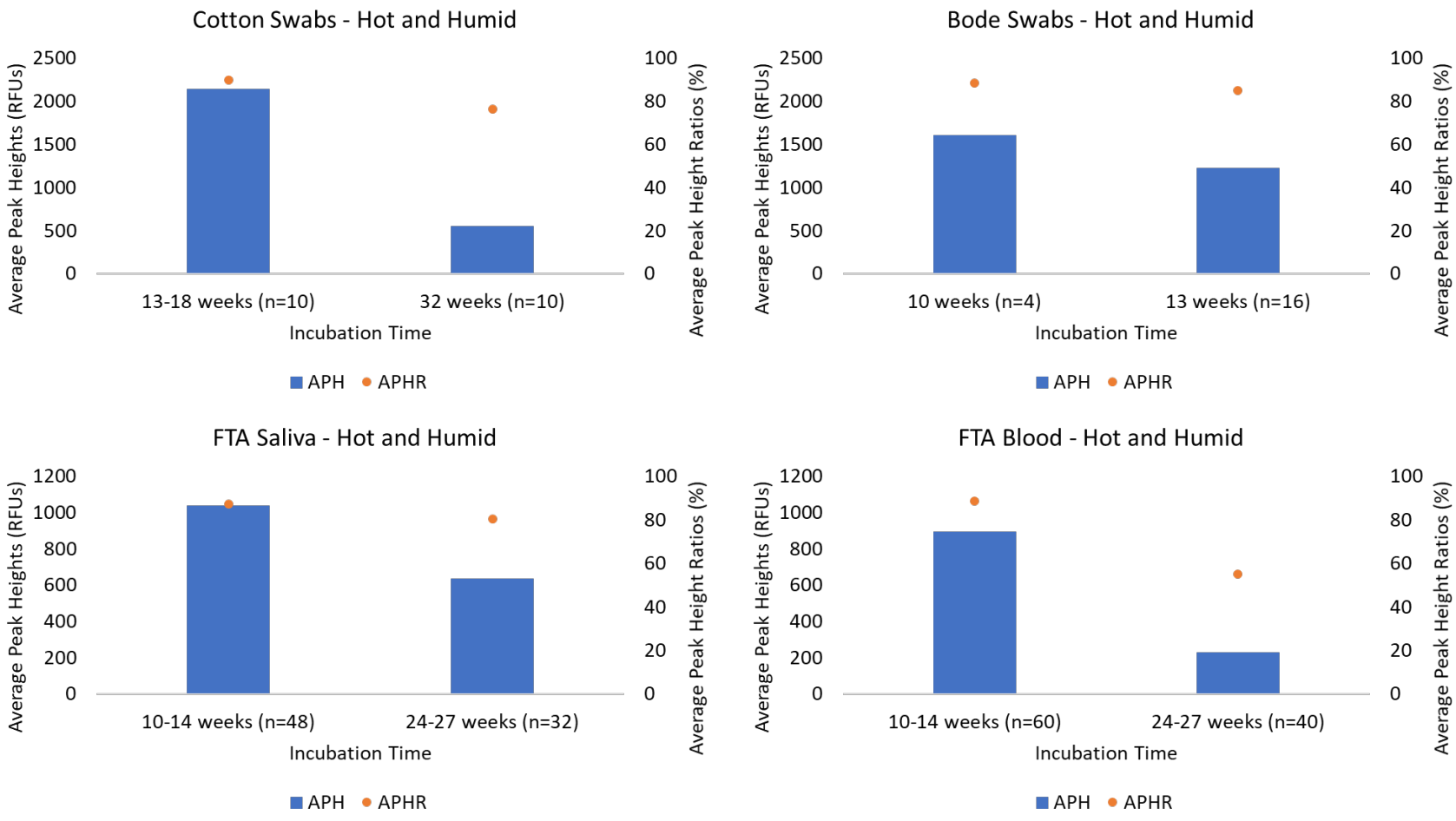
Appendix Figure D2 – Direct amplification workflow used in this study for processing saliva and blood samples on FTA® cards. FTA® cards stored at room temperature were used as controls and insult categories included: storage in hot and humid conditions, UV-exposure (saliva only), and low blood volume samples (blood only) processed with and without STR GO! lysis buffer.



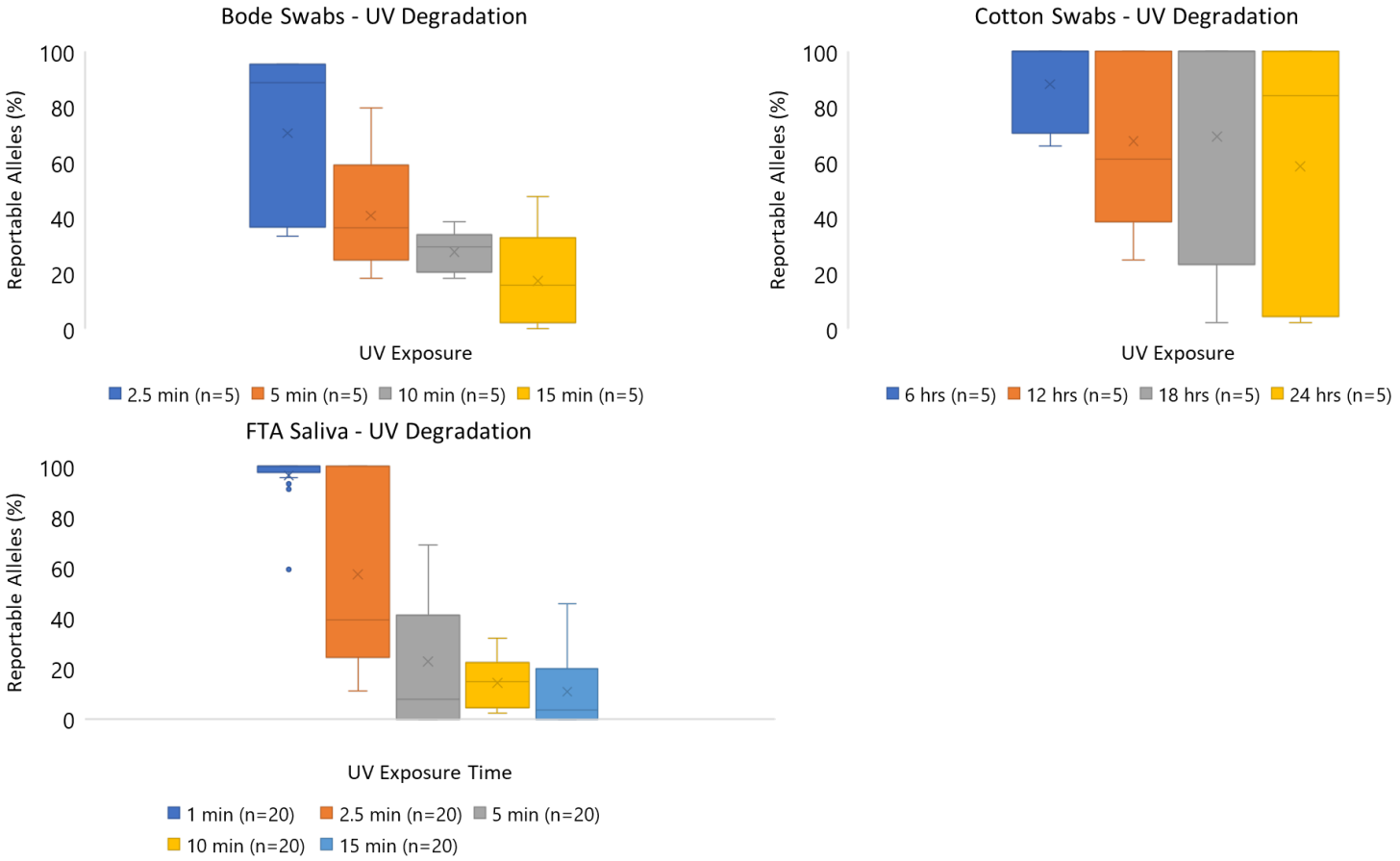
Appendix Figure D3 – Distribution of S/Q ratios for each substrate/sample type stored at room temperature.



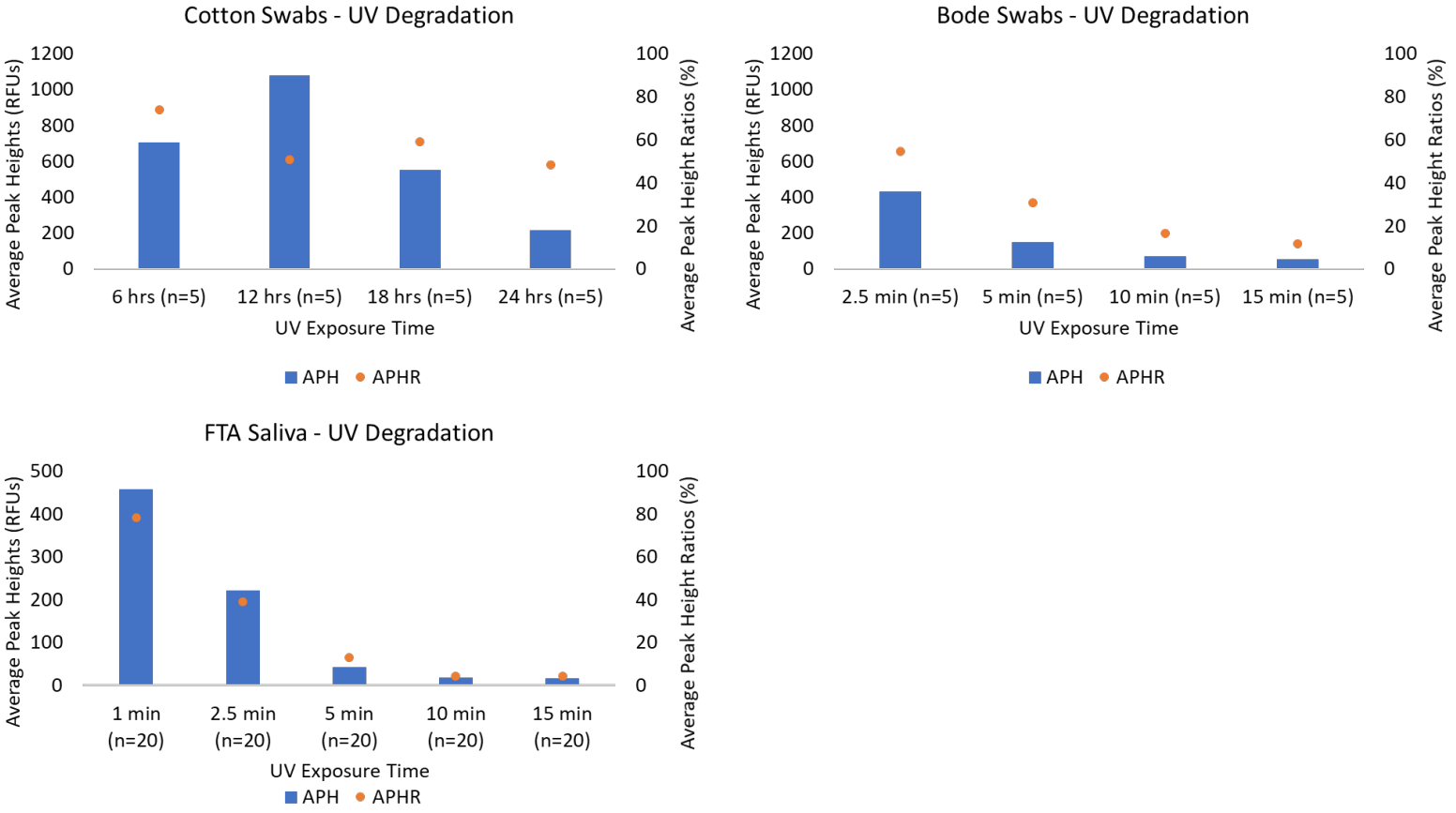
Appendix Figure D4 – Comparison of reportable alleles over time for each substrate type after incubating samples in a hot and humid environment. Substrates include: cotton buccal swabs (top left), Bode buccal swabs (top right), FTA® saliva cards (bottom left), and FTA® blood cards (bottom right). Samples were collected at various time points for up to 32 weeks.



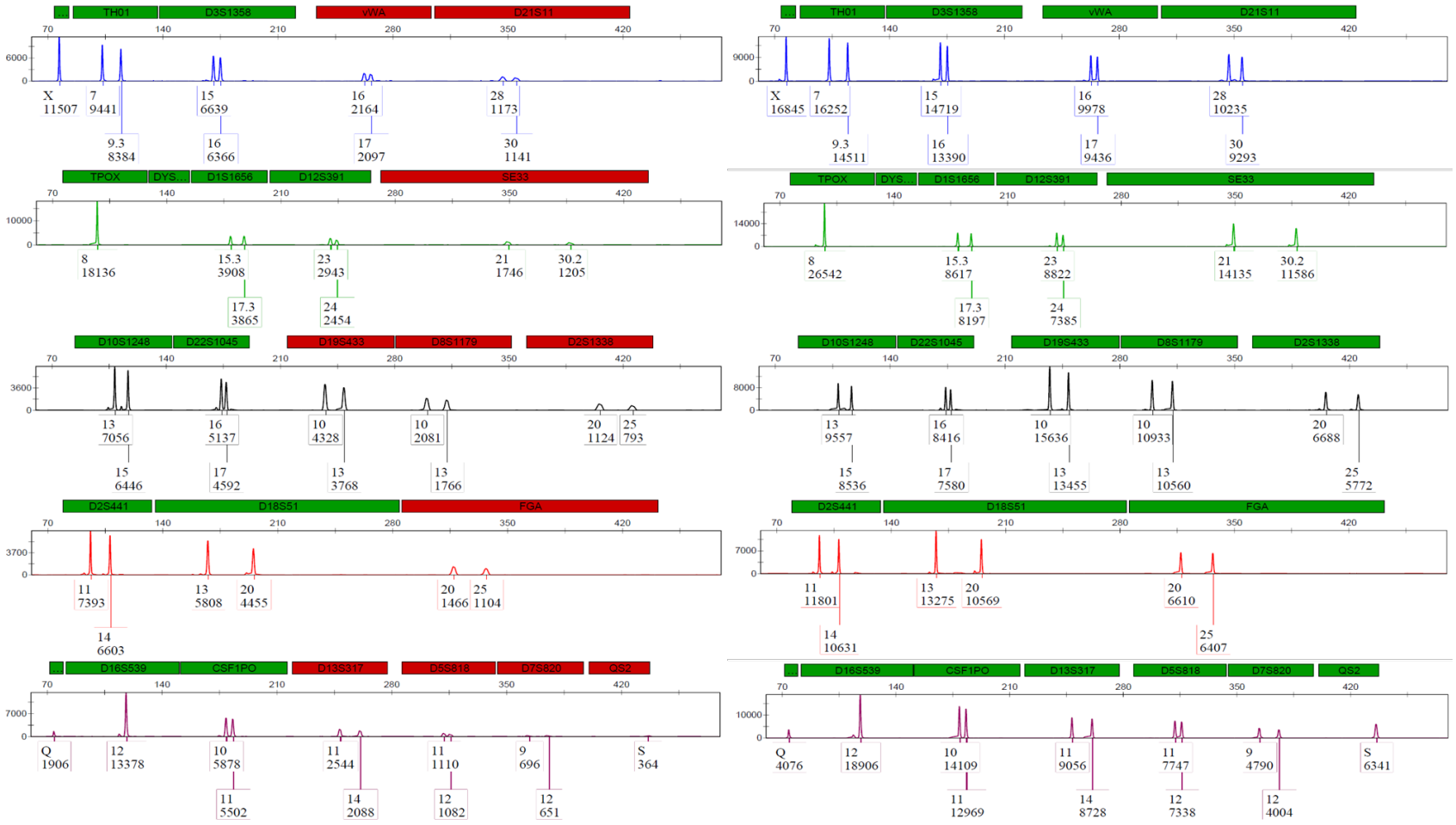
Appendix Figure D5 – Comparison of STR profile quality metrics (average peak heights – primary Y-axis; average peak height ratios – secondary Y-axis) over time for each substrate type after incubating samples in a hot and humid environment. Substrates include: cotton buccal swabs (top left), Bode buccal swabs (top right), FTA® saliva cards (bottom left), and FTA® blood cards (bottom right). Samples were collected at various time points for up to 32 weeks.



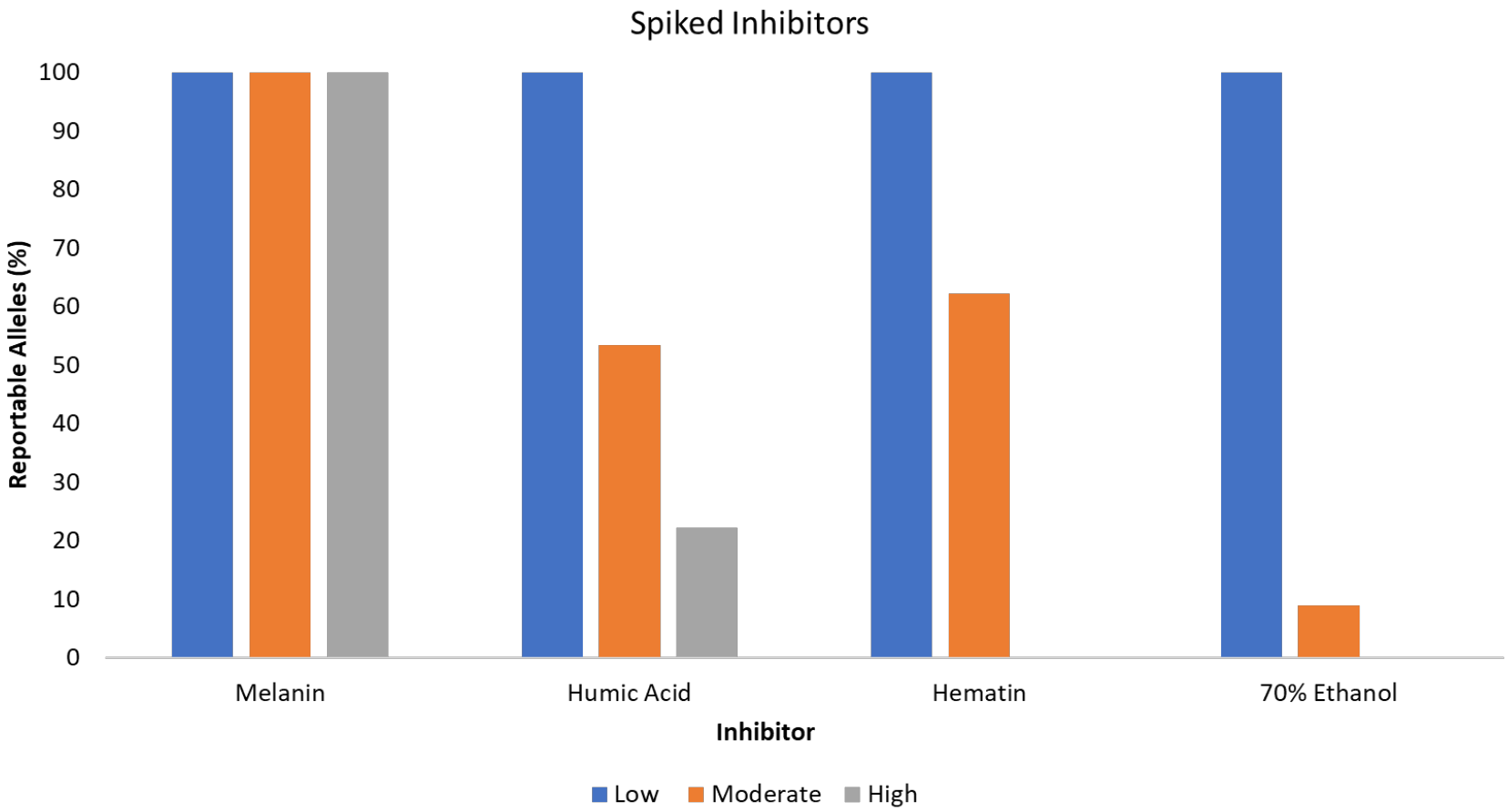
Appendix Figure D6 – Comparison of reportable alleles between different exposure times to direct UV light for each substrate type. Substrates and exposure times include: Bode buccal swabs – 2.5-15 mins (top left), cotton buccal swabs – 6-24 hrs (top right), and FTA® saliva cards – 1-15 mins (bottom left).



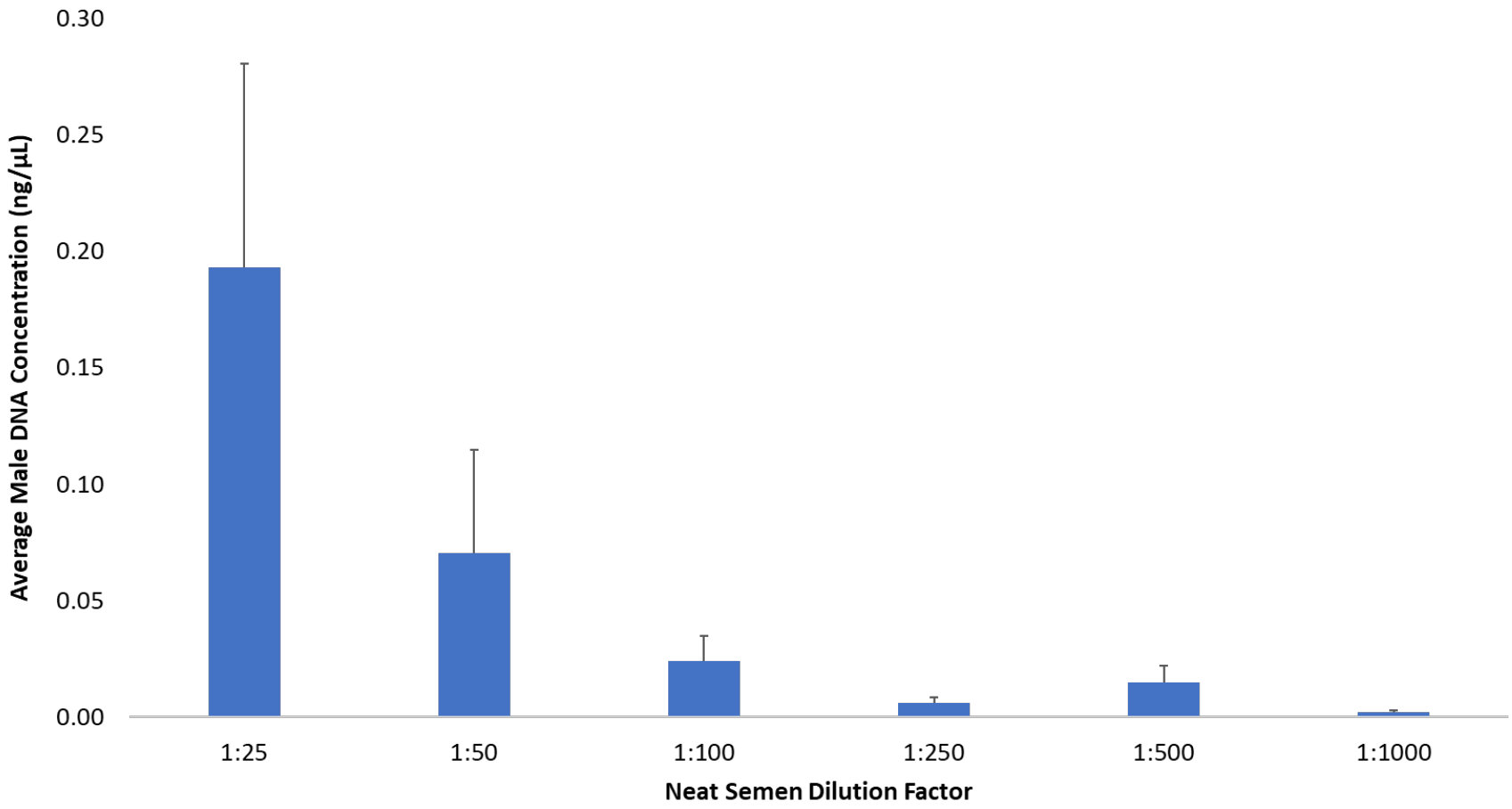
Appendix Figure D7 – Comparison of STR profile quality metrics (average peak heights – primary Y-axis; average peak height ratios – secondary Y-axis) between different exposure times to direct UV light for each substrate type. Substrates and exposure times include: Bode buccal swabs – 2.5-15 mins (top left), cotton buccal swabs – 6-24 hrs (top right), and FTA® saliva cards – 1-15 mins (bottom left).



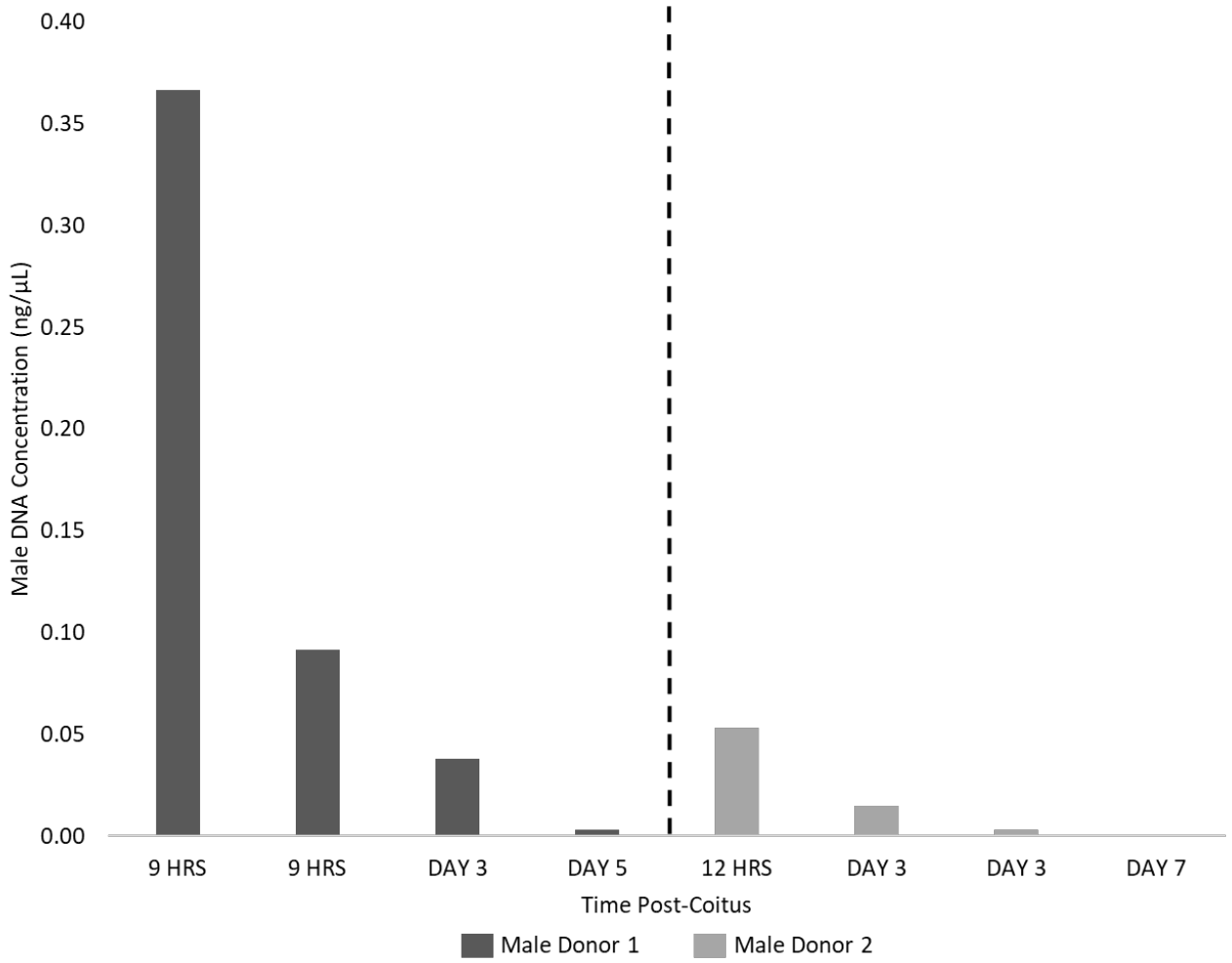
Appendix Figure D8 – Example of a full profile sample with an S/Q ratio below 20%. Left - EPG of sample after original CE injection (S/Q ratio – 19%); Right - EPG of sample after re-injection (S/Q ratio – 156%).



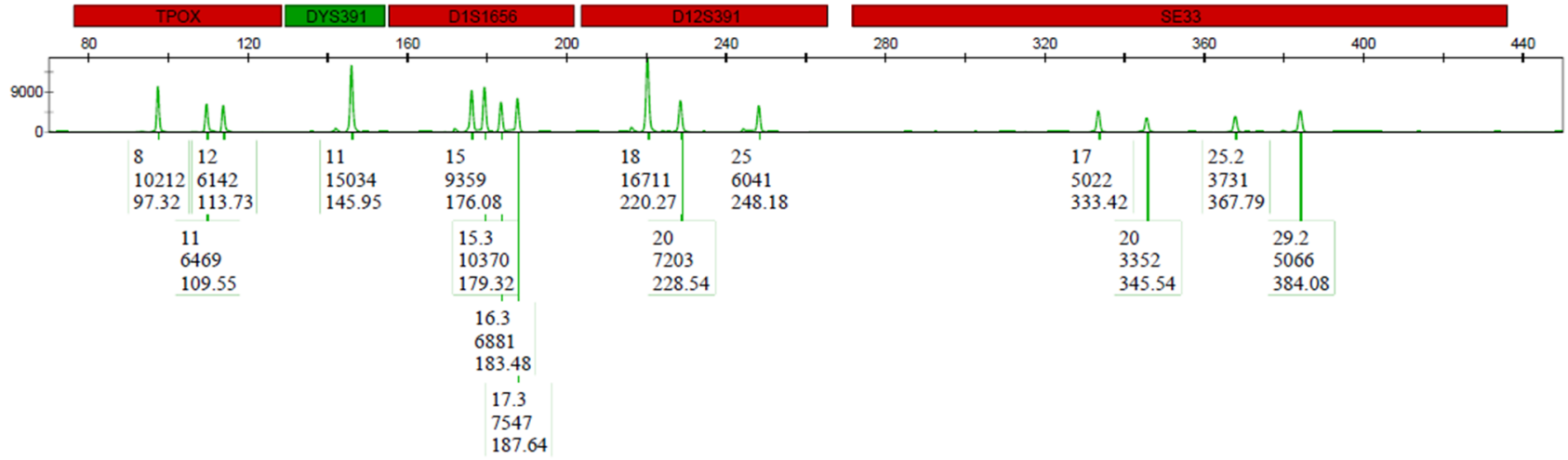
Appendix Figure D9 – Comparison of reportable alleles between three concentrations of each inhibitor tested in this study. Inhibitors and their concentration ranges include: melanin (25-60 ng/µL), humic acid (100-400 µM), hematin (500-1500 ng/µL), and 70% ethanol (1-3 µL/PCR reaction).



Appendix Figure D10 – Average male DNA concentrations (*n*=4) recovered in the sperm fractions of mock sexual assault samples for each dilution factor (1:25 – 1:1000) used in this study. Error bars denote the standard error of the mean (SEM).



Appendix Figure D11 – Male DNA concentrations recovered in the sperm fractions of authentic post-coital samples for each collection time (9 hrs – 7 days) used in this study.



Human (ng/µL)	Human Deg.	Human DI	Male	Male Deg.	Male DI	IPC ΔCT	Mixture Index	Flags
0.09	0.08	1.14	0.06	0.07	0.75	2.56	**1.58**	--

Appendix Figure D12 - Example of an EPG showing a mixture (top) that was not flagged during qPCR (bottom) as determined by a mixture index <2.

APPENDIX E

Application Note

The effectiveness of STR Quality Sensors to inform rework strategies and improve STR success of challenging samples

Carrie Mayes[1], Michelle Harrel[1], Rachel Houston[1], Ryan Gutierrez[1], Sheree Hughes-Stamm[1,2]

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(Published file accessible on QIAGEN's website at: https://www.qiagen.com/us/resources/resourcedetail?id=79c3b384-6e1d-4a7c-9c3a-09d928c34577&lang=en)

Introduction

The Investigator® 24plex GO! and Investigator® 24plex QS kits are 6-dye multiplex assays for the identification of human reference and casework samples, respectively. In addition to the expanded CODIS core and three additional autosomal loci (SE33, D2S1338 and D19S433), these kits also include two quality sensor targets (QS1 and QS2) that serve as internal PCR controls. The presence, absence or relative amplification of these quality sensors can assist the interpretation of STR profiles and direct analysts toward more effective rework strategies (1).

The use of direct PCR for database and reference samples has greatly increased laboratory throughput by employing automated solutions and bypassing the time-

consuming and costly DNA extraction and quantification processes. Due to the predictable nature of databasing samples (buccal swabs and FTA® cards), full STR profiles can be generated from a majority of them. However, lack of purification can leave some samples vulnerable to PCR inhibition, while other complicating factors, such as DNA degradation and low amounts of DNA (LT-DNA), can also affect downstream STR success (1–5). Sources of inhibition can originate from the collection/preservation method used or the sample itself. The main sources of PCR inhibitors in reference blood samples include endogenous hematin and EDTA from blood collection tubes and FTA cards (6). DNA degradation may occur in samples stored improperly or in a facility with limited climate control. For buccal swabs, poor collection and storage techniques as well as exogenous inhibitors from food and beverages can produce low template and/or inhibited profiles (7).

In this study we selected a set of challenging samples (N=53) that generated less than 90% reported alleles in the first amplification round. To assess the values of the QS markers included in the STR kits, the samples were then reworked based on the quality of the electropherogram (EPG) with the QS markers redacted (Figure 1A) and in conjunction with the QS markers (Figure 1B). Results from each of the reworks were analyzed to determine which strategy, if either, improved the profile quality and the number of STR alleles reported.

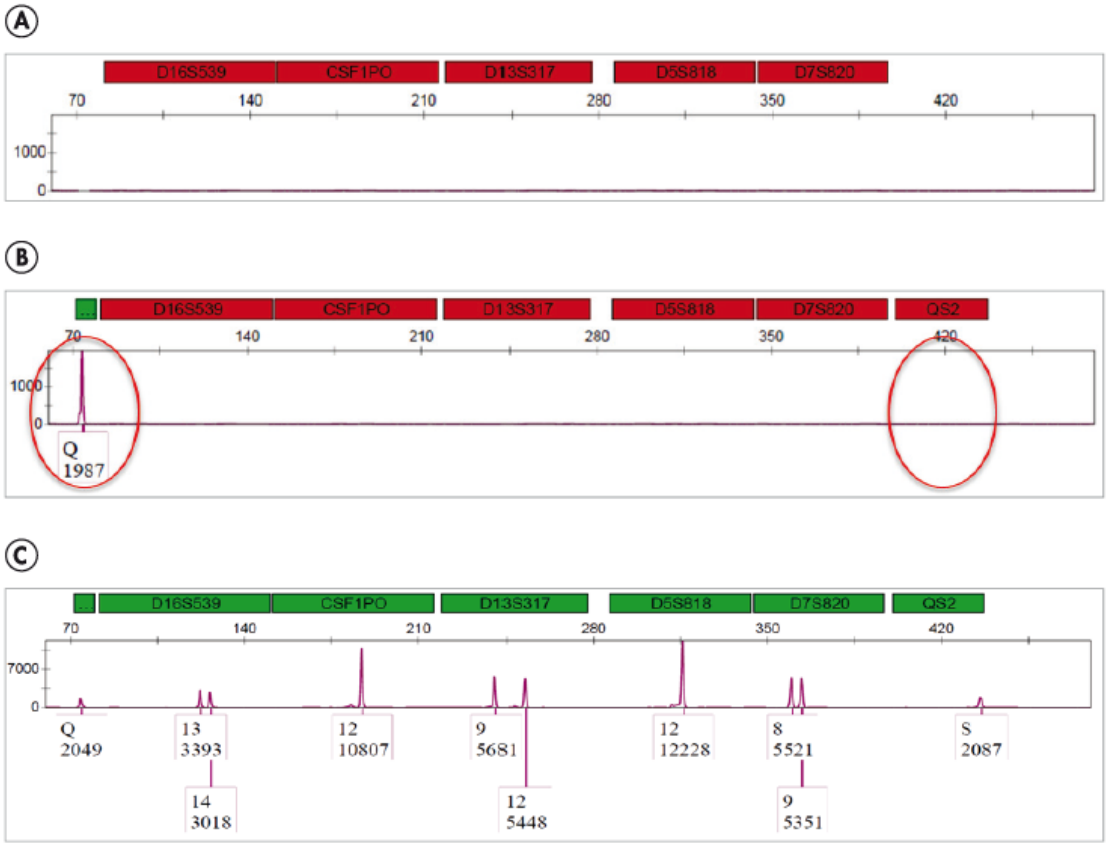

Figure 1. Example EPG (purple channel only) for a low blood volume FTA sample. The sample was analyzed after: **A** first-pass direct amplification (shown with QS markers redacted), **B** secondary direct amplification with new punch (S marker failed), and **C** lysis of new punch with the Investigator 24plex GO! lysis buffer before secondary direct amplification (rework determined by failed S marker in B).

Methods

Sample collection and preparation

Databasing samples

Reference DNA samples were collected from informed and consenting participants pursuant to IRB 2018-05-40949 approved by Sam Houston State University. A subset of samples (N=53) from a larger sample pool were chosen for this study based on STR profile completeness. Blood samples were collected via venipuncture and spotted on Whatman® FTA cards (GE Healthcare). Saliva samples were collected using sterile cotton tipped applicators (Puritan Medical Products Company), the Bode Buccal DNA Collector®

system (Bode Technology) or Whatman FTA cards with Easicollect™ devices (GE Healthcare) using manufacturers' recommended protocols (hereafter referred to as cotton swabs, Bode swabs and saliva FTA samples, respectively).

With the exception of room temperature controls, databasing samples were subjected to a variety of simulated environmental conditions, including incubation in a hot and humid environment for up to 27 weeks, UV exposure for between 1 minute and 24 hours, and poor collection methods, such as single cheek swipes for buccal swabs. Low blood volume samples were simulated using purple-topped blood collection tubes with less than 0.75 ml of blood. Blood from the collection tubes was then deposited onto FTA cards.

Casework-like samples

DNA extracts previously identified as inhibited, low-template or degraded were used in this study. Inhibited and degraded samples were sourced from cadaver muscle biopsies stored in a liquid preservative and unpreserved nylon swabs. In addition, samples spiked with inhibitors (hematin, melanin, humic acid and ethanol) were prepared using neat inhibitors and control DNA.

DNA quantification and amplification

Setup for all quantification and amplification reactions was performed using a QIAGEN QIAgility® liquid handling platform. All mock casework samples were quantified using the Investigator Quantiplex® Pro RGQ kit on a QIAGEN Rotor-Gene® Q and results were analyzed with the QIAGEN Data Handling Tool.

Databasing samples were amplified using the QIAGEN Investigator 24plex GO! chemistry. DNA in both cotton and Bode swabs was amplified according to the

manufacturer's guidelines using 26 PCR cycles. For both saliva and blood FTA cards, single 1.2 mm punches were manually deposited into the wells of a 0.2 ml 96-well plate and 20 µl of STR GO! lysis buffer was directly added to the punches. The plate was then centrifuged and incubated at 95°C for 5 minutes before 2 µl of the crude lysate was added to 20 µl GO! Master Mix. Additionally, a subset of blood FTA samples were directly amplified without use of the GO! lysis buffer. DNA in FTA blood and saliva samples was amplified for 27 PCR cycles.

All sample dilutions for mock casework samples targeted a final DNA input per reaction of 0.8 ng. Samples were amplified with the QIAGEN Investigator 24plex QS Kit according to the manufacturer's protocol on either a ProFlex™ (Applied Biosystems) or Veriti™ (Applied Biosystems) thermal cycler.

Capillary electrophoresis and data analysis

Amplified fragments were separated and detected on an Applied Biosystems® 3500 Genetic Analyzer on a 36 cm capillary array using handbook-defined settings. Data analysis was completed using GeneMapper IDX v1.4 with tertiary analysis being accomplished with in-house Excel® (Microsoft Corp.) workbooks. Stochastic and analytical thresholds were set at 200 RFU and 100 RFU, respectively.

Sample reworks

Strategy determination

To assess the benefits of the QS markers present in STR profiles, strategies for reworking samples were determined by analyzing STR profiles with and without the QS marker information visible. To avoid bias, a forensic DNA analyst from an external crime

laboratory was asked to interpret EPGs with the QS markers redacted. For databasing samples, the analyst was provided with the sample type (blood vs buccal) and substrate (FTA card vs Bode/cotton swab) and asked to indicate their rework strategy. Quantification information was also provided to the analyst during STR profile evaluation for casework samples, as these data would normally be available regardless of the STR chemistry used. The analyst used their experience and their laboratory's standard operating procedures (SOPs) to assess the profile quality and determine the appropriate rework strategy (if any).

In addition to the rework approach indicated by the external analyst (blinded to QS markers), samples were also examined by a different analyst who indicated their rework strategy based on the performance of the QS markers. With the QS marker information, inhibition was suspected when the Quality Sensor S/Q allele ratio was below 70% and confirmed when one or both QS markers dropped out. Samples were classified as low template and/or degraded when the QS markers were balanced and low RFUs were observed consistently throughout the EPG (roughly average peak height of <750 RFUs). The absence of DNA template in the PCR was determined by the presence of balanced QS markers and no other alleles called. In general, rework strategies were determined as follows: adding more template to the PCR amplification (for suspected low or degraded template), diluting the lysate/extract before re-amplifying (for suspected PCR inhibition), or processing a new punch from the same sample (for suspected failed amplification due to no template).

Strategy implementation

Rework strategies for each sample were designated, executed and compared. If the strategies with and without the QS markers were the same, the rework was only performed

once. Databasing samples requiring an increased template included either an additional microliter of lysate (3 µl total) if available or two 1.2 mm punches added to the PCR. For samples identified as inhibited, either a 1:3 dilution or a punch wash with GO! lysis buffer was performed before re-amplification. Inhibited casework samples were diluted as much as 1:15 and re-quantified prior to re-amplification. Finally, samples categorized as having no DNA template in the PCR were reprocessed either as a new punch or from the original lysate/extract when applicable. All PCR cycling conditions and CE parameters remained the same as for the initial amplification.

Results and Discussion

As expected, reworking challenging samples resulted in more complete STR profiles compared to the original amplifications (Figure 2). Compared to the inhibited samples, the degraded/low template samples produced more complete STR profiles but also yielded wider variations in first- pass amplification success rates. Overall, the increase in allele recovery after applying both rework strategies was comparable for degraded/low template samples but differed for those that were inhibited (Figure 2). The greatest improvement in STR success was achieved when inhibited samples were reworked based on information provided by the QS markers (29/29 samples improved) rather than relying on the quality of the EPG alone (20/29 samples improved; Figure 2). Although a trained analyst may be able to identify signs of PCR inhibition within the profile, this study found that inhibition was more reliably and accurately detected based on the behavior of the QS markers.

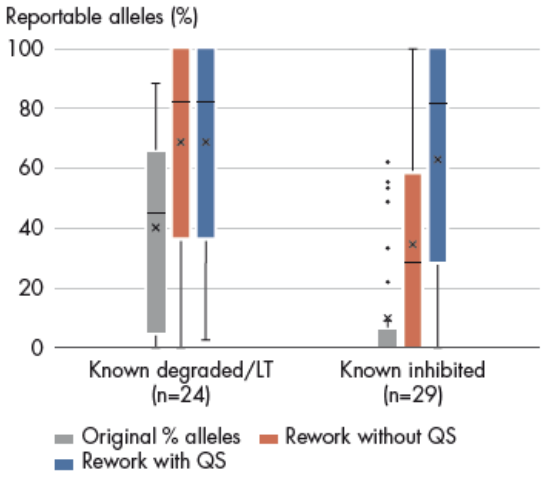

Figure 2. Comparison of STR results before and after reworks with and without QS marker information for challenging samples. Outliers are shown and sample means are represented by the 'x' symbols.

Interestingly, direct amplification of several low blood volume FTA cards resulted in complete amplification failure. We suspect that the accumulation of EDTA in these samples from both the blood tubes and the unwashed FTA cards resulted in high levels of PCR inhibition. Without the QS marker information, the external analyst assumed the punch contained no DNA template and the rework strategy was to process a new punch, which yielded the same results as the initial assays (Figures 1A and 1B). However, with the QS marker information available, inhibition was indicated due to one or both QS markers failing to amplify, and the appropriate rework strategy was identified. In these cases, a wash with GO! lysis buffer was performed and full STR profiles were recovered (Figure 1C).

For samples that had concordant rework strategies regardless of the QS markers, an increase in correct alleles called was achieved 94% of the time. It is important to note that although the rework strategies were identical, the QS markers did provide the analyst with high confidence regarding the level of DNA degradation in the samples. One sample that showed no improvement after rework was highly degraded/low template, and therefore the maximum amount of template was already being amplified. Another sample still showed

signs of extreme inhibition after several dilution attempts, which was confirmed by the QS marker information.

Of the 53 samples reworked, 20 were identified as having different rework strategies based on the characteristics of the DNA profile provided to the analysts, either with or without the QS markers being masked. This demonstrates that although experienced analysts are frequently able to correctly identify the likely cause of a loss of alleles or overall poor profile quality, there are still circumstances where the true issue may be more ambiguous. When the QS markers were used to assess the likely cause of a poor STR result, the reason for PCR failure could be readily identified and the correct rework strategy employed (Figure 3). The QS markers were most beneficial in resolving failed amplifications and highly inhibited samples (Figure 4). The rework strategy based on the QS markers resulted in all but two of those 20 samples having full or nearly full STR profiles (>97%), whereas rework strategies without the QS markers were frequently less successful.

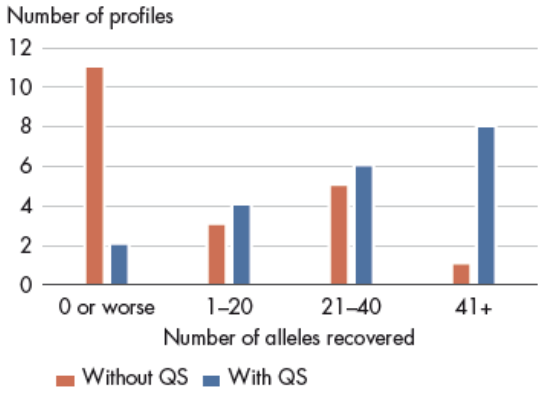

Figure 3. Improvement in profile completeness based on rework strategy. Distribution of the number of alleles recovered (compared to the original amplification) for 20 samples that had different rework strategies based on analysis of the DNA profile with (blue) and without (orange) QS marker information provided.

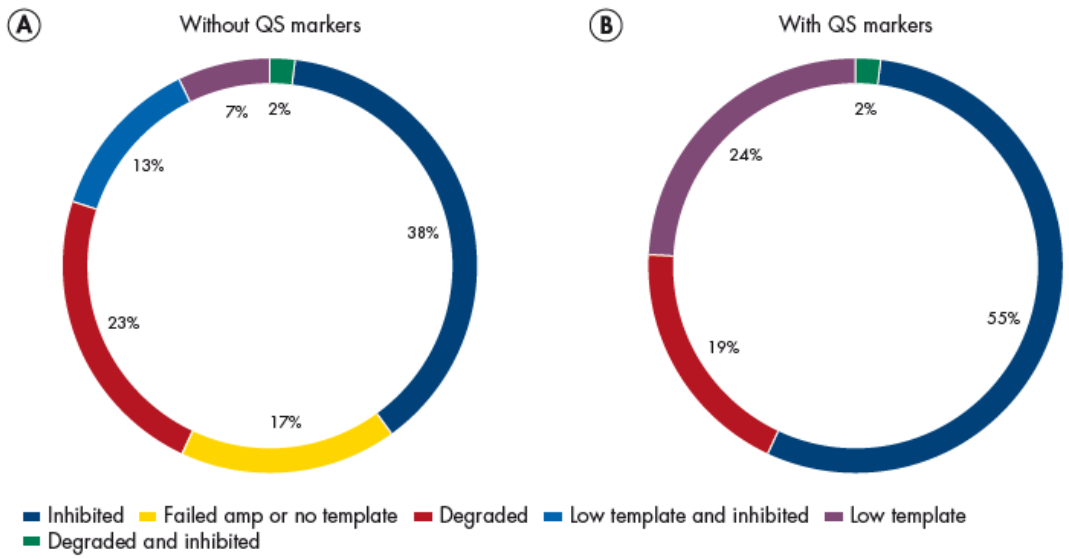

Figure 4. Assignment of the suspected cause of suboptimal DNA profiles. Electropherograms were interrogated **A** without the QS markers visible and **B** With the QS markers visible (N=53).

In addition to recovering more loci, rework strategies based on QS marker information also resulted in improvements in general profile quality. Profile quality of the first amplification was poor, and only 3.2% of samples showed an average peak height ratio (APHR) above 60% and only 13.2% of samples had an average peak height (APH) above 200 RFUs (stochastic threshold). In comparison, 32% of reworks without the QS markers and 47% samples with QS markers showed an APHR above 60%. Also, 47% of samples without QS markers and 55% of samples with QS markers had an APH above 200 RFUs (Figure 5). Overall, when QS markers were used to determine the rework strategy, consistently more alleles were recovered and a greater number of samples with balanced, reportable profiles were obtained.

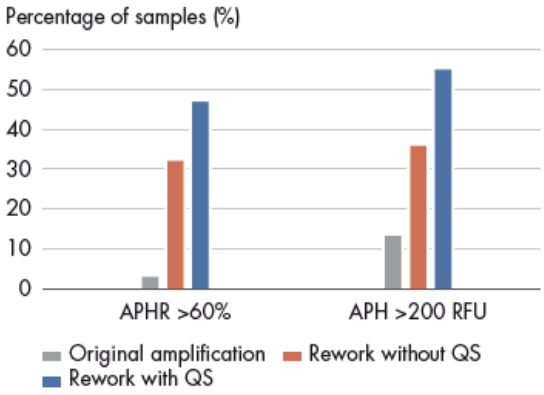

Figure 5. Quality metrics of STR profiles. The number of samples with reasonably balanced and quality STR profiles (APHR >60%, and APH >200 RFUs) before (grey) and after reworks with (blue) and without (orange) QS marker information for challenging samples was determined (N=53).

Conclusion

Most forensic DNA analysts are able to distinguish between degraded, low template and inhibited STR profiles after adequate training and experience. However, there are many instances when the best approach for how to rework a particular sample to improve results may not be apparent. This study demonstrates that QS markers can be used as a straightforward, consistent and valuable tool to assist in the interpretation of STR profiles from challenging samples. The ability to distinguish between highly inhibited samples, severely degraded DNA and failed amplification informs the analyst on the most appropriate rework strategy to eliminate or minimize the number of reworks performed, saving both time and resources, which ultimately improves overall efficiency.

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VITA

LeAnn "Michelle" Harrel

EDUCATION

Forensic Science (Ph.D.)

Sam Houston State University, Huntsville, TX

Expected Completion Date: Dec. 2019

Biology (B.S.)

Double Minor in Chemistry and Forensic Anthropology

Magna Cum Laude; With Honors, Honors College

Sam Houston State University, Huntsville, TX

Graduation Date: May 2015

Irish Studies (International Study Abroad Program)

National University of Ireland, Galway, Galway, Ireland

Courses: Irish Traditional Music and Dance; Gaelic Culture and Literature

PUBLICATIONS

Harrel M, Hughes-Stamm S. A Powder-Free DNA Extraction Workflow for Skeletal Samples. Journal of Forensic Sciences, doi:10.1111/1556-4029.14197 (2019).

Harrel M, Gangitano D, Hughes-Stamm S. The effects of extra PCR cycles when amplifying skeletal samples with the GlobalFiler® PCR Amplification Kit. International Journal of Legal Medicine, 133 (3): 745-750 (2019).

Harrel M, Mayes C, Gangitano D, Hughes-Stamm S. Evaluation of a Powder-Free DNA Extraction Method for Skeletal Remains. Journal of Forensic Sciences, 63 (6): 1819-1823 (2018).

RELEVANT COURSE WORK

Graduate

Forensic Biology, Advanced Forensic DNA, Statistical Genetics, Advanced Genetics: Population Genetics, Pharmacogenomics, Quality Assurance and Ethics in Forensic Science, Scientific Communication, Research Methods, Laboratory Management, Forensic Science Internship, Forensic Science Research, Law and Forensic Science, Forensic Medicine

Undergraduate

Introductory Cell Biology, Introductory Genetics, Organic Chemistry I&II, Quantitative Analysis, Introductory Biochemistry, Advanced Cell Biology, Molecular Biology, Elementary Statistics

RESEARCH EXPERIENCE

Forensic Biology

University of Queensland, St. Lucia, Queensland, AUS (Advisor: Dr. S. Hughes-Stamm) (Aug. 2019 – Present)

Established a new Forensic HID Laboratory at UQ with all necessary tasks including: writing risk assessments and SOPs for all methods and procedures, bringing instruments online, installing software, performing routine maintenance, completing internal-validations for genotyping kits, setting up a staff exclusion database, ordering consumables and reagents.

Performed the laboratory testing, data analysis and writing of case report for one external case submitted to the laboratory for testing. STR typing was performed via direct amplification on swabs used to collect 'touch' DNA from post-blast explosives.

Mentored an Honours student for her research project involving the study of the affect various embalming solutions have on the ability to genotype human tissues over time. Additional investigations examined INDELs as alternate identity markers for severely degraded DNA samples.

Forensic Biology

Sam Houston State University, Huntsville, TX (Advisor: Dr. S. Hughes-Stamm) (2016 – Present)

Explored methods to increase STR success from human skeletal remains such as powder-free bone extraction methods (manual and automated), various digestion methods, and alternate cycling conditions. Performed a comprehensive assessment of the effectiveness of sample quality flags and STR quality sensors to determine rework strategies for databasing and casework samples.

Molecular Genetics

Sam Houston State University, Huntsville, TX (Advisor: Dr. M. Choudhary) (2013–2015)

Characterized a *recA* mutant in *Rhodobacter sphaeroides* 2.4.1 and compared the mutation's effect on the bacterial SOS Response to that of the wild type through PCR, gel electrophoresis, UV exposure analysis, total DNA and RNA sequencing, and Reverse Transcriptase-PCR.

Paleobiology

Sam Houston State University, Huntsville, TX (Advisor: Dr. P. Lewis) (2012)

Identified and sorted fossils from the Koanaka Hills of Ngamiland, Botswana to gain hands-on knowledge of vertebrate anatomy and to catalogue them into a database.

INTERNSHIPS

Fort Worth Police Department Crime Laboratory

During this 10-week internship (May-August, 2016), completed 1-2 week rotations in the following units: Administration, Latent Prints, Chemistry, Firearms and Toolmarks, Evidence Handling and Cold Cases, and Evidence Screening/Biology. Gained experience with chain of custody documentation, Forensic Advantage (LIMS software), evidence screening and processing procedures, QA/QC measures, health and safety procedures, and training methods for each section.

ASSISTANTSHIPS/FELLOWSHIPS

Graduate Assistant

Department of Forensic Science, Sam Houston State University, Huntsville, Texas (September 2017 – Present)

Assisted Dr. Sheree Hughes-Stamm in preparing course material, general administrative and laboratory tasks, reviewing literature, and completing research projects. Assisted Dr. Bobby LaRue in teaching and organizing the graduate Forensic Biology laboratory.

Graduate Assistant

Impaired Driving Initiatives Program, Sam Houston State University, Huntsville, Texas (June 2019 – July 2019)

Compiled participant lists, calculated program critiques, created and updated hundreds of DRE files, maintained databases, and researched current impaired driving statistics.

Doctoral Teaching Fellow

Department of Forensic Science, Sam Houston State University, Huntsville, Texas (September 2018 – December 2018)

Taught and maintained one online section of an undergraduate introductory Forensic Science class.

Graduate Assistant

Impaired Driving Initiatives Program, Sam Houston State University, Huntsville, Texas (September 2016 – May 2017)

Compiled participant lists, calculated program critiques, created and updated hundreds of DRE files, maintained databases, and researched current impaired driving statistics.

Undergraduate Teaching Assistant

Department of Biological Sciences, Sam Houston State University, Huntsville, Texas (Spring 2015)

Taught one section of an undergraduate Introductory Genetics lab. Materials covered: DNA extraction from bacterial cultures, PCR amplification, gel

electrophoresis, sequencing techniques, bioinformatics tools, and Mendelian Genetics through Fruit Fly breeding and maintenance.

PRESENTATIONS AND AWARDS

Harrel, M; Mayes, C; Houston, R; Sorensen Holmes, A; Gutierrez, R; Hughes-Stamm, S*. "Effectiveness of STR Quality Sensors to Inform Rework Strategies for Challenging Database and Casework Samples using a Semi-Automated Workflow." Poster Presentation. 30th Annual International Symposium for Human Identification, Palm Springs, California. (2019)

Harrel, M*; Mayes, C; Houston, R; Sorensen Holmes, A; Gutierrez, R; Hughes-Stamm, S. "Evaluation of a Semi-Automated Workflow and Effectiveness of STR Quality Sensors to Inform Rework Strategies for Challenging Database and Casework Samples." Oral Presentation. Association of Forensic DNA Analysts and Administrators Annual Summer Meeting, Houston, Texas. (2019)

Harrel, M*; Hughes-Stamm, S. "A Faster, Easier, and More Effective Bone Processing Method for DNA Analysis." Poster Presentation. American Academy of Forensic Science 71st Annual Scientific Meeting, Baltimore, Maryland. (2019)

Mayes, C*; **Harrel, M**; Houston, R*; Sorensen Holmes, A; Gutierrez, R; Hughes-Stamm, S*. "Application of QIAGEN Workflow with Quality Sensors and Interpretation: Database and Casework Samples." QIAGEN Human Identity and Forensics Webinar. https://www.qiagen.com/us/resources/e-learning/webinars/webinars%20 on-demand /human-identity-and-forensics/ (2018)

Harrel, M*; Hughes-Stamm, S. "Effective Powder-free DNA Extraction Workflow for Skeletal Samples using the PrepFiler® BTA™ and AutoMate

Express™ Systems." Poster Presentation. 29th Annual International Symposium for Human Identification, Phoenix, Arizona. (2018)

Harrel, M*; Mayes, C; Gangitano, D; Hughes-Stamm, S. "Do we really need to crush? An alternate DNA extraction approach for bone samples." Poster Presentation. American Academy of Forensic Science 70th Annual Scientific Meeting, Seattle, Washington. (2018)

Harrel, M; Mayes, C; Gangitano, D; Hughes-Stamm, S*. "Do we really need to crush? An alternate DNA extraction approach for bone samples." Poster Presentation. 27th World Congress of the International Society for Forensic Genetics (ISFG), Seoul, Republic of Korea. (2017)

Mayes, C; Elwick, K; **Harrel, M**; Gangitano, D; Hughes-Stamm, S*; "Worlds Converge™: A New Approach to Analyzing CE and MPS-based STRs." Oral Seminar Presentation. 3rd Human Identification Solutions (HIDS) Conference, Vienna, Austria. (2017)

Mayes, C; Elwick, K; **Harrel, M**; Gangitano, D; Hughes-Stamm, S*; "NGM Detect™-ing More from Skeletal Remains." Oral Presentation. 3rd Human Identification Solutions (HIDS) Conference, Vienna, Austria. (2017)

Harrel, M*; Hughes-Stamm, S; "The Effect of Using an Extra Polymerase Chain Reaction (PCR) Cycle with GlobalFiler® When Amplifying Skeletal Samples." Poster Presentation. American Academy of Forensic Science 69th Annual Scientific Meeting, New Orleans, Louisiana. (2017)

Tasker, E; Beherec, C; **Harrel, M**; Houston, R; Gangitano, D; Hughes-Stamm, S*****; "Bodies, Bones, & Bombs." Oral Presentation. HID University, OCME, New York. (2016)

Tasker, E; Beherec, C; **Harrel, M**; Houston, R; Gangitano, D; Hughes-Stamm, S*****; "Bodies, Bones, & Bombs." Oral Presentation. HIDS, Barcelona, Spain. (2016)

Harrel, M*; Choudhary, M. "Molecular Analysis of *recA* mutant in *Rhodobacter sphaeroides."* Poster Presentation. American Society for Microbiology General Meeting, New Orleans, Louisiana. (2015)

Harrel, M*; Elings, J; Perrie, C; Workman, E. "Multidisciplinary Analysis of Human Cremated Remains*."* Oral Presentation. Undergraduate Research Symposium, Sam Houston State University, Huntsville, Texas. (2015)

Harrel, M.***** "Molecular Analysis of *recA* mutant in *Rhodobacter sphaeroides."* Oral Presentation. Undergraduate Research Symposium, Sam Houston State University, Huntsville, Texas. (2015)

Harrel, M.***** "Molecular Analysis of *recA* mutant in *Rhodobacter sphaeroides."* Oral Presentation. βββ Biological Honors Society Southcentral Regional Conference, Lake Texoma, Oklahoma. (2015) **– Frank G. Brooks Award for Excellence in Student Research: 3rd Place**

Harrel, M.***** "Molecular Analysis of *recA* mutant in *Rhodobacter sphaeroides."* Oral Presentation. American Society for Microbiology, New Braunfels, Texas. (2015)

Harrel, M*; Choudhary, M. "Growth and Molecular Characterization of *recA* mutant in *Rhodobacter sphaeroides* 2.4.1." Poster Presentation. American Society for Microbiology, Houston, Texas. (2014)

Harrel, M*; Choudhary, M. "*In vivo* construction of *recA* mutant in *Rhodobacter sphaeroides* 2.4.1." Poster Presentation. Undergraduate Research Symposium, Sam Houston State University, Huntsville, Texas. (2014).

Harrel, M*; Choudhary, M. "*In vivo* construction of *recA* mutant in *Rhodobacter sphaeroides* 2.4.1." Poster Presentation. American Society for Microbiology, New Braunfels, Texas. (2014) - **Orville Weiss Award: 1st place**

HONORS and MEMBERSHIPS

- Student Affiliate, American Academy of Forensic Sciences, Sam Houston State University, 2016-Present
- President (2017-19), Treasurer (2016-17), and Member, Society of Forensic
- Science, Sam Houston State University, 2014-Present
- Member, American Society for Microbiology Journal Club, Sam Houston State University Student Chapter, 2014-2015
- Who's Who Among Students in American Universities and Colleges Award, Sam Houston State University, 2014
- Member, Alpha Chi National College Honor Society, Sam Houston State University, 2013-2015
- Member, Golden Key International Honour Society, Sam Houston State University, 2013-2015

- Philanthropy Chair and Regular Member, Beta Beta Beta Biological Sciences Honors Society, Sam Houston State University, 2012-2015
- Member, Alpha Lambda Delta Honors Society, Sam Houston State University, 2012-2015
- PEERS Scholar, Sam Houston State University, 2012-2015
- Dean's List, Sam Houston State University, 2012-2015
- Member, Student Alumni Association, Sam Houston State University, 2011-2015
- Colorguard Tech and Member, Bearkat Marching Band, Sam Houston State University, 2011-2015
- Member, Elliot T. Bowers Honors College, Sam Houston State University, 2011-2015
- President's Honor Roll, Sam Houston State University, 2011

FUNDING and ACADEMIC AWARDS

- Lucas Research Grant recipient, Forensic Sciences Foundation: Fall 2017
- Graduate Bearkat Grant (TPEG) recipient, Sam Houston State University: Fall 2016 - Spring 2017
- Forensic Science scholarship recipient, Sam Houston State University: Fall 2015 - Fall 2017
- Faculty and Student Team (FAST) Research award recipient, Sam Houston State University: Summer 2015
- Undergraduate Research Award, Department of Biological Sciences, Sam Houston State University: Spring 2015

- Elliot T. Bowers Honors College Research Travel Funding, Sam Houston State University: Spring 2015
- James D. Long Biology Endowed Scholarship recipient, Sam Houston State University: Fall 2014 - Spring 2015
- College of Sciences Undergraduate Research Award, Sam Houston State University: Fall 2014
- College of Sciences Undergraduate Research Travel Award, Sam Houston State University: Spring 2014 - Spring 2015
- Elliot T. Bowers Honors College Study Abroad Funding, Sam Houston State University: Summer 2014
- Study Abroad Scholarship, Sam Houston State University: Summer 2014
- Orville Weiss Award, American Society of Microbiology Texas Branch: Spring 2014
- PEERS Scholarship, Sam Houston State University: Fall 2012 - Spring 2015
- Patrick Neal O'Bryant Memorial Endowed Scholarship, Sam Houston State University: Fall 2012 - Spring 2014
- Undergraduate Academic Achievement Scholarship, Sam Houston State University: Fall 2012 - Spring 2013
- Bearkat Grant, Sam Houston State University: Fall 2012 - Spring 2013
- Elliot T. Bowers Honors College Scholarship, Sam Houston State University: Fall 2011 - Spring 2015
- Top 10% Scholarship: Fall 2011 and Fall 2013
- Lola Wright Foundation Scholarship: Fall 2011 - Spring 2012