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Using Direct PCR for Disaster Victim Identification Reference Samples

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Abstract

Forensic genetic testing is an important tool for the identification of victims after a mass fatality event but degradation of remains, presence of PCR inhibitors, and limited amounts of sample can make testing difficult. Standard protocols typically include extraction of genetic material from recovered post-mortem samples, and from ante-mortem reference samples or families of a missing person. This is a time-consuming and laborious process and may result in the loss of trace amounts of DNA available for amplification. Incorporating workflows that bypass the extraction step and directly amplify recovered DNA for short tandem repeat (STR) profile generation has the potential to help expedite the process of victim identification and improve the success rates for small samples. To evaluate the effectiveness of a direct PCR workflow in disaster victim identification (DVI) settings, bone and muscle tissue were subjected to direct amplification for STR profile generation. Furthermore, two possible reference sample types, formalin fixed tissue and slides, and personal belongings such as toothbrushes, hair from hairbrushes, glasses and razors were evaluated with different direct PCR methods. Bone, muscle, hair and toothbrushes were all consistently successful with direct PCR workflows, while razors and glasses were less consistent. formalin fixed samples were found to be inappropriate for use with direct PCR, and should be avoided if possible when constructing reference STR profiles.

Introduction and Literature Review

DNA as a Tool in Forensic Settings

In the field of forensic science today, deoxyribonucleic acid (DNA) testing is considered a very powerful and reliable tool for the identification of people involved in crimes or disaster events. DNA is the genetic material that is inherited from generation to generation. The human genome consists of 3 billion bases, and only one-tenth of a single percent of DNA (about three million bases) differs from one person to the next (NIJ, 2000). DNA is present in every nucleated cell in the body and can be used to identify individuals from the biological material collected from crime scenes (Butler, 2012). In forensic science, short tandem repeats (STRs) are analyzed and used for DNA typing and developing the DNA profile. STRs are polymorphic genetic markers and constitute a very small part of the human genome. STRs are short specific regions (two to six base pairs) on a strand of DNA that are repeating multiple times. These specific regions, also known as loci, are abundant in the genome and different individuals can be distinguished by number of STR repeats at each locus. For each inherited STR genotype, one allele is provided by the mother and the father gives the other. A combination of STR locus genotypes is called a genetic profile and can be used to discriminate between individuals (except for identical twins, as both offspring inherit the same copies of genetic material) (Butler 2012).

After obtaining evidence from a crime scene, any biological material collected can potentially lead to the development of a DNA profile, which is as unique to an individual as a fingerprint. Currently used procedures for recovering genetic material and producing DNA profiles are time-consuming and labor-intensive, and there is a need to utilize more advanced technologies in order to speed up and improve the process for quicker and more successful identifications. Typical forensic DNA typing involves analyzing multiple autosomal STR markers by capillary

electrophoresis to determine the individual's specific allele(s) for each locus. An individual can either have a homozygote or a heterozygote genotype at the different loci. The Federal Bureau of Investigation (FBI) has identified a minimum of 20 specific loci to be used as the standard marker set the for Combined DNA Index System (CODIS) (Hares, 2012, 2015). CODIS is the DNA database created by the FBI that criminal justice departments use to compare DNA obtained from a crime scene to either other crime scene or convicted offender DNA samples that have already been submitted to the database (Butler, 2012).

STR Profile Generation

STR alleles are detected after amplification with the polymerase chain reaction (PCR). PCR is the process by which DNA is replicated exponentially *in vitro* in an instrument called a thermocycler. The process involves three steps: denaturation, annealing, and extension. Denaturation consists in separating the two strands of the DNA double helix. This is done by increasing the temperature of the thermocycler until the hydrogen bonds attaching the two strands together break. Annealing is the process of binding target-specific primers to the target DNA sequence to initiate polymerization. This is done by lowering the temperature of the sample. Extension is the process by which the two separated strands of DNA are used as templates to synthesize complementary strands. Here, DNA polymerase enzyme is used to attach free nucleotides complementary to the originally separated DNA strands (Butler, 2012).

In forensic DNA typing, multiplex PCR is used for the amplification of multiple STR loci simultaneously and is the gold standard for identification of unknown individuals (victim or perpetrator). The commercially available kits used to generate STR profiles in forensic settings all target the same recommended 20 loci included in CODIS and may incorporate additional loci as well. However, each kit differs in the primer sequences used by each manufacturer, which are

specific to the flanking regions of the targeted STR locus to amplify markers (Butler, 2012). For example, the Investigator 24plex QS Kit and Investigator 24plex GO! kit assays developed by Qiagen (Hilden, Germany) have been successfully utilized to amplify the expanded CODIS loci set along with a unique internal control and allows for STR genotyping and human identification. Some kits also include quality indicators which provide information on the quality and integrity of samples being analyzed (Kraemer et al., 2017).

Challenges for Successful Profile Generation in Forensic Settings

For the STR kits to be able to successfully amplify recovered DNA for profile identification, proper ion concentrations conducive to DNA polymerase activity must be achieved. Typically, to achieve these conditions, DNA needs to be extracted and purified prior to amplification. If the sample is not purified prior to PCR, the ion concentrations may not be optimal. Low ion concentration will result in weak primer binding and low PCR efficiency, while high ion concentration will result in nonspecific primer binding and nonspecific amplification (Butler, 2012). Purification of DNA is also required to decrease the potential of PCR inhibition. PCR inhibitors are a broad range of compounds that exert their effects either through direct interaction with sample DNA or interfere with thermostable DNA polymerases. Sample types commonly known to contain inhibitors include blood, fabrics, humic acid, biological tissues, and soil (Bessetti, 2007). In DVI settings, two of the most common sample types recovered, bones and muscle tissue, naturally contain PCR inhibitors. Bones typically contain high concentrations of calcium ions, which can competitively bind to DNA polymerases over magnesium ions and prevent amplification due to reduced enzymatic activity. Furthermore, collagen, another endogenous component of skeletal tissue, is also known to inhibit PCR through multiple means, including inhibiting polymerase activity and interacting with nucleic acids in a way that makes

them inaccessible to available enzymes. Myoglobin, a major protein in muscle tissue, is also known to interfere with certain DNA polymerases that can be utilized in PCR. Some strategies have been developed in order to overcome the effects of such inhibitors when processing these sample types, including the addition of extra magnesium ions when processing bone samples to outcompete the excess calcium inherent in these samples, addition of specific proteases (i.e., collagenase) during sample processing, and the use of thermostable enzymes that are not sensitive to inhibiting substances such as myoglobin (Schrader et al., 2012).

Many inhibiting substances can be introduced to the sample from exposure to the environment, or during sample processing itself. Samples that are recovered from soil may contain humic and fulminic acids, both of which are inhibitory at low concentrations as they adsorb nucleic acids (Schrader et al., 2012). Protocols for minimizing some inhibitors found in soil have been described, such as adding magnesium chloride ($MgCl_2$) and bovine serum albumin (BSA) to amplification reactions (Gomes et al., 2017), however such modifications do not remove or minimize all inhibitors that may be encountered. In addition to environmental inhibitors, swabs and moistening fluids used for sample collection have been shown to influence the sensitivity of PCR reactions. The introduction of powder from the gloves worn by technicians in the field (and in the laboratory) to the sample may also inhibit amplification. Furthermore, certain chemicals commonly used for cellular lysis and nucleic acid purification, such as phenols, salts and Triton X-100, can be inhibitory at certain thresholds. However, issues with the concentrations of these potential PCR inhibitors can be avoided by using commercially available kits for sample processing, as the manufacturers of these kits typically craft their formulations around the limitations and capabilities of their enzymes. That being said, there is no one method for completely neutralizing or removing all PCR inhibitors, and usually the most effective way to

remove or dilute them to workable levels is through extraction and purification of DNA from samples of interest. There are multiple different methods to extract the DNA in order to remove PCR inhibitors, including gel filtration, magnetic silica beads, liquid-liquid extractions, and more (Schrader et al., 2012). These processes all involve purifying the DNA by removing unwanted molecules such as proteins or other cellular material released from cell lysis, as well as exogenous inhibitors that had been introduced to the sample at any point prior to analysis. With all that in mind, the typical forensic DNA typing process involves extracting DNA, quantifying it, amplifying the target STR markers, separating PCR products (amplicons) by capillary electrophoresis, and then analyzing and interpreting the generated DNA profile. This process typically takes about 10 hours to 12 hours per sample (Verheij et al., 2012). Not only is this process time consuming, but it may also lead to a loss of DNA that prevents the amplification and generation of a DNA profile. It is estimated that DNA extraction leads to a 20% to 70% loss of sample DNA initially present in the sample and may introduce foreign DNA into the reaction (Templeton et al., 2013). DNA can adhere to plastic tubes used for processing samples, so extended procedures with multiple transfer steps can cause significant loss of genetic material (Schrader et al, 2012).

DNA-Based DVI After Large-Scale Disasters

DNA analysis plays a vital role in the identification of victims that have perished in mass disaster events. In certain mass disaster situations, such as the attacks on the World Trade Center (WTC) in September 2001 and the Madrid train bombings in March 2004, victim identification through non-genetic methodologies was not feasible due to high levels of sample disintegration and body fragmentation, making it impossible to identify most victims through standard forensic means. In situations like these, as well as the South Asian Tsunami disaster in December 2004

which was spread out over 12 different countries and claimed the lives of over 200,000 victims, the construction of DNA profiles for victim identification had been deemed an essential part of the response by forensic bodies such as Interpol. Mass disaster victim identification (DVI) through STR profiling of the victims claimed by these events is not without flaw, and each situation has presented issues that are continuously being worked on to improve DVI efforts in mass disasters. For example, many of the samples recovered from the WTC attacks were so severely degraded that recovered DNA from these samples were unsuitable for traditional STR typing, as conventional means amplify DNA fragments that are anywhere between 150 bp to 400 bp in length, yet many remains recovered yielded DNA fragments that were under 150 bp long (Alonso et al., 2005). This led to the development of alternate approaches to STR profiling which targeted shorter amplicons, including Mini-STR multiplex kits (Coble et al., 2005) which improved the success rate of STR profile generation from the most highly degraded remains. Other challenges, such as low rate of body recovery, prolonged time before body recovery, and high concentration of family groups among the victim pool make differentiation of samples based on STR profiles difficult (all of which have been observed in the South Asian Tsunami disaster). These issues remain problematic to this day, but the advent of DNA profiling has significantly improved the rate of identification in these mass disaster scenarios and will continue to improve as the technology and recovery methods improve (Alonso et al., 2005).

Due to events like the WTC attacks and the South Asian Tsunami disaster, Interpol has recognized the use of DNA as a crucial element in identifying victims after mass disasters. According to the Interpol guidelines, DVI is performed by DNA profiling and matching evidence found at the scene (post-mortem or PM samples) to the most appropriate ante-mortem (AM) sample that can be collected. In case of AM samples, the Interpol guidelines recommend sample

collection in the following order of importance: first degree relatives (multiple if possible), blood or biopsy samples (biobank specimens), or personal objects that were used by the victim during their lifetime. The first option (samples from first degree relatives) can be problematic in the event where closely related individuals were victims in the same catastrophic event. The second option (biobank specimens) is highly specific if available, however not all individuals will have specimens available for comparison in biobank settings. The third and final option (personal objects) also has its share of advantages and disadvantages. Like biobank specimens, these objects can provide a direct DNA profile comparison to the victim, however, there is a risk of the item not actually belonging to the victim or has been shared between the victim and another individual. In these cases, false claims or misidentifications can potentially be made. To summarize, the key factors in identifying a victim through DNA is in the identification of the appropriate AM samples to be collected, the quality of PM samples collected from the crime scene and being able to match the AM to the PM sample post processing and analysis (Montelius & Lindblom, 2012).

Post-Mortem Samples Utilized for DVI - Overview

The identification of PM samples to be used in generating DNA profiles relies on the nature of the disaster, which dictate sample collection protocols as outlined by the Interpol guidelines. Failure to comply with these procedures may result in loss of genetic material through improper collection and handling technique. Furthermore, environmental factors such as temperature, humidity, exposure to chemicals, or UV-radiation, can also result in the degradation of genetic material, either pre- or post-collection. The integrity of DNA is best maintained in bones and teeth, however there is variability in the success rate of DNA profile generation from different parts of the human skeleton. For corpses that are less degraded, intact muscle tissue can potentially be used. Historically, these samples have needed to undergo an extraction process to acquire DNA,

and then the extracted material must be quantified before genetic profiling can occur (Montelius & Lindblom, 2012).

Post-Mortem Samples Utilized for DVI – Current Practices for Skeletal Remains

Recovered bone samples were used to help identify about 1,500 of the more than 2,700 victims of the WTC terrorist attack within 22 months of the disaster (with most of the identifications coming within the first 12 months). It was possible to identify ~55.6% of the victims in this timeframe because The Bode Technology Group (Bode) had developed a high throughput procedure for skeletal remains that had incorporated DNA extraction, STR amplification, and profile analysis. This procedure was developed to assist the Office of the Chief Medical Examiner (OCME) in New York City with the task of identifying the large volume of samples as quickly as possible The prevailing methods for bone sample processing involved multiple steps, including removing fragments of bone for analysis, cleaning the bone fragment by sanding, pulverization of the fragments into powder, extraction of nucleic acids with organic solvents or silica-based capture, and then further processing of the extracted DNA to purify and quantify recovered genetic material for STR analysis. Each step was labor-intensive, time-consuming, and not suitable for high-throughput processing. Furthermore, traditional cleanup methods were known to be either inefficient at completely removing PCR inhibitors often associated with bone samples or be too harsh and result in excessive loss of genetic material. Because of the extent of the damage to the buildings and victims after the WTC attacks, the OCME decided they would need to test all remains recovered as they anticipated how difficult it would be to locate distinctive remains of each individual from the site of the disaster. When began the process of developing the new high-throughput procedure, the work was performed in two phases. Phase I utilized a newly developed DNA extraction method that reduced the sample preparation time from 20 min to around four

minutes per sample by adjusting the workflow into a high-throughput system with four stations - driller, holder, cleaner/runner, and powder transfer. Furthermore, a 96-well format for DNA extraction (QIAamp 96 DNA Blood Kit from Qiagen) was modified for use with bone shaving samples, including the addition of lysis steps with sodium dodecyl sulfate (SDS) and proteinase K, as well as additional cleanup and incubation steps. This allowed for the processing of over 250 fragments per day. After about 13,000 bone samples were analyzed with the original method, the results were assessed for total success rates, and changes were implemented to begin Phase II (Holland et al., 2003).

Phase II of Bode's attempt to improve bone sample processing for DVI after 9/11 involved analyzing increased quantities of bone powder (Phase I processed 25 mg to 50 mg of bone shavings at a time, whereas Phase II processed 125 mg to 150 mg per sample), adding EDTA to the extraction and purification processes to chelate excess calcium ions, and including collagenase in sample processing to eliminate endogenous interfering proteins, such as collagen and its derivatives. Moreover, new mini-STR multiplexes (BodePlex), were developed to reduce the amplicon size of larger STR loci and overcome the degradation of larger DNA fragments observed due to extensive stress exerted on the bone samples recovered from this tragedy. The improved protocol generated a higher DNA recovery rate and produced 542 additional full or high partial profiles for the over 5000 samples with low partial or no results seen in Phase I. While these advances did improve the timeliness and success rate of DVI using bone specimens in general, it was found that these advances did not improve the recovery of DNA from the most degraded samples. Only 65% of all WTC bone samples tested by Bode produced STR results in phase I and II, which shows how compromised the samples were. For comparison, in another airplane-related mass fatality event that occurred in the same year (the American Airlines Flight 587 disaster on

November 12, 2001) remains were exposed to less environmental insults and 92% of all skeletal remains produced STR results. However, the remains of the victims of the WTC disaster were subjected to extreme degradation conditions, including the sheer force of the collapse of the towers, exposure to extreme heat and fire for weeks to months after the event, subsequent exposure to water and other chemical agents used to try and suppress the ongoing fires, and many more compromising conditions. For especially degraded samples, it was found that STR profiles may not be sufficient by themselves in victim identification but may need to be supplemented with mitochondrial DNA (mtDNA) analysis (Holland et al., 2003).

A review of the DNA results of the WTC skeletal remains conducted by Mundorff et al. (2009) found that the samples consistently resulting in the most complete and successful DNA profiles come from weight-bearing lower limbs, such as patella and metatarsal bones. As explained above, the remains reviews for this study had been subjected to the most extreme conditions human remains can be exposed to prior to victim identification. The patella and metatarsal bones reviewed had resulted in DNA profile success rates of 80.8% and 86%, respectively. The next most successful bones for STR profile generation were the tibia, femur and rib bones, all of which produced successful DNA profiles >70% of the time. Of all bone types evaluated, the skull bones were found to be the least successful bone sample type for profile generation (Mundorff et al, 2009).

Potential advantages for using smaller bone fragments such as patellae and metatarsal, other than their high success rate for profile development, is the relative ease of collection. Unlike femur or tibia samples, smaller bones do not require sampling with a bone saw that may introduce foreign DNA contamination into the sample. During the analysis of the data accumulated from victims of the WTC attacks, it was found that DNA contamination was a significant issue in

generating genetic profiles sufficient for identification and can reasonably be assumed to be an issue in other mass fatality events. Prioritizing samples that require little to no extra sample processing methods can help reduce overall contamination rates, potentially increase successful identification rates, and reduce the overall time required for victim identification (Mundorff et al., 2009; Watherston et al., 2021).

Post-Mortem Samples Utilized for DVI – Current Practices for Muscle Tissue

In disaster events where muscle tissue remains intact, it may be used to develop STR profiles for victim identification as the PM sample. There are several advantages of using muscle tissue over bone samples, including ease of collection, sampling and storage. In a recent study conducted in 2021, a team of forensic scientists at Sam Houston State University proved that it was possible to successfully build STR profiles from decomposing muscle tissue using swabs to collect the required genetic material. Three different swab types were evaluated for collection purposes, two of which were traditionally used in forensic sampling settings (both 4N6FLOQSwabs from Copan Diagnostics (Brescia, Italy), one with an active drying system (ADS) and another with no active drying system (NADS)), and the third swab evaluated was the microFLOQ Direct swab (Copan). The ADS and NADS swabs were subjected to DNA extraction using Qiagen kits, while the microFLOQ swab was subjected to direct amplification (Loockerman et al., 2021). The microFLOQ swab has a small swab head pre-treated with a lysing agent and is designed for direct amplification workflows and had been previously proven to be effective in recovering DNA from bloodstains, saliva and touch samples (Ambers et al., 2018). When samples were collected up to 10 days of corpse decomposition, the profiles generated by traditional (extraction) collection methods and direct methods were comparable, resulting in high partial (90% to 99% average allele completeness) to fully complete (100% average allele completeness) STR

profiles. When the corpse had been sampled after 10 days, the traditional collections methods performed significantly better than the microFLOQ direct swabs (<75% average allele completeness for microFLOQ swabs), while both the ADS and NADS swabs produced STR profiles with >75% average allele completeness when sampled up to 20 days of decomposition. It was also observed that samples collected using the NADS swabs could be subsampled by the microFLOQ swabs and then subjected to direct PCR, resulting in similar profile-generation success rates to what was observed when the NADS swabs were subjected to extraction, but only up to 10 days of decomposition. The success of the subsampling was an important finding, as significant PCR inhibition was observed in samples collected with the microFLOQ swabs and directly amplified. This study highlights the importance of extraction when processing muscle tissue for STR profile generation with currently available PCR technology (Loockerman et al., 2021). Regardless of the PM sample used, the STR profile generated from recovered DNA must be compared to a reference profile generated from an appropriate AM sample in order to confirm the victim's identity.

Reference Profile Generation in DVI – Current Practices with Medical Reference Samples

Biobank samples are sometimes used as AM samples and can be formalin-fixed, paraffin embedded tissue (FFPE). For FFPE tissue it is required to utilize an extraction method that does not break down the genetic material but is efficient in removing paraffin, which is a PCR inhibitor (Montelius & Lindblom, 2012). Furthermore, FFPE tissue has historically been a challenging sample type for constructing genetic profiles for victim identification purposes, due to degradation and modifications (including cross-linkage of biomolecules) of sample DNA that is a result of the fixation step when initially preparing these specimens (Josefiova et al., 2017). Sample DNA is further exposed to caustic elements when these tissues are processed with highly aggressive

chemicals such as xylene and formaldehyde during histological staining. The compounds involved in these processes not only inhibit the PCR reaction if present in the amplification reaction, but also play a role in the degradation of larger gene fragments prior to sample recovery. It can still be possible to obtain STR profiles. A comparison of two commonly used STR multiplex kits (PowerPlex 16 (Promega, Madison, WI) and AmpFLSTR Identifiler PCR Amplification kit (Thermo Scientific, Waltham, MA)) with FFPE slide tissue samples showed that the Identifiler system outperformed its counterpart as it resulted in a higher number of complete profiles for this sample type. Analysis of the data acquired throughout this experiment revealed that this was due to the fact that the size of the loci being investigated for the Identifiler kit are all ≤ 360 bp. The PowerPlex system included larger loci, and when this kit was used to construct STR profiles, it was found that only ~11% of loci > 350 bp yielded profiles, whereas ~88.9% of all loci ≤ 350 bp yielded successful profiles. The difference in the size of the loci evaluated resulted in a 20% increase in the amount of complete profiles obtained by the Identifiler kit over the PowerPlex system (Budimlija et al., 2009).

In the comparison study conducted by Budimlija et al. (2009), the difference in accommodating degradation made one of the compared kits more feasible for aged samples. A statistically significant negative correlation was observed between sample age and amplification success with the PowerPlex system (all samples evaluated by this kit ranged from one to seven years old). However, the relationship between the age of the sample and the success in generating a profile with the Identifiler kit was not significantly linear, as all samples tested with this system between two to five years old resulted in close to 100% recovery of the expected loci. There was a noticeable decrease in profile quality for samples that were between five to 12 years old. However, when comparing the results of cancerous and normal slides from the same patient, it was

found that the Identifiler kit showed a significantly higher level of instability (defined as any difference between the profiles obtained from the healthy and diseased samples) than what was observed with the PowerPlex kit (86% vs. 27%). Instabilities included microsatellite instability (MSI - a size shift in an existing allele), deletions in the targeted loci, or a loss of heterozygosity (LOH; a ≥ 50% reduction in the ratio of allelic intensities for heterozygous loci between healthy and diseased samples). The differences in the strengths and weakness of each kit evaluated strongly implies that there is no one general procedure that can be used for interpreting STR profiles derived from FFPE slides, and any sample processing procedure performed prior to amplification must be evaluated and validated for each different kit that would be used (Budimlija et al., 2009). These findings were corroborated by a later study performed by Funabashi et al. in 2012 where they compared the success of DNA amplification from normal FFPE tissue samples extracted by three different methods - a silica-based DNA extraction kit (QIAamp Mini Kit from Qiagen), a salting-out procedure, and a phenol-chloroform extraction. Of the three methods evaluated, the salting-out procedure, while technically simple and inexpensive, resulted in the lowest yields and purities of DNA extracted across all sample types evaluated. The phenol-chloroform method yielded the most DNA per sample and highest purity of extracted DNA (A_{260}/A_{280} values of ~1.8) regardless of the tissue type processed, yet the commercial kit resulted in the most successful amplification of STR loci using forensic STR amplification kits. A possible reason for the higher success rate for the samples extracted with the commercial kit may be a result of residual phenol, a known PCR inhibitor (Schrader et al., 2012), in the phenol-chloroform extracted samples. Mini-STR kits also provided higher profile success rates than standard STR kits, demonstrating that larger DNA fragments are degraded in the formalin-fixing and paraffin embedding processes. Furthermore, the study also found that increasing age of the FFPE tissue being investigated negatively affected the

amplification of DNA in downstream processes, and that the fixation and embedding processes themselves (time allowed for both the fixation and paraffin embedding processes) have a noticeable effect on obtaining DNA suitable for amplification and analysis (Funabashi et al., 2012). Similar findings have been reported in multiple studies since this report, including an analysis of the formalin fixation process of healthy autopsy tissues conducted by Vitošević et al. in 2018, where they found that the fixative used, time allowed for preservation and DNA extraction method all had significant effects on the downstream success of PCR amplification. One of the most critical factors identified in this study was the buffer state of the formalin solution. Tissues fixed in phosphate-buffered formalin were suitable for molecular analysis for 28 days after fixation, whereas tissues fixed in unbuffered formalin could only be used for molecular analysis for up to seven days after fixation (Vitošević et al., 2018). Variations in sample quality, the extraction method employed, quality of the FFPE tissue and amplicon size targeted all affect the success rate of STR profile generation from FFPE tissue. All these variables make it difficult to regularly obtain suitable DNA for forensic identification purposes from these types of samples.

Reference Profile Generation in DVI – Current Practices with Personal and Touch Items

Besides medical reference samples, personal objects used by the victim during their lifetime, sometimes referred to as direct reference samples, can be used as AM samples in DVI. Prinz et al. (2007) have classified a multitude of direct reference sample types that can potentially be used to generate reference profiles based on the quality of the DNA obtained from these samples into three broad categories: good, fair and poor sources of DNA. These categories are each further divided into commonly available sources and sources that might be available for use in profile generation. Intuitively, good sources of DNA are preferred as they provide the highest success rate of profile generation and can be obtained from several regular household items (i.e., toothbrushes,

razors, hairbrushes or combs). These commonly available sources do have the potential to be contaminated with DNA from other individuals if the item was shared or if the item identified as belonging to the potential victim did not actually belong to them. Good sources of DNA that might be available for use but are not guaranteed to be available include samples found in biobank settings (i.e., bone marrow donor program, blood cards for metabolic disorder screening, criminal databases, sperm bank samples, etc.). These samples may be more specific to the suspected victim than their commonly found counterparts, however these samples are not as readily available or even guaranteed to exist. Fair sources of DNA include lipsticks, used drinking glasses, cigarette butts and eyeglasses, among others. These sources of DNA can provide acceptable DNA profiles in certain circumstances, but if good sources are available, they should be prioritized over these sample types. Poor sources of DNA include jewelry, shoes, dentures, and nail files, and should only be used in events where none of the higher-ranking sample types are available (Prinz et al., 2007). When personal items of the suspected victims are to be used for reference profile generation, it has been found that that both the swab type utilized to collect DNA, as well as the substrate being analyzed, play a critical role in the amount of genetic material recovered, and can help explain why some personal items are considered better sources of owner DNA than others. It has been reported that nylon-flocked swabs can result in up to 30% more recovered DNA than cotton swabs, and plastic substrates generally result in higher recovery of DNA after extraction from metal surfaces (Wood et al., 2017).

Potential for Direct PCR in DVI Settings

The limitations of DNA extraction, including loss of genetic material, time required for sample processing, and potential for contamination, has resulted in an interest in the use of direct PCR for victim identification. Direct PCR is a method that shows high potential in this field

because this method does not require DNA purification and quantitation prior to amplification. It has been proven effective in analyzing samples with trace levels of DNA after placing biological material directly into a PCR tube (Templeton et al., 2013). Detection via direct PCR resulted in the recovery of more alleles and higher relative fluorescent unit (RFU) values when directly compared to the standard DNA extraction technique (Linacre et al., 2010) and subsequently resulted in more complete profiles (Swaran & Welch, 2012).

Direct PCR can be crucial in obtaining results within a few hours of sample collection in cases such as disaster victim identification or mass fatalities. Direct PCR also minimizes opportunities for sample mix-ups or the introduction of foreign DNA. Furthermore, it can be used for analysis during natural disasters, terrorist attacks, and transportation accidents where there will be a multitude of samples that need to be analyzed. Direct PCR avoids the transfer of samples from one tube to another, decreases sample handling, leads to an increase in sensitivity without having to increase the number of amplification cycles, and eliminates the cost of extraction kit reagents (Linacre et al., 2010). Several commercial direct STR kits, including the Investigator 24plex GO! Kit from Qiagen, have been developed and validated according to international regulatory standards for use in forensic settings, in order to capitalize on these advantages and help improve identification workflows (Qiagen, 2015 (1); Zgonjanin et al., 2017).

Direct PCR Successes with Touch Samples

The use of direct PCR in the forensic setting began in the mid-2000s on reference (AM) samples with the release of commercial kits for direct PCR of STRs utilizing buffers that can neutralize the effects of standard PCR inhibitors. Since then, direct PCR has been implemented in ISO 17025–accredited laboratories for high throughput databanking analysis (Cavanaugh & Bathrick, 2018). Furthermore, direct PCR has been performed on fingerprints collected via

sellotape from touched items such as glass and fabrics to obtain evidence that was traditionally difficult to work with due to the limited amount of DNA present on the original sample. The use of direct PCR with these samples also provides the additional benefit of preserving the original specimen, which historically was completely consumed during the standard collection and extraction processes (Verheij et al., 2012).

Cavanaugh & Bathrick (2018) have demonstrated the practical use of direct PCR in constructing STR profiles from touch samples. These samples are generally items that have accumulated skin cells after being handled in one way or another. There are multiple variables that can affect the amount of genetic material deposited on a touch sample, including personal hygiene habits, the surface of the touched element, the nature of the contact, and more. Most of these samples are considered to contain low amounts of DNA but have shown increased success and are valuable in property crime investigations (Cavanaugh & Bathrick, 2018). In typical forensic settings, the standard procedures of extracting and quantifying the DNA from the touch sample would result in significant loss of the genetic material. One of the benefits of performing direct PCR on touch samples is the ability to generate full STR profiles from substrates previously known to be difficult to obtain genetic material from. Templeton et al. (2015) demonstrated that performing direct PCR on swabs used to collect DNA of known concentrations from plastic, glass and brass materials proved to be more effective than extracting and amplifying the DNA collected on the swabs. This was true in terms of both the average RFU peak heights generated and lowest recoverable amount of DNA required to generate a full STR profile. At all amounts of DNA tested in the study (0.1 ng to 1 ng), the average RFU of each allele peak was significantly higher for profiles generated via direct PCR than the average RFU for each allele peak in extracted samples across all three touched substances. The sensitivity of the procedure also increased for two out of

the three touched surfaces when direct PCR was used over traditional extraction. The lowest amount of DNA required to generate a full profile from plastic surfaces was 0.2 ng with direct amplification, while extracted samples required a starting total of 0.5 ng to accomplish the same feat. This phenomenon was also observed in brass samples, as the swabs directly amplified were able to generate full profiles at 0.5 ng of starting material but required 0.75 ng starting material when the sample was extracted (Templeton et al., 2015). Brass substances have been previously characterized as a challenging source for DNA recovery, including the natural presence of PCR inhibitors such as copper and zinc (Gashi et al., 2010; Xu et al., 2010; Horsman-Hall et al., 2009). Clearly, implementing a direct PCR workflow in forensic settings for STR profile generation can provide multiple advantages over standard practice involving DNA extraction.

Direct PCR Attempts on FFPE Tissue Samples

Another study on direct PCR evaluated the proprietary Phusion High-Fidelity DNA Polymerase (Finnzymes Oy, Espoo, Finland) on a variety of FFPE samples (tissues, blocks, and microscopic slides) (Kuusisto & Helminen in 2008. Each sample type evaluated had slightly different sampling instructions, but all sample types were processed in a similar manner - they were subjected to proteinase K digestion (0.2 mg/mL) in Phusion Reaction Buffer and incubated for either one hour or overnight at 60℃, immediately followed by heat inactivation at 98℃ for 10 min. The samples were spun down and 1 µL to 5 µL of the supernatant was used for direct PCR amplification (no paraffin removal step was performed). The Phusion enzyme was compared against a standard *Taq* polymerase for direct amplification, and it was found that the Phusion enzyme resulted in higher amplicon yields for all tissue types evaluated. Furthermore, different sections of the same FFPE breast tissue that was analyzed via direct PCR were prepared for profile generation using a commercially available extraction kit, which included a paraffin removal step

during pre-amplification processing. These samples were also amplified by the Phusion enzyme, and it was found that the enzyme performed equally well on the direct samples as it did on the extracted ones. Across all aspects of the study, it was found that the Phusion DNA Polymerase was most successful in generating STR profiles for FFPE tissue samples when proteinase digestion was allowed to incubate overnight. Again, the authors observed severe degradation and an age effect. The maximum size of successfully generated STR profiles in this study was 300 bp. Older and poorly prepared blocks typically had lower yields than the newer or better-preserved blocks (Kuusisto & Helminen, 2008).

Direct PCR with PM Samples

More recently, Rapid DNA, a type of direct PCR, has been evaluated for use with PM samples that can be expected in mass fatality settings. Turingan et al., (2020) have utilized the ANDE Rapid DNA Identification System (ANDE, Longmont, CO) to amplify and separate STR alleles for bone specimens (specifically femur, phalanx and rib samples). The bones used in this study had fragments of interest removed and thoroughly cleaned, and then ground into a fine powder which was demineralized in ANDE Bone Solution for at least one minute prior to Rapid DNA identification. The bone materials were not extracted and quantified before adding to the Rapid cartridge for lysis, PCR amplification and electrophoresis. In Turingan's Rapid DNA study 95.4% of bone samples yielded useful DNA IDs. The study also demonstrated that the source of bone used plays a significant role in the success rate of profile generation. It was found that phalanx bones consistently yielded the best results of all bone types evaluated. The phalanx bones required less material to generate full DNA IDs than the other bones evaluated, even as the age of the sample being investigated increased. It was observed that rib samples allowed to age six months or longer were more difficult to process than the phalanx and femur bone samples evaluated at

these times, due to the fact that these bones had become fully desiccated, requiring increased amounts of sample to be used for successful generation of a DNA ID (Turingan et al., 2020). The improved sensitivity of the phalanx and femur bones observed in this study align with the findings that Mundorff et al. previously presented in their review of the recovery and preservation of DNA samples at the WTC disaster (Mundorff et al., 2009). Regardless, all three bone types evaluated with the ANDE Rapid DNA Identification system were able to produce useful DNA IDs for at least 12 months after the sample had been exposed to the outside environment (the procedure for older bone samples had to be modified to include an overnight demineralization prior to amplification). In the same study, muscle and organ tissue samples that were evaluated for use with the ANDE Rapid DNA Identification system were only able to produce useful profiles for at most 11 days after the fatal accident (Turingan et al., 2020).

Muscle tissue is another potential PM source of DNA for DVI that has recently been evaluated for use with direct amplification procedures for STR profile generation. Mundorff et al. (2018) successfully generated genetic profiles from fresh tissue by saturating a buccal swab with muscle tissue material and then transferring the sample to FTA collection cards and proved that the average allele recovery rate for the rapid (1.2 mm) and extracted (3 mm) punches were comparable for corpses with decomposition scores of 15 or below according to the Total Body Score (TBS) grading system previously described by Megyesi et al. in (2005). This grading system was used to correlate the recovery of DNA from different specimens by how decomposed the remains were, rather than a set amount of time since the disaster incident. The average allele recovery rate for both direct and extraction methods remained around 100% up until a TBS score of 11, but then slowly decreased to 60% for both methods as the TBS increased to 15. However, the average allele recovery for samples with TBS scores of 16 was only around 30% for both the

direct and extracted punches. Furthermore, the study suggested that the season and/or environmental conditions significantly influenced the stability of the remains, but regardless of how fast or how long it took for the corpse to reach the benchmark level TBS of 15, the same drop off in allele recovery was observed. Mundorff et al. postulate that the universal implementation of a decomposition scoring system like the TBS may help to expedite recovery and identification of victims at disaster sites, as samples that are identified as less degraded (TBS < 15) can be subjected to rapid profile generation techniques using muscle, whereas the more degraded remains (TBS ≥ 15) can be flagged for more extended processing and bone sampling (Mundorff et al, 2018). A later study by Turingan et al. (2020) also found that using swabs to collect DNA from muscle tissue could be amplified directly to produce informative STR profiles, but only for a limited time after the body has begun to decay. In this study, deep red muscle tissue was sampled from both biceps and quadriceps muscles in order to simulate field conditions where the limbs had been separated from the torso. A small fragment of the muscle incision was removed (100 mg to 150 mg) and swabbed until the point of saturation, and the swab was then subjected to PCR amplification using the ANDE Rapid DNA Identification system. Five out of six samples exposed to above-ground conditions were able to generate acceptable STR profiles for up to six days after initial sampling and exposure, and the one sample that did not reach this time point stopped providing useful profiles after day four. One sample was able to generate full IDs for up to 10 days after initial exposure. However, samples subjected to a three-month morgue sub-study (maintained in refrigerated conditions) were able to generate full DNA IDs for the entirety of the sub-study (Turingan et al, 2020).

Roadblocks to Implementing Direct PCR in Routine Fieldwork

While there is evidence supporting the use of direct PCR in DVI, the strict guidelines implemented by forensic DNA regulatory agencies at this time has restricted the use of direct PCR in routine forensic casework. In the United States, the Federal Bureau of Investigation (FBI) Quality Assurance Standard 9.4 requires that all unknown forensic samples are quantified prior to STR amplification (SWGDAM 2020). Such regulations need reevaluation considering the many recent studies demonstrating the benefits of using direct PCR on touch DNA samples, medical reference samples, and simulated PM samples. The following proposed experiment aims to add to the pool of evidence supporting the implementation of direct PCR in DVI settings to improve profile success rates and overall turnaround time.

Hypothesis

To assess the efficacy of direct PCR for forensic profile generation from ante-mortem and post-mortem DVI samples, multiple sample types, including pathology specimens (FFPE), bones, muscle tissue, toothbrushes, glasses, razors, and shed hair were analyzed using this methodology. If the protocol described below succeeds in generating profiles from these specimen types, it will reduce the time needed to process and analyze samples for DNA-based victim identification during mass fatalities. Additionally, the analysis of these samples can potentially provide DNA for developing database entries in the missing person index that can be used as a reference to compare against previously unidentified bodies. This will aid in supporting the victim's families by reducing the time needed to obtain a DNA result and a proper identification.

Materials and Basic Methods

Materials

The tested samples were categorized into post-mortem specimens, medical reference samples, and personal items as sources of ante-mortem DNA. The post-mortem samples were fresh bones and muscle tissue, which were from anonymized UPenn Medical System pathology material. Medical reference samples, namely paraffin-embedded tissue blocks and pathology slides, were also from anonymized UPenn Medical System pathology material. Ante-mortem samples, which came from used razors, glasses, toothbrushes, and combs and hairbrushes with hair, were collected from volunteers at John Jay College of Criminal Justice (IRB approval under #2016-0916). Volunteers also provided buccal swabs to verify recovered DNA profiles.

The following swabs were used to collect DNA from personal items: microFLOQ direct (Copan Diagnostics, Brescia, Italy), nylon FLOQSwab (Copan Diagnostics), Fitzco CEP swab (Fitzco, Inc, Minneapolis, MN), and Fisherbrand polyester swab (Thermo Fisher Scientific, Waltham, MA).

Sample preparation and collection - Post-Mortem Samples

Five bone samples were used for this experiment, and two sets of bone shavings were obtained from each sample (10 total bone shaving samples). To prepare these samples for analysis, the fresh bone samples were each boiled for an hour in sterile nuclease-free water and then irradiated for 45 minutes on both sides to simulate a scenario where the bodies were severely burned, and the flesh had been melted off the bones. A sterile scalpel was used to scrape the surface of the boiled and irradiated bones to remove any remaining tendons and cartilage. Either a 1/32-inch or 3/64-inch drill bit on a Dremel tool was used to carve vertical striations on the bones, and the resulting powder was collected in a 1.5-mL Eppendorf tube. A range of 5 mg to 15 mg of bone

shavings were collected per sample. To avoid burning the bone with the drill and overheating material prior to for analysis, the site of drilling was constantly changed, and the drill bit was allowed to cool down between locations. The drill bit was cleaned after each sample using first 10% bleach, then deionized water, and 70% ethanol. After washing, the drills were also irradiated.

The muscle tissue samples (n=5) were cut open using a sterile scalpel and a 1-by-1-mm segment was taken from the internal part of the tissue and transferred to a clean 1.5-mL Eppendorf tube.

Sample preparation and collection - Medical Reference Samples

Paraffin-embedded tissue samples were cross-sectioned using a sterile scalpel to obtain a central portion of the sample from the inside of the block. The central portion was carefully removed from the block using sterile scalpels, avoiding the paraffin as much as possible.

The histological slides were scraped with a sterile scalpel for at least a quarter of the paraffin ribbon's surface, and the resulting flakes were then collected by dabbing the slide with a polyester swab that had been wetted with STR GO! Lysis buffer (QLB) (Qiagen). The swab containing the slide scrapings was then placed into a 1.5-mL Eppendorf tube.

Sample preparation and collection - Ante-Mortem Samples

For the donated toothbrush samples (a total of seven donated items, five of which were tested on two different occasions), one set of bristles was removed from each toothbrush using cleaned tweezers and placed into individual 1.5-mL Eppendorf tubes. Tweezers were cleaned with 10% bleach, deionized water, and 70% ethanol between each toothbrush.

For the donated hair samples, four to six strands were collected from either the comb or brush (whichever source was donated) with clean tweezers. Strands with intact roots or tissue

flakes were preferentially selected, and then cut and submerged root-first into either 1.5 mL or 0.2 mL reaction tubes.

Genetic material was collected from prescription glasses and razors using several types of swabs, including Copan FLOQ, MicroFLOQ swabs, Fitzco CEP swabs, and Fisherbrand polyester swabs. The donated glasses were swabbed at two locations deemed the most likely to provide a sufficient sample: the nosepiece and the temple tips. Both the glasses and razor samples were swabbed horizontally then vertically. Either a single process wet swab or a two-swab cutting technique were used. The two-swab cutting technique used a wet swab immediately followed by a dry step, as described by Templeton and Linacre (2014). The clippings were then collected in an Eppendorf tube prior to processing for PCR.

Sample Lysis Step

Throughout the experiments, multiple collection methods and lysis treatments were evaluated for optimal recovery of genetic material by direct PCR to generate complete STR profiles. Details for the variation in swabbing procedures are provided in the results section. In addition to direct PCR by adding the sample to the PCR reaction mix, different lysis treatments, with different volumes of lysis buffer and incubation times were evaluated. All the lysis treatments used the Qiagen Investigator QLB were enhanced by enzymatic digestion of sample proteins with a 4 µg/µL solution of proteinase K (Promega, Madison, WI). All enzymatic incubations were performed at 60°C while shaking and the digested samples were then immediately subjected to incubation at 98°C for 10 minutes to deactivate the proteinase K (Kuusisto & Helminen, 2008). Samples were briefly cooled on ice, and then subjected to short-spin centrifugation to bring any condensate formed back into solution. Then 2 µL of cooled lysate was amplified via Qiagen

Investigator 24plex GO! Kit for either 27 or 29 cycles, dependent on the sample type being evaluated.

Polymerase Chain Reaction-Short Tandem Repeats (PCR-STR)

The kit that was used to amplify the DNA recovered from all samples by PCR was the Qiagen Investigator 24plex GO! Kit (Qiagen). This kit was designed for multiplex detection of several targets to create a complete genetic profile, including 21 autosomal STR loci, amelogenin, and a Y-specific STR locus. Each reaction was prepared by adding either the sample or 2 µL of lysed sample to 20 µL of PCR master mix. The PCR master mix was prepared as outlined in the kit's product insert, which involved mixing 12.5 µL of Primer Mix and 7.5 µL of Fast Reaction Mix 2.0 (Qiagen, 2015 (3)).

For each batch of samples processed, positive and negative controls were prepared. The negative control consisted of all PCR reagents and 2 µL of the molecular grade water used for sample dilutions. The positive control included in the kit (Control DNA 9948, 5 ng/µL) was diluted 1:10 down to 0.5 ng/µL with molecular grade water, and 2 µL of the diluted control was added to 20 µL of PCR master mix. All samples and controls were amplified on a GeneAMP 9700 PCR System thermocycler (Applied Biosystems) according to the parameters listed in Table 1.

Table 1: Polymerase Chain Reaction Parameters

Step	Process	Temperature	Time	Number of cycles
1	Denaturation	98°C	30 sec	Perform 2x
2	Annealing	64°C	40 sec	
3	Extension	72°C	5 sec	
4	Denaturation	96°C	10 sec	27 or 29 cycles, dependent on sample type
5	Annealing	61°C	40 sec	
6	Extension	72°C	5 sec	
7	Final extension	68°C	2 min	-
8	Hold	18°C	Infinite	-

Electrophoresis

After amplification, the samples were separated on an Applied Biosystems by Life Technologies Hitachi 3500 Genetic Analyzer. An electrophoresis master mix was prepared by combining 0.4 µL of BTO 550 size standard (Qiagen) and 12 µL of Hi-Di formamide (Life Technologies, Carlsbad, CA). Then 12 µL of the electrophoresis master mix was added to each well of a MicroAmp Optical 96-Well Reaction Plate (Applied Biosystems) that was designated for a sample, and then 1.2 µL of the appropriate sample or control PCR product was added.

For each batch of processed samples analyzed via electrophoresis, a Qiagen 24 plex allelic ladder (included in the PCR kit) was run to provide a reference for the targeted STR allele sizes. If any of the wells in a column with samples remained empty (i.e., only six out of eight wells in a column contained a PCR sample), the empty wells were filled with 12 µL of Hi-Di formamide only (blanks).

Once all samples and blanks were loaded the plate was sealed using a septum and briefly spun down to bring all liquid to the bottom of the wells. The samples were denatured in a

thermocycler for five minutes at 95°C, cooled down for five minutes at 4°C and loaded in the genetic analyzer. Using the Performance Optimized Polymer (POP-4) cartridge (Life Technologies), the injection parameters for the run were set as follows: injection voltage was set to 1.2 kV, with an injection time of 15 seconds.

STR Analysis

The GeneMapper ID-X v1.5 software (Life Technologies) was used to analyze the fluorescent peaks and determine the STR profile of the samples. Sample analysis was based on the Qiagen 24plex macro and used a minimum threshold set to 50 RFU. Each peak that was identified as an allele by the software was further analyzed. True peaks were separated from artifacts (background noise), pulls-ups (carryover), and dye artifacts (consistent signal across all samples at the same locus).

Profile Interpretation and Classification

Volunteer buccal swabs were used to create donor reference genotypes for the ante-mortem samples (toothbrushes, razors, glasses, etc.). The buccal swabs were processed as described in the Qiagen-published protocol for using Bode Buccal DNA collectors with the Investigator 24 plex GO! Kit (Qiagen, 2015 (2)). Sample STR profiles were compared to matching buccal swabs and categorized as follows:

- full profile (F): a full profile describes a STR profile in which all 21 of the autosomal loci are complete and match up with the reference genotype.
- high partial profile (HP): a high partial profile includes 10 or more complete and correctly typed autosomal loci.
- low partial profile (LP): a low partial profile has less than 10 complete autosomal loci.

- not suitable for comparison (NS): a profile not suitable for comparison has no complete locus.
- negative result (N): a negative profile means no allele peaks were detected.

There was a slight difference in classifying the STR profiles for the medical reference (paraffin-embedded tissue and pathology slides) and post-mortem (muscle tissue and bones) samples as compared to the ante-mortem samples. As there were no reference genotypes for these samples, every locus was deemed complete either based on the presence of two alleles (heterozygote) or a single peak of sufficient height to be a homozygote. Samples were categorized as explained above.

The Qiagen Investigator 24plex GO! Kit has two internal quality indicators that provide information for troubleshooting. QS1 and QS2 quality standards were observed as indicators of inhibition and degradation, as shown in Table 2.

Table 2: Quality Indicators with Interpretation

Allele Peaks	**QS1**	**QS2**	**Interpretation**
Present	Present	Present	Successful Profile
Absent	Present	Present	No DNA
Absent	Absent	Absent	Failed PCR
Ski-Slope Profile	Present	Dropdown	Inhibitors Present
Ski-Slope Profile	Present	Present	Degraded DNA

The quality indicators were used to make critical decisions on how to proceed with both individual samples and processing protocols in general. DNA quantitation was performed to explain differences in STR profile quality.

DNA Quantitation

The genetic material recovered for each sample was quantitated using the Quantifiler Trio DNA Quantification Kit (Life Technologies) on the Applied Biosystems 7500 Real-Time PCR System. DNA quantitation standards were prepared by mixing the Quantifiler THP DNA Standard (100 ng/µL) and the Quantifiler THP DNA Dilution Buffer to achieve the following concentrations: 50 ng/µL, 5 ng/µL, 0.5 ng/µL, 0.05 ng/µL, and 0.005 ng/µL.

Quantitation master mix was prepared by mixing 10 µL of Quantifiler Trio THP PCR Reaction Mix with 8 µL of Quantifiler Trio Primer Mix per sample to be run. Then, 18 µL of the master mix was combined with 2 µL of the DNA sample or standard for analysis. Two blanks, or no template controls (master mix only), were included in each analytical run. Samples, standards, and blanks were added to the microplate according to the layout described on the plate map, and the microplate was then sealed and briefly centrifuged to bring all liquid to the bottom of the wells. The plate was then loaded onto the instrument for analysis and run using the parameters listed in Table 3.

Table 3: PCR Parameters for DNA Quantitation

Step	Process	Temperature (°C)	Time(s)	# of Cycles
1	Initial Incubation	95	120	1
2	Denature	95	9	40
3	Anneal/Extend	60	30	

Generated data was analyzed using the Quantifiler Trio program in the Applied Biosystems HID Real-Time PCR Analysis Software v1.2.

Method Optimization and Results

Post-Mortem Samples - Fresh Bone

Two sets of bone shavings were collected from each of the five bone samples received from the UPenn Department of Clinical Medicine and weighed in Eppendorf microcentrifuge tubes. Extraction master mix (180 µL) was prepared by combining 169.2 µL of QLB, 9 µL of proteinase K (4 µg/µL), and 1.8 µL of DTT (0.5 M). From the master mix, 30 µL was added into each sample tube. The samples were then incubated at 60°C for two hours while being shaken at 300 rpm, and then incubated at 98°C for 10 minutes while stationary for enzyme inactivation. Sample tubes were then cooled by placing them on ice and were then centrifuged on short-spin cycle to bring all condensate on the side of the tubes down into solution. Then, 2 µL of the spun-down bone extracts were removed and subjected to PCR analysis as described previously, prior to any DNA quantitation, to simulate what would be done in the field for disaster victim identification. Obtained results are documented in Table 4.

Table 4: Fresh Bone Results

Sample Name	Weight of Shavings (mg)	Quantitation Result (ng/µL)	Total DNA Yield (ng)	DNA Yield per mg Bone (ng)	DNA Input in PCR (ng)	STR Result
BP1a*	8.5	5.9	177	20.82	11.8	Full Profile
BP2a*	5.8	1.86	55.8	9.62	3.72	Negative Profile
BP3*	10.9	0.42	12.6	1.16	0.84	Full Profile
BP4*	14	3.9	117	5.85	7.8	Full Profile
BP5a*	6.9	3.9	117	16.96	7.8	Full Profile

*: Acceptable QS1 and QS2 results

A full STR profile was obtained for four out of the five bone samples investigated. All the samples produced acceptable QS1 and QS2 quality standard results, indicating that all PCR reactions were performed successfully, and the 1 negative profile produced was a result of sample quality issues and not a failure of the reaction. The amount of DNA input for the PCR (determined after samples were amplified) ranged from 0.84 to 11.80 ng, which are all in the acceptable input range for the Qiagen 24plex GO! Kit, again indicating the one failed reaction was due to sample quality and not an issue with the efficiency of the PCR. Due to the success observed in constructing STR profiles with this sample, the second set of shavings for each bone sample were not subjected to further testing or optimization.

Post-Mortem Samples - Muscle Tissue

Several preliminary methods for direct PCR from muscle tissue were evaluated using two of the samples (samples FT5PK and FT6PK). Method 1 involved direct PCR on small tissue cuttings and was performed on two samples initially. One of the samples produced a full profile, while the other produced a low partial profile; however, neither sample had amplification of the QS2 control, indicating inhibition of the PCR reaction.

To dilute out the inhibiting substance, Method 2 evaluated involved indirect amplification, as a small cutting of tissue was placed in a microcentrifuge tube lysed with 20 µL of QLB and 1 µL of proteinase K (4 µg/µL) as described in Material and Methods section for the bone samples. After amplifying 2 µL of the lysed sample, the electrophoresis showed multiple pull-ups for each sample, indicating DNA overload. Therefore, the PCR product was diluted 1:100 before electrophoresis, which then produced full profiles for both samples. Method 2 was repeated once, this time using only 1 µL of the lysed sample for amplification (Method 2A); however, this

modified procedure also resulted in DNA overload and could not produce an acceptable profile unless the PCR product was diluted 1:100 as well.

To compensate for the high yield observed in Methods 2 and 2A, another method (Method 3) was evaluated, now using 200 µL of QLB and 5 µL of proteinase K (4 µg/µL) with the same size tissue cutting. The proteinase K digestion (two hours at 60°C) was shortened to 30 minutes. For this method, 2 µL of the sample was then added to the PCR reaction, which resulted in full profiles for both samples evaluated without requiring further dilutions. This method was used to process five additional muscle tissue samples (Table 5).

Table 5: Fresh Tissue Samples Prepared Using Method 3

Sample Name	Quantitation Result (ng/µL)	DNA Input in PCR (ng)	STR Result	QS Status**
FT5PK	13.12	26.24	Full Profile	PP
FT6PK	19.98	39.96	Full Profile	PP
FT2PK’	14.71	29.42	Full Profile	PP
FT7PK	14.81	29.62	Full Profile	PP
FT8PK*	22.31	44.62	Full Profile	PP
FT9PK	8.62	17.24	Full Profile	PP
FT10PK*	44.66	89.32	Full Profile	PP

*PCR product diluted 1:10
**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

All seven muscle tissue samples eventually resulted in full STR profiles when processed using Method 3. Two of the new samples (FT8PK and FT10PK) yielded large fluorescent peak heights (with too many pull-ups) and the PCR product had to be diluted 1:10 to generate acceptable profiles. DNA quantitation was performed after analysis and the results revealed that the yields of genetic material ranged from 8.62-44.66 ng/µL (Table 5). For the two overloaded samples (FT8PK and FT10PK), the amount of DNA added to the PCR reaction initially was 44.62 and 89.32 ng, respectively, which is higher than the other five samples.

Medical Reference Samples - Paraffin-Embedded Tissue

A small (approximately 1/10th the size of a dime) cutting of paraffin-embedded tissue was placed in a mixture containing 20 µL of QLB and 1 µL of proteinase K (4 µg/µL). The samples were then incubated at 60ºC for 2h as described previously. Two microliters (2 µL) of the lysate were then subjected to amplification. Both samples evaluated resulted in a negative profile, and subsequent quantitation of the lysates revealed that no DNA was retrieved from the procedure listed above. Therefore, paraffin-embedded tissue was no longer considered as a viable option for extraction-less PCR.

Medical Reference Samples - Histological Slides

The histological slides evaluated were each divided into four segments and the mounted sample was scraped off the surface of the slide using a sterile scalpel. A separate sterile polyester swab moistened with QLB was used to collect the scrapings from each segment. Using clean scissors, the tip of each swab was cut off and placed into separate tubes. The four different sections of scrapings collected from each slide were each subjected to different treatment methods. The first treatment method evaluated (Method A) had the swab tip placed in a mixture of 20 µl QLB plus 1 µL of proteinase K (4 µg/µL). Similar to the paraffin-embedded tissue samples, the histological slide samples were incubated at 60°C for two hours, and 2 µL of the lysate was subjected to PCR analysis. Of the two slides, one resulted in a low partial STR profile and the other resulted in a negative profile, but neither sample had successful amplification of the quality indicator QS1 indicating failed reactions for both samples.

For Method B, the swab tips were placed in 20 µL of QLB without proteinase K and incubated at 95°C for five minutes while shaking at 1200 rpm. The heated samples had 2 µL removed and subjected to PCR without being cooled down first. As observed with Method A, one

of the samples resulted in a low partial STR profile and the other resulted in a negative profile. Again, QS1 was not amplified in either reaction, indicating reaction failure.

For Method C, the swab tips were placed in PCR tubes and directly amplified. The samples both resulted in negative profiles, and one sample was missing the quality indicator QS1 (failed reaction), and the other was missing QS2 (indicating PCR inhibition).

In Method D, the swab tips were placed into PCR tubes and wetted 2 µL of the QLB. The samples were incubated at 95°C for five minutes while shaking at 1200 rpm. The PCR mastermix was added for direct amplification as described previously. Again, both samples resulted in negative profiles, and neither sample had acceptable results for the quality indicators. One of the samples was missing both quality indicators, and the other was only missing QS2 (again indicating PCR inhibition).

As Method A was the only treatment method to result in a low partial profile, it was revisited using larger quantities of starting material (all contents of the slide were scraped and collected with the swab, instead of only using a quarter of the slide). This was performed on four samples, and three out of the four produced negative profiles. The other sample resulted in no sizing data and the electropherogram was invalid. Of the three negative samples, two of them had both quality indicators successfully amplified (acceptable results), whereas the other was missing QS1 (failed reaction). The two samples with passing quality indicators were later subjected to DNA quantitation. It was found that one of the samples did not yield any DNA, and the other sample did not result in true amplification, so any quantitative results obtained for that sample were invalid. No further investigation was performed on these samples.

Ante-Mortem Samples - Toothbrushes

The preliminary method evaluated for retrieving STR profiles from toothbrush samples involved removing a tuft of bristles (one clump of bristles originating from the same spot on the toothbrush) and placing the entire tuft into a mixture containing 200 µL of QLB and 5 µL of proteinase K (4 µg/µL). These samples were then subjected to enzymatic digestion by incubating at 60°C for 30 minutes while being shaken at 300 rpm and treated as described above. Next, 2 µl of each sample was removed and used for PCR, initially performing 27 cycles for amplification (steps 4-6 listed in Table 1). Of the first two samples evaluated, one sample resulted in a high partial STR profile while the other produced a negative STR profile. Both samples had acceptable quality standard results, indicating the PCR reactions were successful. Subsequent quantification of the sample lysates revealed that the sample that produced the high partial STR profile had a DNA concentration of 0.17 ng/µL (0.34 ng loaded for amplification), whereas the sample that produced a negative STR profile had a DNA concentration of 0.01 ng/µL (0.02 ng loaded for amplification). The second value of 0.02ng of target DNA is below the recommended range of 0.2 ng to 2 ng of DNA for successful amplification.

To compensate for the low yield from the second toothbrush, the method was modified by extending the incubation time for the enzymatic lysis step from 30 minutes to two hours in addition to two extra amplification cycles (steps 4-6 in Table 1; 29 cycles total) in the PCR reaction. On top of this, a second tuft of bristles from the same toothbrush was removed and placed in another tube containing 200 µL of TE buffer instead of the QLB with 5 µL of Proteinase K (4 µg/µL) and treated the same way. DNA quantitation was performed after samples had been subjected to amplification. The results obtained for the method utilizing 200 µL QLB can be found in Table 6, and the results for the method using 200 µL of TE buffer can be found in Table 7.

Table 6: Toothbrush Sample Results Using QLB and 2 Hour Digestion

Sample Name	Quantitation Result (ng/µL)	DNA Input in PCR (ng)	STR Result	QS Status**	RFU Value Ranges for Loci
T2	0.09	0.18	Full Profile	PP	50-450
T9	1.04	2.08	Full Profile	PP	2,000-6,000
T10	0.87	1.74	Full Profile	PP	1,000-4,000
T13	0.0004	0.0008	Negative Profile	PP	N/A
T14	0.03	0.06	Full Profile	PP	270-1,000

**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

Of the five toothbrush samples evaluated with the QLB and the two-hour digestion method, four of them produced full STR profiles, whereas one of them (T13) resulted in a negative profile. All five reactions had acceptable quality standard results, indicating no reaction failures, or inhibiting substances. Subsequent quantification of the samples revealed that the DNA concentration in sample T13 was only 0.0004 ng/µL (Table 6), which means that only 800 fg (0.0008 ng) of DNA was loaded into the PCR reaction, which is 250x lower than the lowest recommended amount of DNA to use with the STR! Go kit (0.2 ng or 200,000 fg) and explains why this was the only sample to produce a negative profile.

Table 7: Toothbrush Sample Results Using TE Buffer and 2-Hour Digestion

Sample Name	Quantitation Result (ng/µL)	DNA Input in PCR (ng)	STR Result	QS Status**	RFU Value Ranges for Loci
T2	0.06	0.12	High Partial Profile	PP	< 300
T9	2.06	4.12	Full Profile	PP	500-900
T10	0.09	0.18	High Partial Profile	PP	56-1,000
T13	N/A	N/A	Negative Profile	PP	N/A
T14	0.21	0.42	Full Profile	PP	60-700

**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

When using TE buffer for sample digestion, only two of the five samples evaluated produced full STR profiles, as opposed to four out of five observed when the QLB was used. Two samples that previously resulted in full profiles (T2 and T10) produced high partial profiles when subjected to this preparation method. Sample T13 once again produced a negative profile, and DNA quantification resulted in an undetermined result, indicating either no or very little genetic material was recovered from this sample.

Ante-Mortem Samples - Hair

Prior to using shed hair from personal hairbrushes, several direct PCR approaches were evaluated for freshly plucked hair. In the first method evaluated, Hair Method A, two strands of hair were plucked from the root of the donor's head and placed directly in a mixture of 20 µL of QLB and 1 µL of proteinase K (4 µg/µL). The samples were then incubated as before and 2 µL of the processed samples were used for PCR using 27 amplification cycles. Two samples were evaluated using this initial method, and both produced full STR profiles.

In Hair Method B, two hair roots from each donor were placed in microcentrifuge tubes containing 20 µL of QLB only and were then incubated at 95°C for five minutes while being shaken at 1200 rpm. As done previously, 2 µL of the prepared samples were then used for PCR and amplified with 27 cycles. With this method, 1 sample produced a negative STR profile, while the other produced an overblown STR profile; the PCR product had to be diluted 1:10 to obtain a full STR profile.

In Hair Method C, the two hair roots obtained from each donor were placed straight into PCR tubes and direct PCR was performed on the samples (no sample processing). With this method, both samples evaluated produced high partial STR profiles.

When comparing the three preliminary methods for obtaining STR profiles from hair, it was clear that Hair Method A resulted in the most complete and highest quality STR profiles, as well as the highest RFU values for each obtained peak. Hence, Hair Method A was used moving forward when evaluating hair samples obtained from donated hairbrushes. A total of five samples were tested as described for Hair Method A, with the exception that six strands of hair were taken per sample to account for a potential lack of roots, and amplification with 29 cycles instead of 27, again to account for lower amounts of DNA. The results obtained are listed in Table 8.

Table 8: Hair Method A Results

Sample Name	Quantitation Result (ng/µL)	DNA Input in PCR (ng)	STR Result	QS Status**
HA	2.31	4.62	Full Profile	PP
HB*	8.45	16.90	Full Profile	PP
HC	0.15	0.30	Full Profile	PP
HD	0.01	0.02	Negative Profile	DD
HE	0.40	0.80	Full Profile	PP

*: PCR product diluted 1:10

**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

Four out of the five samples evaluated produced full STR profiles (for one sample, HB, the PCR product had to be diluted 1:10 to produce an acceptable result). Only one sample (HD) produced a negative profile. Subsequent quantitation of the processed samples revealed DNA concentrations ranging from 0.01 ng/µL (sample HD) to 8.46 ng/µL (sample HB). The negative sample HD only had 0.02 ng of DNA subjected to amplification, which is 10x lower than the minimum recommended amount for the kit (0.2 ng). Furthermore, the QS status for this sample was DD (Table 8), meaning there was a QS2 dropdown with a ski-slope profile, indicating that the

sample was degraded. The combination of the low yield plus the DNA degradation explains why this sample produced a negative profile while the rest produced full profiles.

Ante-Mortem Samples - Glasses

The first method evaluated, Glasses Method 1, involved cutting the tip of a sterile polyester swab and then using the swab tip to recover DNA around the entire nosepiece (bridge and nose pads) and the temple tips (part that sits around the ears). Each section of the glasses was swabbed horizontally then vertically. A cutting of the polyester swab, instead of a whole swab, was used to ensure all recovered material was processed rather than leaving some behind on the swab. Each cut swab was placed in a mixture of 50 µL of QLB and 2.5 µL of 4 µg/µL proteinase K and incubated at 60°C for two hours. Once again, 2 µL of the processed samples were used for PCR, subjecting the samples to 29 cycles for amplification. Of the 2 sets of glasses, one produced a high partial STR profile, whereas the other resulted in a low partial STR profile. DNA quantitation was performed on these two samples after the fact, and it was revealed that the sample that produced the high partial profile had a concentration of 0.0666 ng/µL (0.1332 ng loaded) and the sample that produced the low partial profile had a concentration of 0.0319 ng/µL (0.0638 ng loaded). Both samples had less than the recommended amount of DNA amplified per reaction (0.2 ng to 2 ng), which explains why neither profile produced was complete.

In Glasses Method 2, the tips of sterile polyester swabs were again cut off the handle before swabbing the glasses, but this time cuttings were pre-wet with 2 µL of QLB prior to swabbing the same areas (temple tips and nosepiece) of the donated glasses. The swab was placed in a PCR tube and directly amplified with 29 cycles. With this method, a total of seven different glasses were evaluated. Of the seven samples, five of them produced negative STR profiles, one resulted in a high partial profile, and the final produced a full STR profile.

Glasses Method 3 was similar to Glasses Method 2, however in this scenario a MicroFLOQ swab was used instead of a polyester one. This swab is very small and designed to be used direct PCR and was not cut prior to collection. A volume of 1 µL of QLB was used to pre-wet the swabs prior to obtaining genetic material. After swabbing, the swab was snapped off at the breakpoint and subjected to direct PCR for 29 cycles. At this time, only one new sample was available for evaluation, and the one sample resulted in a negative STR profile.

The next procedure evaluated (Glasses Method 4) involved cutting a FLOQSwab for each sample and wetting it with 1 µL of a 0.1% (v/v) Triton X solution before swabbing the glasses. Once the glasses had been gone over with the wet swab, a second, dry FLOQSwab was cut and used to go over the areas previously swabbed with the wet one. Both swabs were placed in the same PCR tube and subjected to direct PCR (29 cycles). For this method, two samples were available for evaluation, and one produced a high partial profile while the other produced a low partial profile.

Glasses Method 5 involved the same initial process as described in Glasses Method 4 (one FLOQSwab wet with 1 µL of Triton X followed by a second, dry swab). However, instead of the two cut swab pieces being amplified directly, they were both placed in a solution containing 30 µL of QLB and 1.5 µL of 4 µg/µL proteinase K. These samples were subjected to the same incubation and enzyme inactivation steps described in Glasses Method 1, and 2 µL of the processed samples were used for PCR (29 cycles). With this method, two samples were evaluated. One of them resulted in a high partial STR profile, while the other produced a low partial STR profile. DNA quantitation was performed on these samples after the fact; however, the results were undetermined and not investigated further as it would not advance the goal of this study.

The final method evaluated for obtaining STR profiles from glasses (Glasses Method 6) involved taking cuttings from a Fitzco CEP swab, wetting the cuttings with 2 µL of Triton X (0.1% v/v), and then swabbing the five donated glasses that were available. The cuttings were placed in a mixture of 20 µL and processed as for Method 1 with the PCR for 29 cycles. The results for samples investigated using this method can be seen in Table 9.

Table 9: Glasses Method 6 Results

Sample Name	Quantitation Result (ng/µL)	DNA Input in PCR (ng)	STR Result	QS status**
GA	0.13	0.26	Low Partial Profile	N2
GB	0.4	0.80	Full Profile	PP
GC	Undetermined	n/a	Negative Profile	AA
GD	Undetermined	n/a	Low Partial Profile	N2
GE	Undetermined	n/a	High Partial Profile	N2

**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

Five samples were investigated with this final method. One sample gave a full STR profile (GB), another gave a high partial STR profile (GE), two produced low partial STR profiles (GA and GD), and the other resulted in a negative STR profile (GC). These samples were subjected to DNA quantitation, but only two of the five samples produced acceptable results (samples GC, GD, and GE all produced indeterminate concentrations). Of the two samples with usable quantitation numbers, sample GA (low partial profile) was found to have a concentration of 0.13 ng/µL (0.26 ng loaded into PCR), and sample GB (full STR profile) was found to have a concentration of 0.4 ng/µL (0.8 ng loaded into PCR). Both samples had a sufficient amount of DNA added for amplification (recommended range is 0.2 ng to 2 ng); however only sample GB produced a full

profile. Furthermore, all samples producing partial profiles (GA, GD, and GE) were missing the quality indicator QS2, indicating some degree of PCR inhibition in these samples.

A total of six different methods were evaluated for recovering genetic material from donated glasses for use in building reference STR profiles for the donors. Of all the methods explored for obtaining STR profiles from glasses, none of them showed promise as a viable option for practical use. No Glass Method was able to produce full STR profiles for all samples investigated with the method being evaluated at the time, and only Glass Method 1 (dry polyester swab incubated in lysis buffer and Proteinase K) resulted in 100% of the samples evaluated producing acceptable quality standard (specifically QS2) results. Table 10 gives an overview of all glass methods evaluated and the obtained results for each.

Across all six methods tested, 11 out of 19 total samples (57.9%) from glasses were inhibited, which was portrayed by the quality indicator QS2 missing. The quantifications performed on the samples that were not directly amplified (Glass Methods 1, 5, and 6) were also affected by the inhibiting substances. Only four out of nine of these samples gave conclusive results, and the determined concentrations ranged from 0.03-0.40 ng/µL. Of these samples, only one of them (sample GB from Glass Method 6; see Table 9) ended up producing a full profile, and it was also the most concentrated glass sample quantified at 0.4 ng/µL (0.8 ng loaded into PCR reaction).

Table 10 – Comparison of Different Methods Evaluation for Obtaining STR Profiles from Glasses

Sample Type	Method Used for Obtaining DNA	# of Full Profiles	# of High Partial Profiles	# of Low Partial Profiles	# of Negative/ not suitable Profiles	QS2 detection
Glasses (n=2)	GM1		1	1		2
Glasses (n=7)	GM2	1	1		5	3
Glasses (n=1)	GM3				1	0
Glasses (n=2)	GM4		1	1		1
Glasses (n=2)	GM5		1	1		0
Glasses(n=5)	GM6	1	1	2	1	2

GM = Glass Method

Ante-Mortem Samples - Razors

The first method evaluated for obtaining STR profiles from donated razor samples (Razor Method 1) involved cutting the tip of a polyester swab off and using the tip to swab the razors as described before (horizontally then vertically). The tip was then placed in 100 µL of QLB and 2.5 µL of proteinase K (4 µg/µL) and incubated at 60°C for 30 minutes and processed as described previously with the PCR using 27 cycles for amplification. Of the two samples evaluated, one sample resulted in a low partial STR profile, while the other resulted in a negative STR profile.

Razor Method 2 was nearly identical to the procedure described in Razor Method 1; the only difference was the volume reduction from 100 µL down to 50 µL to concentrate any genetic material potentially recovered and the increase of the number of amplification cycles from 27 cycles to 29. Two more samples were evaluated using this method, and both samples resulted in low partial STR profiles. DNA quantitation reactions were performed on these samples afterwards,

and the yields of the 2 samples were found to be 0.080 ng/µL and 0.028 ng/µL, meaning both samples had less than the recommended amount of DNA (minimum of 0.2 ng) loaded into the reaction.

For Razor Method 3, only one sample was available for initial evaluation. Here, the tip of a polyester swab was cut off its handle and then wetted with 2 µL of QLB before swabbing the sample. The swab was then placed in a PCR tube and directly amplified, again using 29 cycles for amplification. The sample evaluated produced a full STR profile.

In Razor Method 4, a MicroFLOQ swab pre-wet with 1 µL of QLB was used to swab the razor horizontally and vertically (only 1 sample was available). After swabbing the sample, the tip of the swab was broken off into a PCR tube and subjected to direct amplification using 29 cycles. The sample evaluated with this method resulted in a low partial STR profile, and both quality indicators (QS1 and QS2) were absent, indicating PCR inhibition.

The fifth method evaluated for generating STR profiles from razor samples (Razor Method 5) involved the dry/wet swab process described for glasses (Glass Method 4). For each sample evaluated (n=2), two FLOQSwab tips were cut off their handles. Both swab tips were placed in the same PCR tube and subjected to direct PCR (29 cycles). One sample subjected to this method resulted in a low partial STR profile, and the other produced a negative STR profile. The quality indicator QS2 was absent in both samples, indicating reaction inhibition.

Razor Method 6 also involved using two FLOQSwab tips per sample (one wet with 0.1% Triton X-100 followed by a dry one), however this time the swab tips were combined in one tube and lysed in 30 µL QLB plus 1.5 µL of proteinase K solution (4 µg/µL) for 2h. After the subsequent steps 2 µL was again used for PCR (29 cycles). Both samples produced negative STR profiles and were at least missing the quality indicator QS2 (one sample was missing both quality indicators),

indicating PCR inhibition. Subsequent DNA quantitation was performed on the two samples; however, the results were undetermined.

The last method evaluated on donated samples (Razor Method 7) utilized cut FitzcoCEP swabs wet with 2 µL of Triton X-100 (0.1% v/v) for swabbing the razors (only one wet swab was used per sample). The swab tips were placed into a mixture of 20 µL QLB plus 1 µL of proteinase K (4 µg/µL), and then subjected to 2h enzymatic digestion, heat-inactivation, and PCR procedures as listed previously. Five samples were available at this time and evaluated; the results are recorded in Table 11.

Table 11: Razor Method 7 Results

Sample Name	**Quantitation Result (ng/µL)**	**DNA Input in PCR (ng)**	**STR Result**	**QS Status****
R4	0.0005	0.001	Negative Profile	PP
R9	0.0156	0.0312	High Partial Profile	PP
R10	0.0183	0.0366	High Partial Profile	PP
R14	0.0002	0.0004	Negative Profile	PP
R19	0.0009	0.0018	Low Partial Profile	PP

**Quality Sensor Codes: PP = Both present; AA = Both absent; DD = QS2 lower dropped down; N1 = no QS1 detected; N2 = no QS2 detected

Of the five samples investigated, two samples (R9 and R10) produced high partial STR profiles, one sample (R19) produced a low partial STR profile, and the other two (R4 and R14) resulted in negative STR profiles. The DNA quantitation showed that this method did not result in sufficient yields of genetic material for any of the samples. The two samples that produced high partial profiles (R9 and R10) were found to have DNA concentrations of 0.0156 ng/µL and 0.0183 ng/µL, respectively, meaning that about 0.03 ng of DNA were loaded into each of these reactions, which is approximately 6.7x lower than the minimum recommended amount for the kit being used

(0.2 ng). The remaining samples had less than 0.002 ng of DNA loaded into each reaction, values that are less than 100x the lowest recommended amount.

Only one of the seven methods evaluated for obtaining genetic profiles from razors showed initial promise for practical use (Razor Method 3), as this was the only method to produce a full STR profile from any razor sample. Unfortunately, this method was not tested on additional samples.

Seven methods for building STR profiles from razors were evaluated without full extractions, and only one of the seven methods had a 100% success rate (Razor Method 3 – a wet polyester swab that was directly amplified). There was only one sample processed in this manner. Three of the other methods (Razor Methods 4-6) produced only negative or low partial profiles that were unusable anyway due to the absence of amplification of quality indicator QS2, which strongly suggests the presence of inhibiting substances. Razor Method 4 involved direct amplification of a wet MicroFLOQ™ swab, whereas Razor Methods 5 and 6 utilized wet FLOQSwabs™ (direct and indirect amplification, respectively) for STR profile generation. Table 12 gives an overview of all razor methods evaluated and the obtained results for each.

The samples processed with lysis buffer (Razor Methods 1, 2, 6 and 7) were quantified. DNA concentrations for the razors ranged from 0.00-0.08 ng/µL, all of which are very low yields (especially for the reduced volumes of QLB used) and did not result in the generation of any full STR profiles. Some of the quantification results of these samples were undetermined due to inhibition, as were all the amplification reactions for Razor Method 6. This shows that even the indirect amplification processes of genetic material obtained from razors was prone to contamination with inhibiting substances and the recovery process could not dilute them out

successfully. This strongly suggests that if the sample size for razors evaluated using Razor Method 3 (only method to produce a full STR profile) was increased, inhibition would be observed as this was a direct PCR method and there is no opportunity to dilute out the inhibiting substances prior to amplification. The reason the one sample evaluated with this method produced a full profile is more likely due to chance than the method being superior to the others investigated.

Table 12 – Overall Results for Different Razor Methods Evaluated

Method	Collection/ Pre-treatment	# of Full Profiles	# High Partial Profiles	# Low Partial Profiles	# Negative/ Not Suitable Profiles	QS2 detection
RM1 (n=2)	Wet Polyester swab - 100 µL QLB			1	1	2
RM2 (n=2)	Wet Polyester swab - 50 µL QLB			2		2
RM3 (n=1)	Wet polyester swab - Direct PCR	1				1
RM4 (n=1)	Wet MicroFLOQ™ - swab Direct PCR			1		0
RM5 (n=2)	Wet FLOQSwab™ followed by dry FLOQSwab™ - Direct PCR			1	1	0
RM6 (n=2)	Wet FLOQSwab™ followed by dry FLOQSwab™ - 30 µL QLB				2	0
RM7 (n=5)	Wet FITZCO swab - 20 µL QLB		2	1	2	5

QLB = Qiagen Lysis Buffer
RM = Razor Method

Investigating Makeup as a Source of PCR Inhibition

Since PCR inhibition was noted at a high rate in both glasses and razor sample evaluations, a quick experiment was set up to confirm if make-up or any other facial product used could be the source of inhibition. The experiment was set up as follows: Control DNA 9948 (5 ng/µL; included in Investigator 24plex GO! Kit) was diluted down to 250 pg/µL (0.25 ng/µL) in both sterile water and QLB, separately. Then, two polyester swabs were taken, and both were inoculated with 500 pg of DNA (2 µL) from the control diluted with water. One of the two swabs was also dabbed in compact foundation make-up. Finally, a total of five PCR reactions were set up for amplification using the parameters outlined above for 29 cycles: sample SCD, which was the swab inoculated with DNA but no make-up; sample SCMD, which was the swab inoculated with DNA and make-up; sample DNA, which used 2 µL of the DNA diluted in water (250 pg/µL; 500 pg total) and no swab; sample DLB, which used 2 µL of the DNA diluted in QLB (250 pg/µL; 500 pg total) and no swab; and sample LB, which was only 2 µL of stock QLB (no DNA – negative control). Table 13 summarizes the results.

Table 13: Summary of Make-Up Inhibition Experiment

Sample ID	**DNA input (pg)**	**Profile Status**	**Average RFU Values**	**Sample Issues**
SCD	500	Full	2,000	N/A
SCMD	500	Failed	N/A	No Sizing Data
DNA	500	Full	10,000	N/A
DLB	500	Full	1,000	N/A
LB	0	Negative	N/A	N/A

From this experiment, it was clear to see that make-up can inhibit PCR. When comparing the two swab samples (SCD and SCMD), the swab without make-up (SCD) produced a full profile with both quality indicators intact, whereas the swab dabbed in compact foundation (SCMD) resulted in a failed reaction. Inhibitory substances from the make-up hindered the reaction so that no sizing data could be procured.

It was also observed in this experiment that both QLB and the polyester swabs themselves contribute to some degree of PCR inhibition. When investigating the effects of the polyester swabs on profile generation, it was observed that sample SCD (swab with 500 pg of DNA on it) did produce a full STR profile with peaks around 2,000 RFUs. However, the sample labeled as DNA (which used the same control DNA diluted in water, except the DNA was added directly into the master mix and not onto a swab) produced a full profile with peaks around 10,000 RFUs – a five-fold increase in intensity. As postulated previously, the polyester swab may be sequestering components of the PCR master mix, or it may be that the genetic material captured by the swab in sample SCD was not fully released during direct amplification. Either way, it is obvious that the presence of the polyester swab did significantly impact the quality of the data generated.

As far as the QLB is concerned, there was a 10-fold decrease in allele intensity for the sample diluted in QLB (sample DLB; average RFU value of 1,000) as compared to the sample diluted in water (sample DNA; average RFU value of 10,000). This result was unexpected, as it was observed in earlier experiments with toothbrush samples that QLB provided superior stability and allele intensity as compared to TE buffer, an aqueous solution. No further investigation was performed at this time, but it was clear that all these components were mitigating factors in building reference STR profiles from the ante-mortem sample types evaluated.

Discussion

Post-Mortem Samples - Fresh Bone

The QLB protocol utilized for recovery of genetic material from bone samples was mostly successful. A full STR profile was obtained from four out of the five bone samples investigated. All five bone samples yielded a sufficient amount of DNA for the PCR to be successful, but one sample failed even though the quality indicators passed. Failure to amplify the DNA recovered may be due to degradation prior to analysis. No further investigation was performed at this time to identify the source of the amplification failure.

As far as the practicality of the recovery process is concerned, cleaning the bones of blood and tissue was the most time-consuming step. The use of bleach as a cleaning agent was avoided due to the possibility of PCR inhibition, instead the bones were boiled in deionized water, which was time consuming. The irradiation of the bones was a standard procedure that can be replicated in most crime labs, and the collection of shavings with a Dremel tool was not a technically complicated process and can be implemented with any type of drill tool. The amount of DNA yielded for each sample was normalized by calculating the amount of DNA yielded in nanograms per milligram of bone shaving collected (ng_{DNA}/mg_{bone}). In this experiment, it was found that the yield of DNA per mg of bone varied greatly for each sample (1.16 ng/mg to 20.82 ng/mg) even though all bones were fresh clinical samples not exposed to environmental degradation. This intraindividual variation is typical for human tissue (Butler 2012). Drill shavings from bone can also work for more compromised samples. During the World Trade Center victim identification effort 44% of bone samples gave good partial or full profiles from a small amount of shavings (Holland et al. 2003). Even though bone samples found in forensic casework typically have PCR inhibitory components and require an extraction and purification step to generate an STR profile

(Ludeman et al., 2018), the lysis in QLB without purification described here worked well. This is consistent with recent findings where Rapid DNA systems were successfully used to generate STR profiles from bone samples after a short demineralization and extraction step (Turingan et al. 2020). The method described in this study would also be suited for immediate disaster victim identification. This method could be implemented into a high throughput system as described by Bode for victim identification after the attacks on the WTC, bypassing the extraction step and reducing the total turnaround time for victim identification and notifying family members (Holland et al., 2003).

Post-Mortem Samples - Muscle Tissue

Recovery of DNA for the generation of STR profiles from muscle tissue samples proved to be feasible and efficient without the need for extraction. Adding small portions of muscle samples to the PCR mix for direct PCR resulted in the generation of at least partial profiles, and the lack of amplification of the QS2 quality standard implied the presence of inhibitory substances. To dilute out the inhibitory substances, muscle tissue was first incubated with a low amount of QLB. This approach yielded so much DNA that amplifying 1 µL or 2 µL without prior quantitation overloaded the reaction resulting in off-scale peaks and pull-ups in the generated STR profiles unless the PCR product was diluted down 1:100 prior to electrophoresis. The final muscle method used a shorter incubation time and larger volume of the QLB buffer, and still yielded significant quantities of DNA in all samples evaluated. Two of the seven samples investigated with this method still had to be diluted after the PCR. When quantitation of these two samples was performed, it was found that the samples requiring dilution had been amplified with a DNA input of 44.62 and 89.32 ng of DNA which is over 20x above the manufacturer-recommended range for

amount of DNA of 0.2 ng to 2 ng (Qiagen 2015b). The 1:10 dilution of the PCR product generated STR profiles without artifacts that met all acceptance criteria.

The final muscle tissue method was so successful that in all samples investigated, the recovered amount of DNA was still higher than what was needed for STR profile generation using the STR GO! kit. During the sample preparation process, it was found to be difficult to take a muscle tissue cutting small enough to prevent potential overloading. This confirms the feasibility of the processes described by Mundorff et al. (2018) and Turingan et al. (2020) introduced in the literature review section.

Post-Mortem Overview

Overall, recovery of genetic material without full extraction from both post-mortem sample types evaluated (bone and muscle) proved to be successful in the experimental set-ups described above. The results observed for STR typing without extraction show promise for eventual field use in DVI. The yield of DNA recovered from some muscle tissues was too high for immediate use with PCR amplification, while one of the bone samples failed. Further method optimization studies would need to be performed on both sample types to see if it is possible to normalize the amount of genetic material recovered per sample. The results observed from both sample types investigated in this study, as well as the studies conducted by Mundorff (2018) and Turingan (2020), show that these post-mortem sample types are amenable to direct amplification for field use in DVI. Furthermore, the efficacy of each method would need to be evaluated with increasing age of samples and exposure to different environmental conditions, as these factors cause increased sample degradation and higher potential for introduction of PCR inhibiting compounds.

Medical Reference Samples - Paraffin-Embedded Tissue

The paraffin-embedded tissue samples tested all resulted in negative STR profiles. This result was not surprising for an extraction-less method of DNA recovery due to the processing that paraffin-embedded tissues undergo. Both the formaldehyde and the paraffin, two critical compounds in the fixing process, could have played a part in inhibiting the PCR. Even if the concentration of these compounds was not sufficient for PCR inhibition, formalin is known to cause DNA degradation (Budimlija et al., 2009). Especially since the paraffin blocks used had been stored for 10 years, the age of the samples themselves might have contributed to the failed STR profile generation with little to no DNA left due to degradation. The fact that the lysates of the samples evaluated were found to have no DNA and that the GO! Kit PCR successfully amplified both quality indicators, implies that the inhibitory effects of formaldehyde and paraffin had been diluted out in the processing stage. The lack of DNA and the STR profiling failure was either due to degradation of the genetic material over time or the DNA-modification effects of formalin (Josefiova et al., 2017). Regardless of the root cause of the failure, paraffin-embedded tissues should not be considered a viable option for a rapid generation of a reference STR profile in DVI; this sample type requires more extensive extraction and subsequent DNA clean up and/or concentration steps. Josefiova et al. (2017) successfully generated STR profiles from FFPE tissue samples (5 mg) using a commercially available DNA tissue extraction kit. However, they observed that the staining formulation, stain time, and age of the tissue being stained all affected the success rate of STR typing.

Medical Reference Samples - Histological Slides

As with the paraffin-embedded tissue samples, the histological slides evaluated did not result in acceptable reference STR profiles, and again this result was not unexpected. Histological

slides are prepared from paraffin-embedded tissue and are therefore subjected to the same formaldehyde and paraffin processing procedure, which as stated before, can result in PCR inhibition. Moreover, the slides are also stained using hematoxylin and eosin stain, which also can result in PCR inhibition. Of the multiple methods evaluated for recovering DNA from this sample type, only the first produced a partial profile. When repeated with increased amounts of the sample used for processing, no acceptable profiles were generated. Throughout the process of evaluating different methods for microscope slides, most of the samples evaluated (n=12) had the quality indicator QS1 missing, two had QS2 missing (indicating PCR inhibition), and two had both quality indicators missing. Only two samples had both quality indicators present, of these two samples one was negative and did not show any DNA after quantification, the other sample had detectable DNA and a partial profile. As with the paraffin blocks this sample type only contains low amounts of degraded DNA and requires specialized protocols for successful results (Silva Funabashi et al. 2012). Furthermore, the slides resulted in high background noise levels in the generated negative STR profiles. This may be a result of paraffin scrapings being introduced into the PCR reaction, and then being detected in the end-point analysis (pass through the laser leading to the artifacts. Previous studies have also shown that the slide preparation process from paraffin-embedded tissue can also degrade genetic material (especially larger fragments of DNA) in addition to inhibiting downstream amplification reactions (Budimlija et al. 2009).

Medical Reference Sample Overview

Neither of the medical reference samples evaluated showed promise for use as reference profiles in DVI without extraction. Both paraffin-embedded tissues and histological slides are processed the same way and treated with chemicals that inhibit PCR. To remove these compounds,

a full DNA extraction is necessary. The objective of this study was to find suitable samples for rapid reference profiling; neither of these sample types can be used for that purpose.

Ante-Mortem Samples - Toothbrushes

All methods evaluated for building reference STR profiles from donor toothbrush samples had varying degrees of success. Toothbrushes were initially considered to contain sufficient amounts of DNA and tufts of bristles were only amplified with 27 cycles. Even with longer incubation times and 29 cycles one sample had insufficient amounts of DNA and did not yield an STR profile. As can be seen by the range of DNA results, there is clearly variation in how much DNA is left behind on a used toothbrush, which may be related to how well the brush is cleaned after use. There may also be an effect on the amount of DNA recovered based on the tuft selected for analysis. When comparing the two buffers, the QLB to a TE buffer, the QLB buffer performed better, even though some of the quantitation results were lower. It appears as if the lysis buffer helps to preserve the integrity of the recovered DNA, as there was a higher full profile rate and higher RFU values for this method even with less starting material.

Based on the results observed in this study, it would appear that using toothbrush samples is a feasible tool for building reference STR profiles during DVI, even without extensive extraction protocols.

Ante-Mortem Samples - Hair

The preliminary methods for producing STR profiles from hair samples were performed with freshly plucked hair, and all three tested methods were able to successfully generate at least 1 high partial profile per batch. Hair Method A (treatment with QLB and proteinase K) was the most successful and used to investigate hair samples obtained from donated hairbrushes, to simulate what would be available for building reference STR profiles of suspected victims. The

only modification made to this method was that for the brush samples, 6 strands were investigated per patient, as opposed to the original two strands of freshly plucked hair. This was done to account for the advanced age of the brush samples and potential degradation of these samples after exposure to the outside environment. Four out of the five samples produced full profiles making this a suitable method for building reference STR profiles in DVI cases requiring rapid turnaround times.

For this analysis, the hair recovered from brushes were all telogen phase and preferentially selected if the roots and/or tissue flakes were visible; at least six strands were added to only 20 µL of QLB to achieve a sufficiently concentrated extract. A great deal of caution had to be taken to ensure full submersion of the root ends for each individual strand in the lysis buffer. To further complicate the preparation process, the different samples showed great variability in the degree of difficulty for handling each strand. This procedure proved to be more technically challenging than anticipated, and the technologist handling the sample would need to be diligent to make sure all steps were performed correctly so acceptable reference profiles could be produced. A rapid screening method for identification of DNA-rich hair roots by directly staining nuclei has been described by Lepez et al. (2014). This method can identify strands of hair that would most likely result in successful STR profile generation. It was observed that at least 20 nuclei are necessary to obtain partial STR profiles after the strands were extracted and amplified, and that roots containing 50 or more nuclei resulted in the generation of full STR profiles. Furthermore, it was found that roots with no nuclei seen resulted in negative STR profiles 96% (94/98) of the time. This procedure could be implemented into a direct PCR workflow when analyzing hair samples for reference profile generation in order to select the most appropriate samples for processing, which could reduce turnaround time and improve identification rates (Lepez et al., 2014). Furthermore, it was

found in a study attempting to optimize procedures for direct amplification of DNA recovered from hair that decreasing the number of amplification cycles in PCR and/or diluting the subsequent PCR products during electrophoresis have helped to minimize PCR artifacts and improved the overall success rate of profile generation (Ottens et al., 2013). Direct PCR workflows on hair samples can be incorporated into any forensic laboratory and improve the current processes for STR profile generation from these sample types.

Ante-Mortem Samples - Glasses

The majority of DNA samples recovered from glasses were affected by the presence of inhibitory substances, almost 60% of samples had quality indicator QS2 missing. The source of PCR inhibition could be from several different points in the recovery process. For the samples subjected to direct PCR, the swabs themselves may have absorbed too much of the PCR reaction mix, not allowing for the proper concentrations of any component for proper amplification. Furthermore, the swabs that were directly amplified could possibly have released inhibitors or adhesive material while the samples were being heated. Our added inhibitor test showed that this may have been the case for the polyester swab. For all samples, collected materials on the glasses (i.e., suntan lotion, make-up, lotion, etc.) could have contributed to PCR inhibition as well. Again, preliminary testing was able to show that makeup prevented DNA amplification. Several components present within cosmetics such as detergents, fats, alcohols, and pectin could interfere with the efficiency of the PCR, which will require a suitable DNA extraction method capable of overcoming PCR inhibition (Kim et al., 2018). With all this in mind, glasses do not appear to be a suitable candidate for use as a tool in quickly building reference STR profiles for DVI situations.

Ante-Mortem Samples - Razors

As with the glasses, there are multiple opportunities for PCR-inhibiting substances to be introduced onto razor samples that could prevent the successful generation of an STR profile. Inhibition was found for some of the samples, but most of the samples simply had insufficient amounts of DNA for profile generation. There is an expected individual variation of DNA deposited on a razor. This may be related to the shedder status of the donor, which also plays a role in touch DNA recovery (Lowe et al., 2002). This makes it difficult to standardize protocols for recovery of genetic materials from these sample types. Furthermore, the fact that the number and size of the razor blades present on different makes and models of razors varies greatly; the difference in the amount of surface area available could change the amount of recoverable DNA. Lastly, DNA may be rinsed off the razors during repetitive washing steps while the donors were shaving. With all these variables considered, it is obvious that razors are not the most reliable source of rapid reference DNA in DVI settings. It could still be attempted to use a small clipping and direct PCR, as in Razor Method 3. This method gave a full profile but unfortunately was tested only once and not repeated.

Conclusion

In the experiments described above, there was mixed success with the different sample types evaluated using direct PCR for STR profile generation. Both PM sample types evaluated, bone and muscle tissue, proved to be conducive to direct PCR protocols, and the findings presented here have been corroborated through the success of multiple other scientists in the field being able to produce STR profiles from these sample types with different STR kits and protocols. These findings are especially vital for future DVI scenarios after mass fatality events where evidence is limited or severely degraded.

The AM samples evaluated in this study performed variably as compared to the PM samples. As for direct reference AM samples, the hair recovered from donor brushes and the bristles of donated toothbrushes were repeatedly successful in STR profile generation. The razor and eyeglass samples investigated failed to produce STR profiles consistently for different reasons. The razor samples often did not contain enough DNA to amplify, while the donated glasses frequently contained inhibitory substances that prevented successful profile generation. Future modifications to sampling protocols may help to improve the amount of DNA recovered from these sample types, or help minimize the amount of PCR inhibitors collected, as evidenced by the success in generating DNA profiles from various touch samples, including the use of sellotape for sample collection, in other studies.

All medical reference samples (FFPE slides and tissues) evaluated failed to produce STR profiles with direct amplification, both due to the presence of inhibitory components in these samples and the degradation of genetic material due to the formalin fixation process. These compounding factors are known to be problematic for extraction-based protocols and could not be overcome with direct procedures. The failures observed in this study, as well as the other studies referenced, insinuate that FFPE tissue is not a desirable choice for developing STR profiles, even when the sample DNA is extracted. Other medical reference samples (i.e., blood cards for metabolic disorder screenings or bone marrow donor programs) should be evaluated for use with direct PCR, or other AM sample types should be prioritized over these samples if available.

Direct PCR has tremendous potential for use in DVI scenarios to speed up the identification process by reducing processing time and increasing overall success rate for profile generation. Furthermore, direct amplification preserves the integrity of recovered samples and also reduces the opportunities for both sample contamination and sample mix-up during extended processing

steps. The successful generation of STR profiles from bone, muscle, hair and toothbrush samples through direct PCR suggests that other sample types encountered in routine forensic casework may also be amenable to direct amplification. As more studies demonstrate the efficiency and efficacy of direct amplification, the current regulatory roadblocks preventing implementation of these procedures into daily casework should be updated to reflect the evolving state of the technology.

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