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Degraded Samples in DNA Profiling: A literature review

Senior Project

In partial fulfillment of the requirements for

The Esther G. Maynor Honors College

University of North Carolina at Pembroke

By

Lee Bartalone

Biology

5/10/2023

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Lee Bartalone Date

Honors College Scholar

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Dr. Maria Pereira Date

Faculty Mentor

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Joshua Kalin Busman, Ph.D. Date

Senior Project Coordinator

**Acknowledgments**

I would like to thank my mentor, Dr. Maria Pereira, for her expert advice in the field of genetics, and for her advice in tackling such an extensive list of resources. I would also like to thank my colleague, Elliot Stewart, for his encouragement in this endeavor. I am forever thankful for the opportunity that the Honor’s College of UNC Pembroke provided for me to put the time and effort into this research.

**Abstract**

This literature review covers multiple aspects of degradation in samples that may result in an altered DNA profile. The main elements that may impact a profile include but are not limited to temperature, moisture, burial depth, nucleic acid type, kits used, extraction procedure used, collection and preservation method, the presence of inhibitors, and tissue origin. All these factors may contribute to the value obtained during quantification that indicates the level of degradation in a sample otherwise known as the Degradation Index (DI). The DI of a sample has been suggested to influence the quality of a DNA profile, and in most cases, a high DI may lead to a partial/incomplete profile or even no profile at all.

Keywords: DNA degradation, DNA profiling, DNA fingerprinting, STR, PCR, NGS, SNP

**Background**

Roughly less than 5% of eukaryotic DNA codes for proteins. Its compacted form is known as “chromosomes,” of which humans have 23 in the haploid genome (46 in the diploid), which equals around 6.1 pg of DNA (Kobilinsky *et al.* 2005). A gene site in a homologous chromosome, known as a locus, may be homozygous or heterozygous based on whether the alleles are the same or different, respectively. Stepping out to look at the DNA double helix structure, the two strands run antiparallel to each other. Each strand is a linear sequence of nucleotides which are made of a phosphate group, a pentose sugar, and a nitrogenous base. The nitrogenous bases can be categorized as purines with 2 ring conformations (A or G), or pyrimidines with one ring (T or C). The purines and pyrimidines on both strands are linked together by hydrogen bonds and can be broken with temperatures at or above 95 °C process known as denaturation of DNA. If the nucleotides linked on the same strand by phosphodiester bonds are separated the process is known as degradation and it will split the DNA into smaller fragments (Kobilinsky *et al.* 2005).

DNA profiling mostly focuses on the analysis of DNA repetition (Kobilinsky *et al.* 2005). Alec Jeffreys developed Restriction Fragment Length Polymorphisms (RFLP) in the UK in 1984 (Newton 2008). RFLP was the first kind of DNA profiling technology developed and relies on minisatellites. Minisatellites are based on a Variable Number of Tandem Repeats (VNTR), which are repeating units in the noncoding region of DNA (Kobilinsky *et al.* 2005). VNTRs are adjacently connected regions that are approximately 7-25 bp per repeat, and they have a high degree of polymorphisms. Polymorphism means that the variations found in the repeat units are in each population. VNTR regions are often repeated 5 - 50 times, so the fragments generated are often of high molecular weight (Butler 2005). Despite their prevalence, high molecular weight fragments degrade the quickest, so RFLP typing can become problematic to use in forensic cases (Butler 2005). The reduction in peaks (where height indicates quantity of material) in relation to molecular weight is referred to as the “ski slope effect,” and is often described as a linear progression that shows degradation (Bright *et al.* 2013; Westen *et al.* 2013). Despite this, there has been evidence to suggest that an exponential curve may be more relevant to showing the exact progression in the decrease of peak heights with molecular weight increases (Bright *et al.* 2013).

In 1986 Kary Mullis developed Polymerase Chain Reaction (PCR) as a DNA profiling technique. PCR is used to produce multiple copies of a DNA area of interest. This technique depended on the use of primers and microsatellites [short-tandem repeats (STRs)], which are approximately 2-13 bp each (Helm *et al.* 2016; Kobilinsky *et al.* 2005). STRs are repeated at a rate of about 40 or more times at a single locus, although, they do not have to be adjacently connected (Kobilinsky *et al.* 2005). When DNA is damaged, particularly long STRs become difficult to read and have a lower peak height on average (Westen *et al.* 2013). Damaged DNA can also potentially result in the complete loss of an amplicon/potential PCR product. If the region is damaged, that area may not be able to be amplified properly. These amplicons may be able to be retrieved with another amplification kit if there is an extra DNA sample left. If there is no sample left over, performing extra PCR cycles, increasing Capillary Electrophoresis (CE) voltage, or applying size-selective post-PCR purification to samples may allow for recovery (Westen *et al.* 2013). Though the STRs themselves are small, the primers in PCR must anneal to the surrounding sequence. The average size of a PCR primer is 400 bp (Kobilinsky *et al.* 2005).

Analysis with smaller primers enhances the capabilities of typing smaller amounts of DNA. MiniSTRs allow for this by reducing the average primer size from 400 bp to 94 -167 bp (Butler 2005; Helm *et al.* 2016). A problem with this is that it is associated with an increased likelihood of spontaneous point mutations (insertions, deletions) on the flanking area outside of the primer site which can display differences in a heterozygous allele (Butler 2005). Another more recent analysis method is the use of Single Nucleotide Polymorphisms (SNPs). SNPs occur at a much higher frequency than STRs in eukaryotic DNA, which makes them prime for analysis (Kobilinsky *et al.* 2005). They are also population-specific and not prone to preferential amplification in comparison to STRs (Kobilinsky *et al.* 2005).

# Next Generation Sequencing

## CE is a common method for profiling forensic casework; however, it comes with its limitations. CE has a typing maximum of 25 - 30 loci, making the multiplex capabilities limited (Senst *et al.* 2022). Additionally, the larger loci are less likely to be amplified, with the common fragment length being 80 - 500 bp. In comparison, Massively Parallel Sequencing (MPS)/Next Generation Sequencing (NGS) can allow for the sequencing of up to 231 markers (such as in the MiSeq FGx system) in a single run (Cho *et al.* 2020; Senst *et al.* 2022). With this method, DNA fragments do not have to be separated based on size. This allows for sequencing smaller amplicons which can aid in typing forensic samples (Cho *et al.* 2020). Some limitations to this method are that it requires a lot of time, it is expensive, and its use requires qualified staff (Senst *et al.* 2022; Zupanič Pajnič & Fattorini 2021).

Cho *et al.* (2020) compared 24 common loci between MPS with CE. The DI that Cho *et al.* (2020) used lists samples DI<1 as no degradation, DI 1-10 as slight to moderate degradation, and DI>10 as severe degradation. DI was determined by finding the ratio between the small and large fragments generated in a sample. The DI of the 92 samples that were tested ranged from 2.9 - 85.6, which indicated slight to severe degradation. Degradation in MPS samples showed 17 ± 4.6 loci recovery from DI<10, and 12.5 ± 4.4 loci recovery from DI>10, whereas CE recovered 8.1 ± 4.2 loci from DI<10 samples and 5.9 ± 2.7 loci were recovered from samples with a DI>10. The overall recovery of samples showed 57.4 ± 15.2 samples with adequately recovered alleles in MPS, while CE showed 31.1 ± 29.5 sample recovery (Cho *et al.* 2020).

Fattorini *et al.* (2017) examined the reliability of NGS on STR and SNP markers after sample incubation at 70 °C for 6, 8, and 10 h. The researchers found that there was a higher reading in the 6-h incubated samples in comparison to the controls by 3 STR markers and 10 SNP markers, potentially because of the higher DNA yield. Despite this, the rate of allelic imbalances was found to be higher in the high molecular weight STR samples, some of which had less than 30 reads. Additionally, following incubation for 10 h, there was an average of 33-44% allelic dropout, with the longer allele dropping out 60% of the time in the STR samples, which is a crucial point of concern. Fattorini *et al.* (2017) also examined dilutions of the 6-h incubation samples and found that an increased dilution negatively correlates to the number of correctly typed loci with the 1:3 dilution having the highest frequency of no results.

As such, NGS is not without its limitations, especially since it is still a relatively new technology in the world of DNA profiling. There is also the chance that it may have disagreements with the traditional CE methods. One example of this is the comparison of 57 femur samples from the Konfin 1 Mass Grave in Slovenia by Zupanič Pajnič and Fattorini (2021). The PCR/CE method (using the GlobalFiler™ CE kit) produced 40 full profiles, 2 near full, 12 partial profiles, and 3 no profiles. The full and nearly full profiles had an average DI of 30.8, while the remaining groups had an average DI of 41.8. Thirty-eight samples were also replicated, with only 10 out of 38 being the same as their previous profiles, with an average DI of 13.9, which shows that the low replicability may be due to the degraded nature of the samples (Zupanič Pajnič & Fattorini 2021).

The NGS procedure (GlobalFiler™ NGS STR panel v2.0) processed only 19 samples, 15 of those being the no/partial profiles from the CE run, and the remaining 4 were controls (Zupanič Pajnič & Fattorini 2021). With the degraded samples, 66.8% were on target, 79.3% were uniform, and 71.6% were variable; whereas with the nondegraded samples, 87.5% were on target, 97.2% were uniform, and 7.8% were variable. Overall, this shows low replicability because of disagreements with the CE method. When genotyped, there was a higher frequency of homozygous alleles in the NGS procedure than the consensus sequence, indicating allelic dropout. There were also drop-in alleles determined by stutter peaks that read above the threshold, and it was determined that 25-30% of these were isometric artifacts. Out of the 15 samples, 2 were inconclusive due to degradation, 1 had less than 3 loci, 2 were partial profiles of 8 - 12 loci, and the remaining 10 were successful with 20 - 31 loci (Zupanič Pajnič & Fattorini 2021). Overall, NGS picked up where CE failed; however, CE also confirmed allelic presence in the NGS dropout cases. It is unknown if this variance was due to the high DI in all samples, or whether it was due to differences in the NGS and CE procedures.

There is also the possibility that NGS may increase the chance of contamination because of the multiple-step procedures. Kieser *et al.* (2020) compared the NGS procedure (using the ForenSeq™ software) to the even more novel Reverse Complement PCR (RC-PCR) technique using a population study, dilutions, and mock forensic samples. RC-PCR is a single-step PCR procedure that is designed to amplify degraded DNA. The population study showed that concordant results between the ForenSeq™ procedure and the RC-PCR procedure except for 4 troubled samples. The dilutions were 83.7% concordant down to 60 pg. With the mock forensic samples, the buccal samples were concordant, while the touch samples showed that all but one had a high number of SNP alleles. With the 4 troubled samples from the population study, the RC-PCR method showed a complete SNP profile while ForenSeq™ identified 59.6 - 96.8% of the 27 SNPs (Kieser *et al.* 2020). This shows that while NGS may be effective, there are still new techniques being developed that may increase the efficiency of DNA typing.

## Collection and Preservation of Samples

In cold environments samples may be able to be preserved, however, the specimen can degrade because of the moisture when exposed to snow (Biggin *et al.* 2022). The collection of samples from snow, requires the specimen to be kept frozen during transport to prevent degradation from freeze-thaw cycles. This is not always feasible, however as many forensic identification field kits may not have the tools required for preserving a frozen state (Biggin *et al.* 2022). Some common alternatives to freezing samples include 20% ethylene glycol-propylene glycol (PEG) solution, TNES (which may partially dissolve tissue; however, it decreases nuclease activity), desiccation (such as the use of NaCl), and in emergency situations, detergents may be used (Cox 2008). Buffered formaldehyde is another preservative to consider. While formaldehyde is helpful in retaining/preserving tissue structure, it is also known for being degradative to DNA (Allen-Hall & McNevin 2013). Despite this, Konarzewska *et al.* (2019) found that when formaldehyde was buffered to be a neutral pH, severe DNA degradation was prevented in tissue samples (particularly the brain), especially following paraffin embedding, although there still needs to be more studies on this phenomenon before considering putting it into practice on a larger scale.

NaCl in a solid form desiccates the sample, which inactivates the nucleases and slows microbial growth, while in an aqueous form, it can denature proteins (Allen-Hall & McNevin 2013). EDTA is a chelating agent that binds to metal ions, inactivating the nucleases by not allowing them to bind to metal ions. Concurrently, detergents can also inactivate nucleases by lysing the cells. Ethanol protects against bacterial degradation by removing water from the sample and denaturing proteins/nucleases. The buffers, on the other hand, stabilize the pH, reducing low-pH damage (Allen-Hall & McNevin 2013).

FTA™ paper is a common preservative/collection method reviewed by Allen-Hall and McNevin (2013). FTA™ paper is used for dried samples (treated or not) and can be directly inserted into PCR. Previous research suggests that it can preserve and produce effective profiles in avian blood for up to 4 years. Biggin *et al.* (2022) also examined the effectiveness of FTA™ paper for collections in comparison to filter paper and swabs on blood samples from freshly fallen snow. There was no diffusion of the blood into the snow, likely due to the low moisture content since the snow would not have had time to melt. All samples produced sufficient qualities and quantities of DNA, with DI values being less than 1, which indicates no degradation or inhibition. The swab samples produced an average of 3.573 ng μl-1 of DNA, the filter paper samples produced an average of 28.01 ng μl-1 of DNA, and the FTA™ paper samples produced an average of 5.439 ng μl-1 of DNA. These results showed that the filter paper provided the best quality and quantity of samples for collection in this instance (Biggin *et al.* 2022)

NaCl is a popular desiccant and can preserve tissue samples at 37 °C for 38 weeks, producing full STR profiles at 35 °C after a month and full Powerplex® 16 profiles in a year (Allen-Hall & McNevin 2013). Sodium aluminum silicate beads are another popular desiccant that can produce a full profile with no signs of degradation after using the QI Amp DNA Minikit for extraction after 5 m of storage at RT. Dimethyl sulfoxide (DMSO) is a type of organic solvent, and there have been multiple studies done on how it preserves different tissue types. In mouse livers, DMSO produces HMW DNA products after 2 years of storage at RT. Preservation of avian blood is achieved with good quality DNA from both RT storage and 65 °C, although the length of storage was not specified. Human muscle tissue is slightly different, as full AmpFlSTR Identifiler™ profiles were achieved after 1 m at 35 °C. Ethanol was found to be as effective as DMSO after 6 m at a concentration of 70% in fresh or decomposed porcine tissue, although avian tissue showed degradation (Allen-Hall & McNevin 2013).

LST is a type of buffer that contains sodium azide, is highly toxic, and it has been successful in the preservation of elephant leukocyte DNA for 8 weeks at RT with no signs of degradation but, in human lymphoid tissue, DNA could only be recovered after 4 weeks at RT (Allen-Hall & McNevin 2013). Longmire is another buffer, but it was found to be weaker than DMSO in its preservation capabilities. Standard lysis buffer is another option, however, only partial profiles were ever recovered after storage of human muscle tissue at 35 °C for 1 month (Allen-Hall & McNevin 2013).

Brand-specific preservatives were also considered by Allen-Hall and McNevin (2013) in their review. RNAlater was found to be as successful as DMSO and 70% ethanol for the storage of fresh and decomposed porcine tissue. Oragene was found to be more successful than LST buffer at preservation for 52 weeks at RT. Genotek was found to produce full STR profiles after 1-month-long storage at 35 °C in human muscle tissue. DNAgard+SampleMatrix also showed no difference between year-old rehydrated samples and untreated samples frozen at -20 °C (Allen-Hall & McNevin 2013).

With all these different types of preservatives, the practical application of each in the field depends on whether they are safe to use, widely available, cost-effective, and easily transported (Allen-Hall & McNevin 2013). Since LST contains sodium azide, it is considered field-unsafe, and since ethanol is flammable, its ease of transport is significantly reduced. The researchers suggested that DMSO, Genotek, and DNAgard were best used for direct sampling; but in the case of freshly deceased remains, FTA™ paper is the most useful, while NaCl is the best for ready use if the remains are extensively decomposed. While DMSO and lysis buffer can also be used in the case of extensive degradation, both require previous preparation before being used in the field (Allen-Hall & McNevin 2013).

Technical Comparisons: Extraction Methods, Profiling Kits, and Inhibition

DNA must go through isolation/extraction because some of the extraneous material may cause the DNA to degrade (Rudin & Inman 2002). There are multiple methods of extraction. Chelex extraction is used if there is a minute amount of sample. To extract this way, the sample is boiled in Chelex, which will break open the cells and denature the DNA. The Chelex then binds to the extraneous materials from the cell, effectively isolating the DNA. This method works well with reverse blots and PCR. QIAamp® is a silica-column-based extraction method, where the DNA passes over and adheres to the column, allowing the extraneous material to be washed away. Afterward, the DNA is eluted off the column and collected (Rudin & Inman 2002).

Organic extraction maintains larger fragments of DNA and cleans it more thoroughly than Chelex extraction (Rudin & Inman 2002). This method isolates DNA with organic solvents and purifies and concentrates it via filters or precipitation. The phenol-chloroform extraction method is one such type of organic extraction. Heathfield *et al.* (2021) compared the QIAamp® extraction method and the phenol-chloroform extraction method on 52 tooth samples from three individuals. Out of the subjects, subject one produced 0% profile with the QIAamp® method, and 43% with the phenol-chloroform method, subject 2 produced 4% with the QIAamp® method, and 4% with the phenol-chloroform method, and subject 3 produced 84% with the QIAamp® method and 89% with the phenol-chloroform method. With DNA concentration for each tooth type, the phenol method produced higher quality profiles from canines (which had the highest DNA concentration) and premolars, while the phenol and QIAamp® methods were comparable between incisors and molars. The profiling success was 41% with the phenol method and 33% with QIAamp® for full profiles, and 35% and 28% for failed profiles, respectively (Heathfield *et al.* 2021).

Organic extraction methods are most useful if sperm is not expected or suspected to be in the sample (Rudin & Inman 2002). Differential extraction, in comparison, is used to isolate DNA from samples that are mixed with sperm and non-sperm cells. This extraction method uses two sets of chemicals; the first set lyses the non-sperm cells, of which the supernatant is collected and goes through organic extraction, while the second set lyses the sperm cells. The result is one tube with non-sperm DNA and a second tube with sperm DNA (Rudin & Inman 2002).

There is also a call to streamline the extraction process. While organic extraction methods are effective and produce relatively high-quality DNA, the methods are often time-consuming, use toxic reagents, and require multiple tube transfers that may increase the chance of sample loss or contamination (Calacal *et al.* 2021). When comparing the phenol-chloroform method to the semi-automated DNA IQ™ extraction software and the automated Maxwell® 16 LEV extraction software, the DNA IQ™ method showed an improved allele recovery rate when compared to the phenol-chloroform extraction, although their mean DNA yields were comparable. In contrast, the Maxwell® 16 LEV extraction method was more efficient than the DNA IQ™ and phenol-chloroform methods at removing inhibitors, with no evidence of cross-contamination. Overall, the average allele recovery rate was 84% ± 15.3% for the phenol-chloroform method, 88% ± 14.5% for the Maxwell® 16 LEV method, and 90% ± 12.7% for the DNA IQ™ method (Calacal *et al.* 2021).

The quality of the profiles produced often depends on the kits that are used during the procedure as well. There are a multitude of specialized kits to choose from depending on the tissue that is typed. The Powerplex® Fusion 6C kit is one that is often used in forensics, as it can be applied to a multitude of different tissue types. Hakim *et al.* (2020) compared the effectiveness of profiling using the Powerplex® Fusion 6C kit or the GlobalFiler™ Express kit on buccal samples. Their results showed that the Powerplex® Fusion 6C kit called 30 out of 39 full profiles, and 9 partial profiles, while the GlobalFiler™ Express kit called 22 out of 39 full profiles and 17 partial profiles. Neither process resulted in unreadable profiles, with the Powerplex® Fusion 6C kit having the highest average peak height and allele calls out of the two kits (Hakim *et al.* 2020). The GlobalFiler™ PCR Amplification kit is also a useful one for forensic caseloads. This is because it has 10 miniSTR amplicons, which are most helpful for amplifying degraded samples (Vernarecci *et al.* 2014). In that study, in comparison to the AmpFlSTR NGM SElect™ PCR amplification kit, the GlobalFiler™ PCR amplification kit was able to amplify a larger number of loci, which increased the quality of the resulting profiles from samples with a DI>4. Additionally, the power of discrimination in samples with a DI>10 reached upwards of 1 in 37 million using the GlobalFiler™ kit, whereas the NGM SElect™ kit had a power of discrimination of 1 in 1 million (Vernarecci *et al.* 2014).

The success of profiling also depends on whether there are inhibitors present. While the kits and extraction procedures may assist in isolating the DNA, some inhibitors will interfere with those processes. Griffin *et al.* (2022) examined the effects of 18 illicit drugs, diluents, and adulterants on DNA samples to determine whether profiling DNA from seized drugs would be effective in aiding in identifying those involved in the production, preparation, and distribution of the drugs. The compounds tested were nicotinamide, acetaminophen (tablet and powder), caffeine (tablet and powder), mannitol, glucose, icing sugar, DMS, cocaine, DXM, heroin, ketamine, GHB, MDMA, methamphetamine, PMA, and Pseudoephedrine. When the STRs were evaluated with IQC, none of the amphetamine-type stimulants showed inhibition. Glucose, DXM, Ketamine, and GHB showed some inhibition, but it was mitigated following dilution. Heroin also showed some inhibition but was not mitigated following dilutions (Griffin *et al.* 2022).

When evaluating the results by the peak heights, PMA MDMA, and methamphetamine had low peaks, but after dilution, PMA and MDMA peak heights rose to the normative range, whereas methamphetamine remained unchanged (Griffin *et al.* 2022). Pseudoephedrine, GHB, and cocaine had even lower peaks following dilution, and it is suggested that these drugs may prevent DNA and primers from binding. Glucose and DMS had significantly low peaks, however, they improved once diluted, with DMS’s peak heights increasing drastically. Caffeine had a nonsignificant but high peak, indicating little to no inhibition. DXM, ketamine, and heroin all had extremely low peaks; after dilution, DXM and ketamine were improved but still low with heroin remaining unchanged (Griffin *et al.* 2022). This all indicated that generating usable STR profiles from illicit drugs would be difficult but not impossible.

There has also been reported success in Belgium regarding DNA typing on residual fingerprint skin debris, which brings forth the question of whether it is possible to profile a sample that has been covered with a dactyloscopic powder. Van Hoofstat *et al.* (1999) examined 11 dactyloscopic powders and they were classified as white, black, magnetic, or metal. The white powders included BVDA white, Sirchie indestructible white, and Faurot white. The four black powders include BVDA blower black, special black, concentrated black, and Sirchie volcano. The two magnetic powders include BVDA magnetic black and BVDA magnetic grey, while the two metal powders are BVDA special silver and Faurot aluminum bronze. After powdering, the metallic powders produced no results, as they totally inhibited analysis. In contrast, good intensity of peaks and profiles were obtained after the application of Faurot white, BVDA white, magnetic black, and special black, while another good profile (with low intensity) was recovered from Sirchie Volcano. A low-quality profile was found with blower black, concentrated black, and magnetic grey while differing alleles with dropout were found with indestructible white (Van Hoofstat *et al.* 1999). This showed that typing can be successful from fingerprints, however, the fingerprints should be taken before typing, as DNA typing will destroy the evidence.

#### Comparison of Nucleic Acids

It is generally assumed that RNA is more easily broken down than DNA because RNA degradation is tissue-specific and there are multiple endogenous and exogenous nucleases that can take RNA apart in or out of a cell (Fattorini *et al.* 2020). RNA is also very susceptible to hydrolytic breakdown, especially at higher temperatures such as 90 °C (Lacerenza *et al.* 2022). The additional hydroxyl group in RNA implicates weaker phosphodiester bonds at a neutral pH, but in acidic conditions, DNA degradation is promoted (Fattorini *et al.* 2020). Storage time in previous studies has had mixed results on whether it influences the level of DNA degradation, but evidence has suggested that saliva stored at RT loses detectable RNA (Lacerenza *et al.* 2022).

To evaluate which collection method was the most effective in catering to both RNA and DNA during simultaneous extraction, Lacerenza *et al.* (2022) evaluated whole blood, diluted blood, semen, saliva, and skin samples with swab collection of either RNase-free water, ethanol or a commercial stabilizing solution. During DNA retrieval, water outperformed ethanol in both whole blood and semen stains. RNAlater also outperformed ethanol in the semen stains. In skin samples, the swabs treated with ethanol yielded more DNA than the ones treated with water, and RNAlater also outperformed water. In contrast, RNA retrieval was best with RNAlater. The profiling success of DNA was highest in ethanol (99.6%), followed by RNAlater (98.7%), and water (71%). RNA profiling success was highest in ethanol and RNAlater (100%) from whole blood, and highest in RNAlater from saliva [87.5% (50% ethanol, 37.5% water)], with similar findings in the other sample types (Lacerenza *et al.* 2022).

The effect on storage found by Lacerenza *et al.* (2022) suggested that saliva RNA was best retrieved after one day, while semen RNA retrieved was best after seven days of storage. This phenomenon was not fully discussed; however, the high microbial activity of saliva samples may be the cause for a decrease in RNA retrieval. Overall, it is suggested that DNA outperformed RNA during simultaneous extraction. Despite these results based on storage and collection methods, Fattorini *et al.* (2020) evaluated amplifiable products after induced depurination due to hydrolysis in both DNA and RNA samples by evaluating the copies of the GADPH target gene in a pooled sample of blood from 42 volunteers. The DI of the pooled sample was 5.6. There were 286 copies of the GAPDH target per ng of DNA while there were 5,822 copies of the GAPDH target per ng in RNA. The AU% was also higher in RNA samples and became up to 3x higher by 24 h of incubation. After 36 h, 0.8% of the RNA samples were amplifiable, while none of the DNA samples were amplifiable. These results suggest that RNA may provide better results than DNA after prolonged incubation at 70 °C when targeting GAPDH. (Fattorini *et al.* 2020). Overall, the success of sequencing is suggested to depend on the marker and samples.

Mitochondrial DNA is about 16.5 kb long and is extrachromosomal and circular (Robertson 1999). The circular structure of mitochondrial DNA makes it less susceptible to exonuclease degradation (Foran 2006). Mitochondrial DNA is made up of a coding sequence (that codes for rRNA, tRNA, and 13 proteins) and a noncoding sequence (1,123bp). The two strands are the H chain, which is purine-rich, and the L chain, which is pyrimidine-rich. It is also monoclonal, except for the few individuals who have heteroplasmy (Robertson 1999). Mitochondrial DNA is also maternally inherited and does not go through recombination. Because it does not go through recombination, the genome is more susceptible to spontaneous mutations and has a lack of repair mechanisms. All mitochondrial DNA sequences are compared with the Anderson sequence, with differences being referred to as “polymorphisms with respect to the Anderson sequence.” Most polymorphisms are in hypervariable region 1 (16,024-16,365), and hypervariable region 2 (73-340) (Robertson 1999).

With hair samples, the analysis of nuclear DNA is only available if the follicle is available. The follicle contains sheath cells, which enclose the follicular root tag and is a source of polymorphic proteins. Most hair encountered at a crime scene, however, does not have the sheath/follicle. As of 1999, DNA typing became more sensitive than protein markers, which allows for the amplification of DNA by PCR. Telogen phase hair, however, has very little nuclear DNA that can be detected even with PCR, but it has over 5K copies of mitochondrial DNA. To decide if mitochondrial DNA or nuclear DNA should be extracted, the DNA should be quantified on a slot blot (Robertson 1999). If the slot blot shows sufficient DNA, then nuclear DNA is typed, whereas if the nuclear DNA is insufficient, then mitochondrial DNA is typed.

Foran (2006) compared the degradation of whole or homogenized brain, liver, and muscle tissue at various temperatures (-20 °C through 37 °C) that had been extracted with one of three primers - Cytochrome b (mitochondrial), Myelin Basic Protein (single copy gene), and 28S (rDNA multicopy gene). In the whole tissue samples, the Cytochrome b products degraded the slowest on average, with 28S degrading the fastest. This is concordant with the expectation of the outcome that mitochondrial DNA should degrade at a slower rate than nuclear DNA due to its structure. Once homogenized, however, the reverse was true. This was shocking because their results suggested that once the cellular components are homogenized and nuclear and mitochondrial DNA occupy the same space, the mitochondrial DNA degrades the fastest. One theory of why this may occur is that homogenization removes the nuclear DNA from an environment with multiple polymerases with exonuclease activity, however, the reason for mitochondrial DNA degrading at such an accelerated rate is not explained by this theory (Foran 2006).

**Mass Disasters and Mixed Samples**

During mass disaster situations, many samples must be processed and profiled. The challenges to profiling these samples are the sheer number of victims, how decomposed/destructed the body is, the rate of DNA degradation, accessibility to the body, and the type of DNA available for reference (Alonso *et al.* 2005). When recovering samples from victims, it is advantageous to collect numerous samples from each body, as this allows the association of body parts to the body and aids in future analysis in case of any doubt in identification. These samples are recommended to be taken with photo documentation and from areas of least-affected material. Where allowed, it is preferential to obtain soft tissue samples since soft tissue is easiest to type, but this may be compromised by the comingling of remains in disasters (Alonso *et al.* 2005).

During comingling of DNA, it may be hard to determine which alleles belong to which subject. To do so, the sample should be processed as usual, but it is crucial to examine the peaks that result from the analysis. For two-person mixtures (which is the most common mixture in forensic casework), the number of peaks can reach up to four (presuming both individuals are heterozygotes with non-matching alleles, any more will indicate a more complex mixture) (Butler 2005). Kim *et al.* (2019) examined a two-person mixture sample from a placenta found on a discarded male newborn in Seoul, South Korea. Under normal circumstances, the maternal genotype can be obtained by isolation of the decidua, but this is no longer applicable if the placenta is degraded. Since the victim was abandoned in water, the maternal components outside of the placenta were washed away, leaving the mixed genotype in the placenta as the only evidence. This means that the fetal cells were a source of contamination for determining the maternal DNA.

The fetal cells from the victim’s body were used as a reference sample for the mixed profile generated from the placenta. To purge the sample of the fetal DNA, the mixed sample was pelleted and lysed three separate times. The results showed that the first lysate removed significantly degraded DNA with a DI average of 15.4. The second lysate had a high male DNA quantity and a low Male (M): Female (F) ratio while the third lysate had a low male quantity and a high M:F ratio, which generated a better maternal profile despite somewhat damaging the maternal cells. This shows that the damaged cells were more susceptible to lysis. Each placental piece also varied wildly in degradation, presumably because of their different proximities to the fetus, with the closest pieces being the most significantly degraded (Kim *et al.* 2019).

To corroborate the identification of the victim, reference samples can be taken either directly (such as antemortem comparison from used razors, toothbrushes, bloodstain cards, etc.) or through kinship analysis. If a direct reference is used, it should always be confirmed through kinship analysis. During DNA analysis, multiplex PCR amplification is preferred because it can easily evaluate direct matches with high discriminatory power. Mini-STR multiplexes are particularly useful if there is evidence of DNA degradation in the samples, and SNP typing uses even smaller amplicons to type DNA that STR data may not have pulled a profile from (Alonso *et al.* 2005). There are also automated systems such as Bode Technology that can process up to 250 bone samples per day. If less than 15 STR loci are considered, a large number of false positives may be considered, which decreases the significance of the matches. The number of fortuitous hits will decrease the more loci are considered, so the better the reference sample, the more significant the match may be. However, if the DNA is significantly degraded, it is likely that only partial profiles may be determined, which further complicates the number of false positive matches, especially in mass disaster situations (Alonso *et al.* 2005).

In the aftermath of the World Trade Center, the initial recovery period lasted 9 months. During this time, the remains were exposed to UV radiation, humidity, moisture, heat, fire, and mold, and many remains were left with only skeletal elements (Mundorff *et al.* 2009). Of these elements, studies suggest that the clavicle is better than the rib bone for DNA preservation, the long bones are preferred to the skull and rib bones, the weight-bearing bones are preferred to the non-weight-bearing bones, and the compact bones are preferred to the cancellous bones (Mundorff *et al.* 2009). As such, DNA typing success varies wildly between skeletal elements.

Mundorff *et al.* (2009) grouped samples from the ‘World Trade Center Human Remains Database’s Resampled DNA database’ and the ‘Entire sample database’ into four groups: head, trunk, lower limbs, and upper limbs. The head group comprised of the skull, maxilla, and mandible; and the trunk group was comprised of the clavicle, scapula, rib, sternum, vertebra, sacrum, and innominate. Whereas the upper limb group was comprised of the humerus, radius, ulna, carpals, metacarpals, and hand phalanx; and the lower limb group was comprised of the femur, patella, tibia, fibula, tarsals, metatarsals, and foot phalanx. On average by body part group, the lower limbs were 69% identified, the trunk was 63% identified, the upper limbs were 54% identified, and the head was 49% identified. This suggests that the lower limbs have a higher DNA identification rate on average, presumably because they are compact and weight-bearing (Mundorff *et al.* 2009).

**Tissue Comparison**

The tissue choice is crucial to obtain suitable DNA for analysis. DNA is easier to extract from soft tissue such as organs and muscles in comparison to bone and teeth samples. This is because the dentin, enamel, and cementum on teeth and the carbonated hydroxyapatite and collagen in bone promote DNA preservation (Heathfield *et al.* 2021; Raffone *et al.* 2021). As such, hard tissue is only used once the soft tissue has gone through severe degradation. Thakar *et al.* (2019) showed the difference in typing hard and soft tissues by comparing the success rate and analysis of brain, prostate, and teeth samples from 20 decomposed corpses with a postmortem interval of approximately 25 d. The brain and teeth yielded full profiles with the exception of two non-amplifiable brain samples. The prostate samples all yielded partial profiles, with 6 samples amplifying more than 15 out of 21 loci, and the remaining 14 samples having multiple dropouts. The peak height ratio is indicative of profile quality, as it compares the peak heights in a heterozygotic pair with the standard peak/height ratio being around 0.7. The brain had a peak height ratio of 0.62 - 0.92 and the teeth had a peak height ratio of 0.72 - 0.85, indicating that both peak height ratios were near to the normative range with some outliers that may indicate lower profile quality (Thakar *et al.* 2019). Since the prostate samples only showed partial profiles, their peak heights were not calculated.

The DNA retrieval will also vary wildly between soft tissue types, as shown by the collection of 81 samples with varying DI values by van den Berge *et al.* (2016). Of these samples, 14% had no degradation, 56% had moderate degradation, 9% had severe degradation, and 22% dropped out (indicating severe degradation, 41% of these were skeletal muscle). Based on averages, the brain, heart, and lungs showed the least degradation, whereas the skin showed the most degradation (van den Berge *et al.* 2016). Helm *et al.* (2021) had similar results, with the STR profiling success being 99.5% for the aortic wall, 99.3% for the urinary bladder wall, 95.5% for brain tissue, 96.8% for liver tissue, 89.8% for the skeletal muscle tissue, and 74.2% for oral tissue. Evaluation of the Relative Fluorescent Unit (RFU) values showed that the liver samples had the highest degradation while the aortic tissue had the least degradation. RFUs are the level of fluorescence obtained by binding a fluorescent protein to the DNA to indicate quantity at a certain locus. The oral samples had the second highest degradation values, though this is likely due to microbial contamination, insect contamination, and liquid decomposition products that accumulate in the mouth (Helm *et al.* 2021). As such, buccal and saliva samples (though cost-effective and simple to analyze) are not considered as effective as other liquid samples such as blood.

The effectiveness of DNA typing blood samples directly depends on quality because of the components of red and white blood cells (Kaur *et al.* 2020). Red blood cells have no nucleus and therefore have no DNA. Despite this, they contain hemoglobin, which can be used in testing for the presence of blood. If the sample is poorly preserved, the blood antigens can change structure and hemoglobin can turn into hemichrome. Conversely, white blood cells contain a nucleus, which is what DNA analysis depends on. Thirty-eight samples were evaluated and classified based on their level of blood preservation. Seven of the samples were categorized as “best preserved,” were reddish brown in color, and were dried before they were placed in a paper envelope for storage. Seven moderately preserved samples were dark brown and semi-dried before they were stored in a paper envelope. Fourteen were poorly preserved and yellow/greenish in color and were wet when they were stored in a small container. Finally, the worst preserved samples (10) were a greenish/blackish color and were wet when stored in a small plastic container. Two of these worst preserved samples had maggots hatch in the container. The results as found by Kaur *et al.* (2020) showed that all samples generated adequate DNA profiles, including the worst-preserved samples but, the best-preserved ones, had the highest quality and quantity of DNA.

Reid *et al.* (2019) compared blood and buccal sample analysis of adults and infants with varying postmortem intervals at the Salt River Mortuary in South Africa. Though the postmortem intervals were not statistically significant, as an 887-day-old sample produced a full profile, the age of the deceased and the type of samples were significant. All the blood samples yielded full profiles with a DI average of 0.804 ± 0.34, indicating nondegraded DNA. In comparison, the buccal samples had an average DI of 6.35 ± 8.45, indicating moderately to severely degraded DNA. The adult buccal swabs showed 64.86% full profiles, 21.62% partial profiles, and 13.51% failed profiles. The infant buccal swabs had no failed profiles, of which the samples had 84.21% full profiles, and partial profiles made up the remaining 15.79% (Reid *et al.* 2019). Though Kulstein and Wiegand (2018) examined how well blood and saliva samples held up after washing on specific fabrics, their averages from STR profiling showed a similar trend to Reid *et al.* (2019) results. The STR profiles were 91.1% successful from blood samples, whereas the saliva samples were only 52.1% successful (Kulstein & Wiegand 2018).

##### **Environmental Effects**

The preservation of DNA exposed to the environment is reliant on temperature, moisture, pH, organic matter, microbes, and groundwater (Raffone *et al.* 2021). The higher the temperature, the slower the hydrolysis is, which can preserve RNA, however, this also increases the likelihood of the chemical degradation of collagen, which harms DNA (Raffone *et al.* 2021). Humidity also has a dissolving action, which can penetrate through organic substances, leaving holes and increasing the likelihood of inhibition. The likelihood of nucleic acids going through hydrolysis increases with high water content/humidity (Alaeddini *et al.* 2011). This can take the form of the breakage of apurinic sites, or cleavage of pyrimidine-glycosyl bonds, which separates the pyrimidines from the sugar-phosphate backbone (Alaeddini *et al.* 2011). The outcome of the profiles of samples exposed to high humidity depends on the sample type and quantity. Sirker *et al.* (2016) found that a 5 μl blood sample can produce a detectable profile after 71 weeks, whereas a 0.05 μl sample can only produce a detectable profile after 33 - 34 weeks. In comparison, a 5 μl semen sample can produce a detectable profile after 20 weeks, whereas a 0.05 μl sample can only produce a detectable profile after 8 weeks, and a 5 μl saliva sample can only produce a weak but detectable profile after 8 weeks (Sirker *et al.* 2016).

Alternatively, dry storage, ambient storage, and frozen storage are much more conducive to nucleic acid preservation. Sirker *et al.* (2016) found that in dry storage both blood and semen samples produced a detectable profile after 71 weeks even at concentrations of 0.05 μl, whereas saliva produced a detectable profile after 71 weeks at a concentration of 5 μl, with the 0.05 μl sample being unreliable but detectable. Ambient storage is described by Alaeddini *et al.* (2011) as storage that is at room temperature with little to moderate humidity. Alaeddini *et al.* (2010) found that when examining bone DNA survival, ambient and frozen (-70 °C) samples resisted severe DNA depletion. Both environments showed a consistent DNA intensity and neither had evidence of microbial contamination.

In acidic conditions, bone preservation decreases as the minerals dissolve, contrarily, if the conditions are neutral or slightly alkaline, the bone and DNA preservation increase (Raffone *et al.* 2021). With organic matter, decomposition produces acidic compounds in the soil such as acetic, formic, oxalic, humic, and fulvic acids, which are all strong PCR inhibitors. Organic matter (such as plant leaves) can also leave behind porphyrin residues, which can also inhibit PCR, and nitrogen components in the soil can also increase mineral dissolution, which reduces the protective capabilities of the bones. Microbes can cause bioerosion, which can cause extensive damage to collagen. Bacteria and fungi can also be sources of contamination, leading to acid production and accelerated decay (Raffone *et al.* 2021).

The effects of groundwater on bone preservation depend on whether the groundwater has restricted water table movement or continuous wet/dry cycles (Raffone *et al.* 2021). Restricted movement means that the water table is either waterlogged (such as a marshland), or the soil is not water dense. This environment is often rich in calcium and phosphorus, which promote bone preservation. Cyclic water tables are associated with the upper layer of soil being rich in oxygen and food, which increases the level of bioactivity. This cycling and high bioactivity often form large pores in bones, which accelerates DNA degradation and decomposition. The lower layer of soil, however, has reduced oxygen, and therefore less activity and slowed decomposition as it is below the body of water. This continuous cycling, however, negatively impacts bone and DNA survival (Raffone *et al.* 2021).

In shallow burial sites, the surface of the site is likely to have soil depressions, variation in plant growth and composition, as well as intermixing of the soil layers that may prove suboptimal for DNA preservation by increasing the ease of which the remains can be accessed by animals, plants, and microbes. Areas that are cold or arid with permafrost and with neutral to alkaline pH soil are optimal for DNA preservation. In contrast, hot and mid areas with acidic pH soil show the worst rates of preservation by increasing the activity of DNases (Thomas *et al.* 2019). As an example of shallow burial, Alaeddini *et al.* (2010) shallowly buried rib bone samples at a depth of 10 cm with a PMI ranging from 103 - 445 d and performed a Southern Hybridization Blot procedure on the samples with a focus on the Alu sequence to determine the DNA origin. The Alu sequence was chosen because of its specificity to primates and the high copy number of the sequence. The Southern Blot showed that there was severe depletion in human DNA in the buried samples when compared to the controls. The blot also showed that there was a high quantity of non-human contaminants in the samples, which was expected given the microbial activity below ground (Alaeddini *et al.* 2010).

**Temperature**

Pyrolysis is the rapid molecular degeneration during heat changes and will often result in difficult-to-process or read profiles (Emery *et al*. 2020). This is because, under high heat changes, only the bone and teeth remain, which are notoriously more difficult than soft tissue to extract DNA from (Emery *et al*. 2020). The DNA in teeth is particularly well protected because it is surrounded by dentin, enamel, and cementum (Lozano-Peral *et al*. 2021). The DNA quantity and quality will also depend on the level of heat damage present on the samples/remains.

Emery *et al.* (2020) took 51 samples of radii, cranial bones (parietal and temporal), phalanges, metacarpals/tarsals, tibiae, and first premolars. Each sample was categorized according to how burnt they were. Eight samples were labeled as Category 1, which is characterized by white/yellow bones that have been heated at temperatures <200 °C. Nine samples were labeled as Category 2 which is characterized by yellow/brown bones that have been heated at temperatures 200 - 300 °C. Ten samples were labeled as Category 3 which is characterized by carbonized/black bones that have been heated at temperatures 300 - 350 °C. Six samples were labeled as Category 4 which is characterized by grey/blue bones that have been heated at temperatures 550 - 600 °C. Seven samples were labeled as Category 5 which is characterized by calcined bones/white ash that have been heated at temperatures >650 °C.

The average DNA retrieval varied depending on the retrieval process. Emery *et al.* (2020) used either the Dabney aDNA extraction process or the Loreille demineralization extraction process. The average degradation index values for the Dabney protocol varied by category from 2.01 - 4.62, surprisingly with the highest value of degradation being for Category 3. The average degradation index values for the Loreille protocol varied by category from 1.78 - 9.45, with the highest average being from Category 4. The average DNA yield of the aDNA procedure in order by Categories 1-5 was as follows: 5.98 ng μl-1, 2.4 ng μl-1, 0.27 ng μl-1, 0.018 ng μl-1, and 0.0003 ng μl-1, respectively. The average DNA yield of the demineralized procedure in order by Categories 1-5 was as follows: 4.66 ng μl-1, 6.27 ng μl-1, 0.37 ng μl-1, 0.016 ng μl-1, and 1.14x105 ng μl-1, respectively. The profiles generated as per the Dabney protocol were 87.5% full profiles for Category 1, 88.9% full profiles for Category 2, 80% full profiles for Category 3, 16.7% full profiles, and 16.7% partial profiles for Category 4, and 28.6% partial profiles for Category 5. The profiles generated as per the Loreille protocol were all 8 full profiles for Category 1, 77.8% full profiles for Category 2, 80% full profiles for Category 3, 16.7% full profiles for Category 4, and no profiles for Category 5. This shows that both extraction processes were comparable by burn category (Emery *et al.* 2020).

The three bone types with the highest DNA yield for the Dabney protocol were the metatarsals, metacarpals, and phalanges, whereas, with the Loreille protocol, the highest DNA yield came from the parietal bone, the metatarsals, and the tibia. For both protocols, the premolar, radii, and temporal samples produced the lowest DNA yield. Interestingly, although the phalanges were one of the highest DNA yields for the Dabney protocol, they also had the highest degradation value at 15.6. Emery *et al.* (2020) suspected that the reason behind this may have been because of the differential preservation during exposure to the high temperatures, where the phalanges may have been the most exposed.

In comparison, Lozano-Peral (2021) examined 40 samples total of molars and premolars at temperatures of 100 °C, 200 °C, or 400 °C; these samples then went through either a Quantifiler® or Qubit™ detection. At 100 °C, the Quantifiler® detected DNA 100% of the time, and Qubit™ detected DNA 60% of the time. At 200 °C, the Quantifiler® detected DNA 80% of the time, and Qubit™ detected DNA 30% of the time. At 400 °C, the Quantifiler® detected DNA 20% of the time, and Qubit™ detected DNA 0% of the time. These findings are concordant with the findings by Emery *et al.* (2020) because DNA typing became significantly difficult once the temperatures reached above 300 °C. Though the DNA was detected at higher temperatures, only 9.38% of the loci were able to be amplified after exposure to 200 °C, and only 4.38% of the loci were ample to be amplified after exposure to 400 °C (Lozano-Peral *et al.* 2021).

Though the aforementioned studies were focused on high-temperature exposure, DNA often goes through multiple freeze-thaw cycles during collection, handling, and storage, which can lead to a decrease in the molecular weight of DNA samples (Shao *et al.* 2012). The researchers used whole blood samples and put them through one of three protocols: Protocol A cycled the temperature to -70 °C gradually, Protocol B immediately cycled to -70 °C, Protocol C cycled to -20 °C, and Protocol D was the control. Protocol D showed no changes in DNA size, which showed the effectiveness of the control group. Protocols A, B, and C all showed progressive degradation, with Protocols A and B being of similar molecular weight and Protocol C with a slightly slower reduction in molecular weight (Shao *et al.* 2012).

Though freezing DNA is commonly used to preserve the sample, Fattorini *et al.* (2023) found that after extended periods of storage at -80 °C, DNA degradation was not blocked. Fattorini *et al.* (2023) collected samples from either mitochondrial DNA data obtained in 2012 or from a sample stored in 2012 and extracted in 2022. The sample that was stored at -80 °C for 10 years had two times less mitochondrial DNA copies per gram of tissue than the sample that was processed immediately after it was obtained in 2012. The long target during qPCR was also not obtained in the extended storage sample, which is indicative of DNA degradation (Fattorini *et al.* 2023). Otherwise, samples obtained from cold environments show a trend of DNA preservation that is superior to samples obtained from hot environments (Thomas *et al.* 2019).

##### **UVRadiation**

The length of time exposed and the type of UV radiation exposure can impact the quantity and quality of the DNA extracted from a sample. UVA (365 nm) is the type of UV radiation biotic organisms are most exposed to. Despite this, it is the least damaging of all the types, although it can produce free oxygen radicals (Rahi *et al.* 2021). UVB (302 nm) is known for its negative effects on living organisms, as some but not all this radiation is absorbed by the ozone layer. UVB causes damage by crosslinking covalent bonds either on the same strand (intrastrand) or on the opposite strand (interstrand). UVB can also crosslink DNA and proteins. Despite its total absorption by ozone, UVC (254 nm) is the most damaging since it causes rapid DNA degradation. UVC exposure is also associated with mutations and the formation of pyrimidine dimers (Rahi *et al.* 2021).

Research conducted by Tie *et al.* (2021) showed that with cyclical exposure to UV radiation (16 h in light and 3 h in darkness repeated 75 times), long assay amplification from 20 donors’ whole blood samples became difficult after 15 cycles. By 75 cycles the short assay amplification became difficult and the long assay became unobtainable. Depending on the kits used for detection, the STR profiles obtained had variable success. The AmpFlSTR Identifiler Plus™ PCR Amplification kit only showed partial DNA profiles past 45 cycles, and by 75 cycles, only 3 out of 21 loci were detectible. In comparison, the GLB PCR Amplification kit was able to detect 5 out of 21 loci by the 75th cycle. Tie *et al.* (2021) also studied SNP detection at dilutions of 0.02 ng - 0.14 ng of the sample. By the 75th cycle, all 12 loci were detectable.

Rahi *et al.* (2021) also investigated the effect of UV radiation on blood samples, though this time the samples were obtained from a single donor. Radiation exposure was done in 20-minintervals for a total of 120 min. The natural solar exposed samples and the UVA samples showed no noticeable DNA damage. The UVC samples showed the complete degradation of the HMW markers and partial degradation of MMW at 20 - 40 min. The MMW markers showed total degradation by 60 min, while the LMW markers remained unaffected. Because of the loss of HMW and MMW markers, the DNA was unsuitable for a full STR profile. UVB showed complete degradation of HMW markers at 80 min, while the MMW and LMW markers both showed partial degradation after 100 min. These partially degraded samples were unsuitable profiles (Rahi *et al.* 2021).

Gršković *et al.* (2013) examined the effects of UVC radiation on naked DNA, blood, saliva, and semen. The authors split their study up into examining UVC radiation of the aforementioned substances as either diluted or undiluted, as well as, done on surfaces with or without decontamination. Their results showed that the naked DNA was unable to generate a profile after 2 min of UVC exposure, no matter the circumstances. Similarly, all the diluted bodily fluid samples resulted in the inability to generate a profile after 1 h. With undiluted blood, a partial profile was obtained after 8 h of exposure on a decontaminated surface; but a full profile was obtained after 90 h of exposure on a contaminated. The undiluted semen samples followed a similar trend with obtaining a partial profile after 8 h of exposure on a decontaminated surface, a full profile after 42 h of exposure on a surface without decontamination, and a partial profile after 90 h of exposure on a surface without decontamination. Gršković *et al.* (2013) continue their analysis of undiluted saliva samples fared worse than the undiluted blood or semen samples. A partial profile was able to be obtained on a decontaminated surface after 1 h, but the profile was completely lost after 4 h. On a surface that was contaminated, however, a full profile was obtained after 1 h of exposure, and a partial profile was able to be obtained after 42 h.

**Ballistics**

DNA found on casings is thought to go through complete degradation once firing. This is because in the chamber, temperatures can reach upwards of 1800 °C, and there is extraordinarily high pressure. Additionally, PCR can be inhibited by the metal ions found on the cartridge cases or gunshot residue. There is limited data on the profiling of casings, although casings are common forensic evidence (Radojicic *et al.* 2018). This may in part be because the DNA recovered from casings is based on touch DNA, which is still not wholly understood. Residual touch DNA comes from the top layer of skin, known as the “horny layer”, or stratum corneum (Winnepenninckx *et al.* 2022). The keratinocytes from this layer are flat cells and often result in a partial or fully degraded nucleus once shed, therefore, they have low DNA content to start with, even before going through a gun barrel (Winnepenninckx *et al.* 2022). Despite these results, multiple other studies have shown that profiles can still be recovered.

Because of the low quantities of DNA, traditional swabbing methods, such as the rinse and swab method, have been reported to have varying levels of success (Prasad *et al.* 2021). An alternative method for collecting DNA involves soaking the cartridges in ATL buffer and incubating them for a variable amount of time depending on the specific procedure (Radojicic *et al.* 2018; Prasad *et al.* 2021). Despite some reservations about typical swabbing methods, Radojicic *et al.* (2018) found that 55 out of 399 (13.78%) of the soaked cartridges successfully produced interpretable profiles, while 49 out of 269 (18.22%) of the swabbed cartridges successfully produced interpretable profiles. This study suggests that in certain circumstances, the swabbing method, though old, should not be overlooked in forensic techniques as it can still be effective in comparison to the soaking technique.

In comparing DNA recovered from fired vs from unfired cartridges, Winnepenninckx *et al.* (2022) showed that the average DNA yield 24 h after handling was 0.022 ng ± 0.025 ng fired and 0.078 ng ± 0.074 ng unfired. The success rate of profiling the fired cartridges was 40.4% ± 26.06%, while the success rate for the unfired cartridges was 73.9% ± 25.5%. The DNA yield a week after handling was 0.009 ng ± 0.01 ng fired and 0.035 ng ± 0.039 ng unfired, with the success rates being 21.9% ± 21.1% and 49.4% ± 32.8%, respectively (Winnepenninckx *et al.* 2022). Prasad *et al.* (2021) showed a similar trend between fired and unfired, but the exact percentages depended on the metal that the casing was made from. Of the usable profiles, the brass had 75% success unfired, and 25% success once fired; whereas nickel had 100% success unfired, and 100% usable (but partial) profiles once fired. The unfired nickel showed the least degradation with the DI ranging from 0 - 10, but the fired brass showed the most degradation, with the DI reaching higher than 10, indicating severe degradation. This could be due to the indication of free copper ions shown by the ATL buffer changing color to blue. Free copper ions are known for destabilizing the DNA structure, as well as being a strong profiling inhibitor (Prasad *et al.* 2021).

##### **Case Studies and Legal Use**

The first conviction using DNA evidence that was upheld by a higher court was *Andrews v. State* in 1987. His DNA was evaluated by the Lifecodes laboratory, which found that his semen matched the DNA found in the rape kit and estimated that there was a 1 in 1.0x1010 chance otherwise, which led to his 22-year sentence (Newton 2008). Two years later in 1989, *State v. Woodall* was the first case affirmed by the highest state court, further showing the rapid progression of DNA evidence being accepted by the law. However, this near-blind acceptance of DNA evidence had its downfall due to the *People v. Castro* case. In *People v. Castro*, Lifecodes estimated that there was a 1 in 1.0x108 chance of another match, but the defense brought the validity of the DNA evidence into question. The defense did so because the evidence did not qualify under the scientific evidence criteria. The scientific evidence criteria include if the expert’s theory/technique can be challenged if the theory/technique has been peer-reviewed, the potential error rate, if there are standards/controls for comparison, and if this theory/technique is widely agreed upon in the scientific community. Additionally, Lifecodes had made a huge technical error, the probability was not as high as they made it out to be, especially because the blood found on the suspect’s watch did not match the victim’s DNA at all, which led to the first exclusion of DNA evidence in law. Following this debacle*, United States v. Jakobetz in 1992* was the first case where the DNA evidence met all the scientific criteria (Newton 2008).

A case discussed by Pilli *et al.* (2016) entailed a deeply lice-infested elderly woman who was admitted to the hospital and died soon thereafter. Because of the extensive nature of the infestation, a house call was required to determine if the cause of death was neglect. Lice were found in both the victim’s room and the guest’s room. The victim’s caretakers claimed that they had stayed in the guest room, so 30 lice from the guest room were evaluated to determine their stomach contents. For genetic analysis, all appendages were removed from the insects, and 20 were sectioned to leak their GI material while the others were crushed. Of the sectioned insects, half had dark abdominal staining (which were positive for Hb), and the other half had transparent abdomens (which were negative for Hb). No human DNA was obtained from the non-abdominal stained or the non-sectioned insects (Pilli *et al.* 2016).

There were only autosomal markers detected, which indicated female DNA. DI<2 indicated little to no degradation (Pilli *et al.* 2016). Only partial profiles were obtained with traditional STR methods, and so NGS techniques were used. When using NGS, the reference sample showed no degradation and generated a nearly full profile, save for 2 loci with one allelic drop-in, and the replicate only had a single nucleotide dropout. The results of the profile showed that the reference sample and the two tested samples had complete overlap, indicating that the DNA found in the lice was only from the victim, with no evidence of the presence of another person (Pilli *et al.* 2016).

Besides the use of DNA profiling for crime-related casework, it is also often used for the identification of unknown victims of disasters. One such example is the frozen left forearm found at the 1948 Flight 4422 impact sight in 1999. At the time of its discovery, the forearm did not initially go through DNA profiling, with the ones who discovered it opting to attempt to obtain a fingerprint match (Loreille *et al.* 2010). Due to the poor quality of the sample and references, however, this attempt at identification failed and the forearm was then embalmed. In 2002 there was an attempt to STR type the arm, but the profiles failed due to the volume of formaldehyde which had degraded the DNA. Further attempts were put off until the attempt at identification of the remains by Loreille *et al.* (2010). They examined data collected from the sample using two techniques from two different laboratories: Simon Frasier University/SFU (an ancient DNA lab, which forgoes the decalcification process), and the Armed Forces DNA Identification Laboratory/AFDIL (which put the sample through the decalcification process).

The references were compiled with saliva and blood collection from the 24 maternal relatives of missing seamen and 6 maternal relatives of missing crew members. These references were extracted via the QIAamp® minikit (Loreille *et al.* 2010). Additionally, only 2 paternal relatives were found and tested in the same manner. The maternal relatives accounted for 19 victims, the paternal relatives accounted for 2 victims, and the fingerprint cards accounted for 16 victims. Of which there was one overlap between maternal and paternal relatives, and 6 overlaps between the genetic relatives and the fingerprint cards, accounting for all 30 victims in total. AFDIL and SFU compiled 697 bp and 681 bp mitochondrial DNA profiles respectively, of which they overlapped, which showed the validity of both techniques. These profiles matched the maternal relative of one victim, with all other possible matches differing at 5 or more positions. The two potential paternal references showed one reference differed at 10 loci from the remains, while the second was consistent with the remains. The Y haplogroup was found to be rare as well and had not previously been observed (Loreille *et al.* 2010).

Though the DNA analysis only pointed towards one victim of the impact, Loreille *et al.* (2010) also rehydrated the fingerprints and examined them in comparison to the fingerprint cards. The fingerprint analysis of obvious visual dissimilarities (such as fingerprint type as well as unique characteristics) narrowed the potential match pool down to two victims, of which the fingerprints were run through a program to further conclude whom the arm belonged to. The arm was therefore positively identified as Francis Joseph con Zandt by mitochondrial DNA analysis, and Y-STR analysis and was affirmed through fingerprint analysis (Loreille *et al.* 2010).

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