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A Nondestructive Procedure for the Extraction of DNA from Human Teeth

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A NONDESTRUCTIVE PROCEDURE FOR THE EXTRACTION OF DNA FROM HUMAN TEETH

by
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ABSTRACT

This research focuses on constructing a procedure that allows for the nondestructive extraction of mtDNA from teeth for forensic DNA analysis. Currently, only a destructive method for extracting DNA from teeth is used in the forensic laboratory to identify an individual. A disadvantage of extracting DNA through a destructive method is the complete or partial loss of the tooth. To extract DNA through a destructive method, the tooth must either be cut horizontally or vertically to access the pulp, or the root ground into a powder to extract DNA from the dentine-cementum. With the destruction of the tooth, additional forensic or structural information that could have been retrieved from the same tooth is lost.

A nondestructive method would preserve the tooth since no invasive procedures are needed to access its DNA. Instead, the tooth would be subjected to a series of buffers, including extraction, washing buffers 1 and 2, and elution buffers. The series of buffers will remove the DNA from the dentine and cementum of the tooth without damaging the tooth.

Three nondestructive method trials were conducted in the laboratory that yielded no detectable levels of DNA. Positive and negative controls were added to the nondestructive method to monitor each step of the procedure along with fresh reagents and PCR kits. Unfortunately, the results suggest that the DNA in the teeth samples might have already been degraded since the control electrophoresis data displayed the expected electrophoresis band at ~383 base pairs. Due to the Covid-19 pandemic, research progress for this procedure was halted abruptly before the procedure could be tested on intact DNA samples.

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LIST OF ABBREVIATIONS

bp	Base pairs
CODIS	Combined DNA Index System
CR	Coding region
DNA	Deoxyribonucleic acid
GuSCN	Guanidine thiocyanate
HPLC	High performance liquid chromatography
HOMO	Highest occupied molecular orbital
HV1	Hypervariable region 1
HV2	Hypervariable region 2
HV3	Hypervariable region 3
LUMO	Lowest unoccupied molecular orbital
mtDNA	Mitochondrial DNA
PCR	Polymerase chain reaction
RFLP	Restriction length fragment polymorphism
RNA	Ribonucleic acid
STR	Short tandem repeat
UV/Vis	Ultraviolet/visible

A NONDESTRUCTIVE PROCEDURE FOR THE EXTRACTION OF DNA FROM HUMAN TEETH

1. Introduction

1.1 History of DNA

Although James Watson and Francis Crick are historically credited with the 1953 discovery of the double helix structure of deoxyribonucleic acid (DNA), the road to the discovery of DNA began in 1866 with Gregor Mendel, the Father of Genetics. Mendel experimented with pea plants and shined light on the process of genetic inheritance. Mendel also coined the terms dominant and recessive. He noted 'invisible' factors that allowed for the prediction of traits, which we now know today as "genes"[1]

In 1869 Friedrich Miescher, while separating the components of a white blood cell, discovered a new molecule which he called nuclein[2]. He noted that the new molecule consisted of hydrogen, oxygen, nitrogen, and large phosphorus content[1]. Unbeknownst to him, he discovered the basis for all life – DNA.

After Miescher, Albrecht Kossel continued research on nucleic acids. In 1881 he managed to isolate and describe the five basic organic compounds of nuclein: adenine, cytosine, guanine, thymine, and uracil, which is found in RNA. With his discovery, he was awarded the Nobel Prize for Physiology or Medicine[2].

Several scientists expanded on Miescher's and Kossel's work, such as Theodor Boveri and Walter Sutton, who created the Boveri-Sutton chromosome theory. Their work became fundamental to the understanding of the chromosome. In 1902, Sir Archibald Edward Garrod published his findings of recessive inheritance of genes in humans. In 1944 Oswald Avery noted

that DNA is responsible for transforming cell properties. A few years after Avery's discovery, Erwin Chargaff discovered DNA's role in heredity. Using his research he created Chargaff's rules: (1) In DNA, the number of guanine and cytosine units are equal, and the number of adenine and thymine units are equal. (2) The composition of adenine, cytosine, guanine, and thymine differ between varying species[1]. In 1951 Rosalind Franklin, while working with X-ray crystallography, was able to photograph DNA. After studying her photographs, she hypothesized the helical structure of DNA. Finally, in 1953, James Watson and Francis Crick managed to solve the puzzle and confirmed the helical structure of DNA, and in 1962, they were awarded the Nobel Prize in Physiology or Medicine[1]. Thus, the discovery of DNA dates back over a hundred years and is not the work of only two scientists. Many brilliant minds came together, built off of each other's work, and laid the foundation for the future of DNA.

1.2 Nuclear DNA and Mitochondrial DNA

DNA is a helical structure found in the nucleus of cells composed of two strands called polynucleotides, which can be broken down to its simple monomeric form – a nucleotide. A single nucleotide is composed of three components: a deoxyribose sugar, a phosphate group, and one of four nitrogenous bases (adenine [A], cytosine [C], guanine [G], and thymine [T]).

Each nucleotide is connected by phosphor-diester linkages between the phosphate group of one nucleotide to the sugar of the other nucleotide, creating a sugar-phosphate backbone of the polynucleotide. The nitrogenous bases of one polynucleotide strand connects to their complementary base on a separate polynucleotide strand (A connects to T; C connects to G) via hydrogen bonds to construct the double-stranded helical structure of DNA. The size of DNA is

measured in base pairs (bp), and in humans, there are a total of 6 billion bp within our chromosomes[3]. **Figure 1** shows how the nitrogenous bases and separate nucleotides connect.

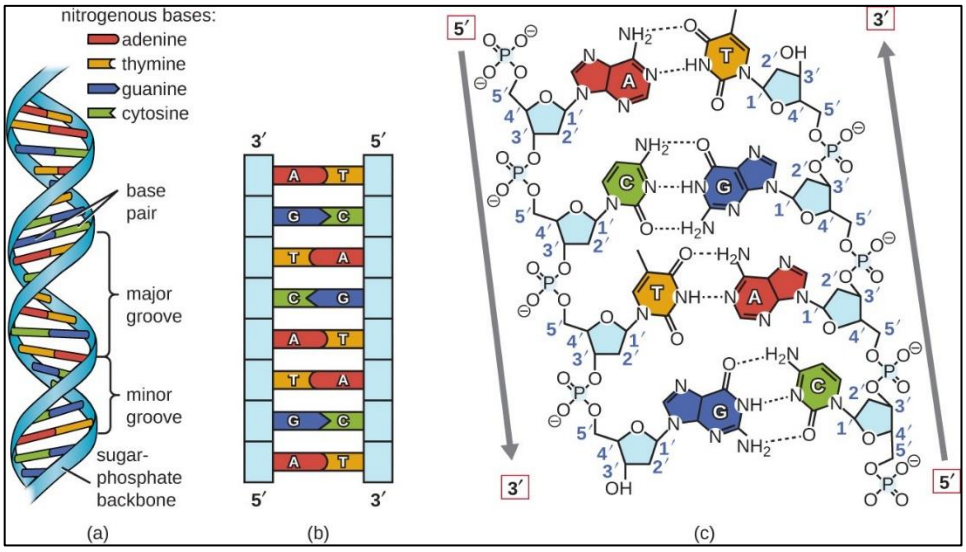

Figure 1. The structure of DNA and the bonds of the nitrogenous bases and sugar-phosphate backbone[4].

Mitochondrial DNA (mtDNA) is another source of genetic information. Each mitochondrion contains between 2 and 10 copies of mtDNA, and each cell can contain up to 1,000 mitochondria[5]. Mitochondrial DNA, unlike nuclear DNA, is arranged in a small circle called a plasmid, which contains 16,569 base pairs. mtDNA stores the genetic instructions for the production of thirteen proteins which comprise the oxidative phosphorylation complex of cellular respiration. While nuclear DNA is inherited from both parents, mtDNA is maternally inherited[6].

Along with nuclear DNA, mtDNA can also be used to identify individuals. With human remains, forensic laboratories can use mtDNA, especially in older skeletal remains, along with other evidence, to establish a connection. Due to the abundance of mitochondria in each cell, as well as the ability to match the mtDNA with a living relative, scientists believe that mtDNA is more suitable to identify older skeletal remains where nuclear DNA might be degraded or only present in small quantities[4]. While unique identification of individuals is not feasible due to

multiple individuals having the same mtDNA type, the information is still useful in cases where nuclear DNA analysis is unavailable[5]. In mtDNA, there are two regions with a high variation in sequence known as hypervariable region 1 (HV1, found between 16,024 bp and 16,365 bp) and hypervariable region 2 (HV2, found between 73 bp and 340 bp) that can be used to determine the relationship of the mtDNA to other evidence. Hypervariable region 3 (HV3, found between 438 bp and 574 bp) can be used to confirm identity when the HV1/HV2 regions are indistinguishable[8]. An image showing the mitochondrial genome can be seen in **Figure 2**.

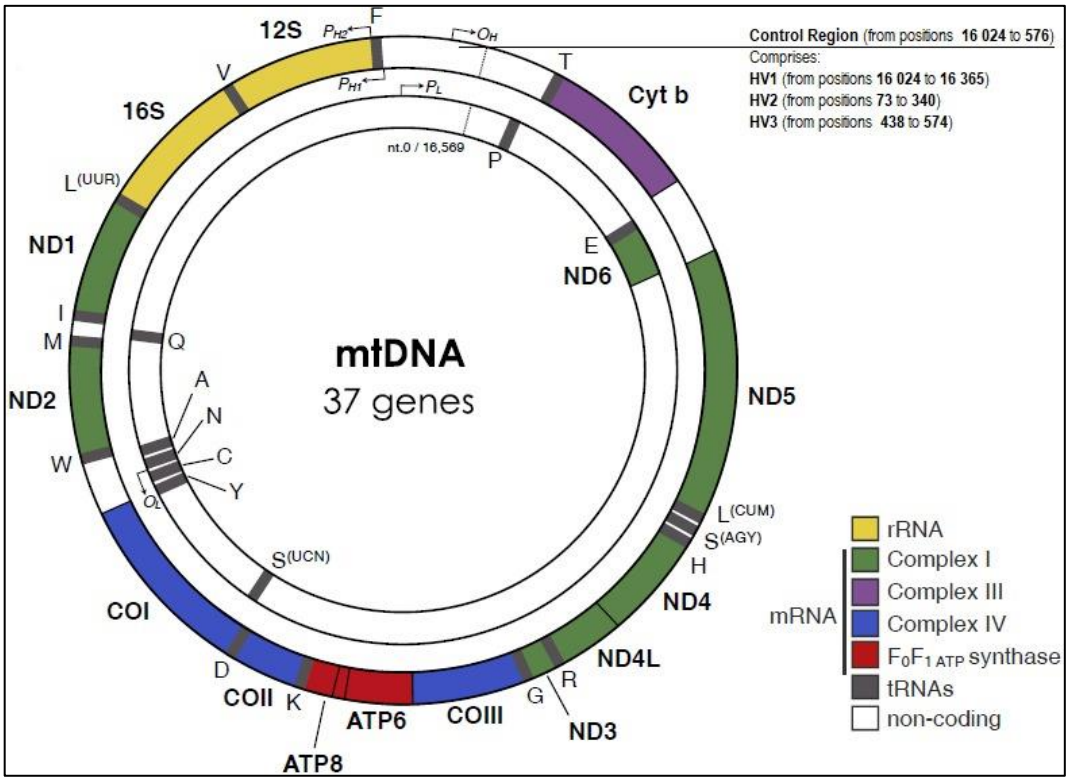

Figure 2. The human mitochondrial DNA genome with genes and control regions labeled[8].

The first instance of using mtDNA in human identification dates back to 1986. In October 1984 a 3-year old child went missing, and in March 1986, the remains of a human child were found in the desert. The forensic scientists were able to match the mtDNA of the remains to the mother, thus confirming the identity of the child[8].

1.3 Structure of the Tooth

In humans there are four types of teeth: incisors, canines, premolars, and molars; used for cutting, tearing, and crushing food, respectively. The tooth is divided into two major anatomical parts: the crown and the root. The crown of the tooth is usually covered by the enamel, and it is visible above the gums. The root is hidden within the gums and anchors the tooth to bone of the upper and lower jaw[9]. The structure of the tooth, along with its supporting structures, can be seen in **Figure 3**.

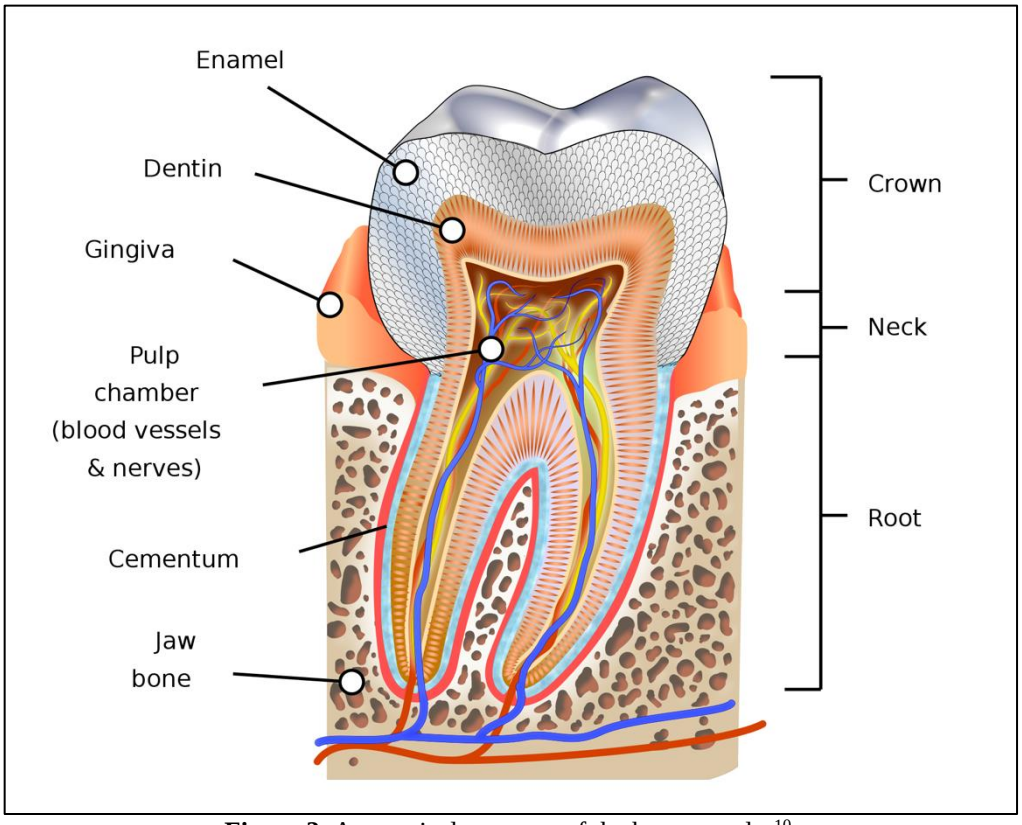

Figure 3. Anatomical structure of the human molar[10].

The enamel is the hardest substance in the human body and is visible covering the crown of the tooth. Mineral constitutes 96% of the enamel, with the remaining composed of water and organic material. The bulk of the tooth is dentin, which is found under the enamel and primarily

functions to support the enamel. Dentin is composed of 70% inorganic materials, 20% organic materials, and 10% water. Beneath the dentin is the pulp chamber, which houses the blood vessels and nerves of the tooth. The cementum is the protective structure that surrounds the root of the tooth, which also functions as the attachment point of periodontal ligaments to anchor the tooth. The cementum is composed of 45% inorganic material (mostly hydroxyapatite), 33% organic material, and 22% water[11].

Around the tooth are the supporting structures: periodontal ligaments, gingiva, and alveolar bone. The periodontal ligament is the tissue that connects the cementum to the alveolar bone and also functions as support for the tooth. The gingiva, also known as gums, cover the bones of the upper and lower jaw. Finally, the alveolar bone is the bone that forms around the tooth. Unlike normal bone, the alveolar bone can be broken down and rebuilt if pressure is applied to a tooth[11].

1.4 Genetic Information in Teeth

For DNA extraction, soft tissues or blood is preferred, but when human remains are damaged from fires or explosions teeth are an excellent candidate due to the protective features of the enamel, as well as their location in the jaw[12,13,14]. The enamel has a higher acellular content, allowing it to protect the tooth from environmental factors as well as contamination from other external DNA[12].

In the human tooth, several structures in the tooth or surrounding structures can be sources of DNA. Specifically, the pulp chamber, dentin or cementum ground to a powder, alveolar bone, and periodontal ligaments can be used. Researchers have found the dentine-cement powder to be the best candidate for either nuclear or mitochondrial DNA extraction[15].

Both nuclear and mitochondrial DNA have the ability to stay in human remains for hundreds or thousands of years. Nuclear DNA is the most common type of DNA used in forensic application, and teeth are an excellent source of nuclear DNA. Due to mtDNA's increased resistance to decomposition, it will degrade slower than nuclear DNA[12]. When extracted nuclear DNA samples are degraded or insufficient, forensic scientists resort to mtDNA for genetic information. Under these conditions, mtDNA has a higher chance of obtaining a DNA profile than nuclear DNA, because of the large number of mitochondria per cell[16].

1.5 Current Analysis of DNA from Teeth

Currently, the standard procedure in DNA extraction from teeth is through destructive means. Contemporary methods include crushing the entire tooth, conventional endodontic access, a vertical cut, and a horizontal cut through the cervical root. The preferred method is the horizontal cut through the cervical root due to the ability to independently test the cementum and preservation of the crown for morphological identification[15].

While the crown is preserved, the pulp that is extracted from the pulp chamber and the root that is ground into a fine powder are placed in tubes. The process of DNA extraction is both tedious and time consuming, with steps consisting of placing the samples in extraction buffers, centrifuging the samples multiple times throughout the extraction process, and extracting and transferring the supernatant into different tubes repeatedly[15].

After the isolation of DNA, the samples must undergo polymerase chain reaction (PCR) amplification to rapidly increase the number of copies of DNA to be able to study in greater detail. In 1983, Kary Mullis invented PCR, revolutionizing how DNA is studied. PCR techniques involve the DNA undergoing thermal cycling, which is repeated cycles of heating and cooling to

promote different temperature dependent reactions. Two main components are added to the sample for PCR – a primer and a DNA polymerase, usually in the form of the heat-stable Taq polymerase[17].

PCR consists of three basic steps – denaturation, annealing, and elongation (**Figure 4**). The first step, denaturation, usually occurs at around 94 ºC for thirty seconds. The denaturation step allows DNA to denature from its double-stranded form to a single stranded form, which allows replication. The second step, annealing, usually occurs at 54 ºC for one minute. The annealing step allows for the primers to connect to the single stranded DNA templates. The third step, elongation, usually occurs at 74ºC for one minute. The elongation step allows the DNA polymerase to synthesize new double-stranded DNA by adding deoxyribonucleotide triphosphates. Each step constitutes once cycle during PCR, and the cycle is repeated between 30-40 times, creating millions of copies of DNA[17,18].

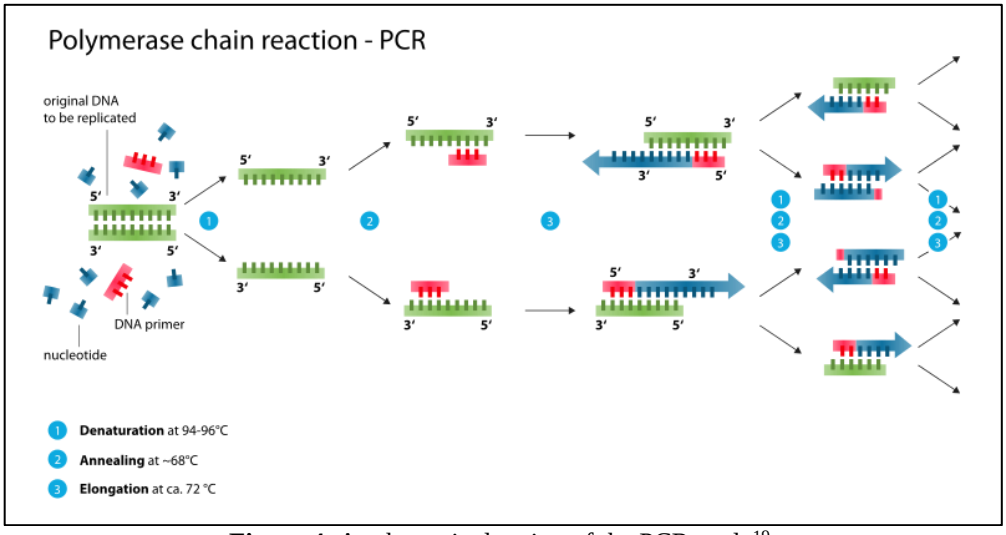

Figure 4. A schematic drawing of the PCR cycle[19].

After DNA amplification, the samples undergo agarose gel electrophoresis for analysis. Gel electrophoresis is the separation of DNA fragments by size using an applied electrical current. The gel is placed into the electrophoresis tank, then a buffer solution, usually containing ethidium bromide, is poured in to submerge the gel. Before current is applied, the samples and a

positive control ladder are inserted into the wells of the gel. The DNA ladder contains predetermined lengths to help approximate the size of the samples. Prior to being pipetted into separate wells within the gel, the samples are loaded with a fluorescent dye for visualization. An electrical current is then applied to the electrophoresis tank. Since DNA is negatively charged, when a current is passed through the gel, the DNA will travel towards the positively charged electrode. The smaller the DNA fragment, the further it will travel through the permeable gel matrix. After the DNA has separated, the gel can be visualized under a UV transilluminator, where the DNA will appear as bright bands[20].

By altering the extraction, isolation, and purification steps in the destructive method, a nondestructive method can be performed. Instead of crushing or splitting the tooth, it can be placed in a guanidinium thiocyanate (GuSCN) buffer, and the buffer extracts the DNA to be analyzed, leaving the tooth undamaged[21]. GuSCN is used as a protein denaturant, with its properties of inactivating RNase and DNase enzymes that would ultimately break down RNA and DNA, respectively[22]. By submerging the tooth in a similar extraction buffer as the destructive method, with a few alterations DNA can ultimately be extracted from the tooth into the buffer. There is no need for cutting the tooth, which preserves its integrity for future reference.

1.6 UV/Vis Spectroscopy

Ultraviolet-visible (UV/Vis) spectroscopy is a versatile analytical technique that allows for the determination of the identity of a compound or functional group by comparing sample absorbance spectra to a spectral library. Molecules that contain bonding and non-bonding electrons have the ability to absorb UV and visible light. Most molecules absorb light within the

UV wavelength range (100-400 nm) and the visible light range (400-700 nm). When a photon is absorbed by a molecule, the electrons in the ground state are excited and promoted into a higher energetic state[23]. Electrons go from the highest occupied molecular orbital (HOMO) to the lowest unoccupied molecular orbital (LUMO) and the energy difference between the HOMO and LUMO, known as the band gap, must be exactly matched by the energy of a photon to excite the electron into a higher energetic state as seen in **Figure 5**.

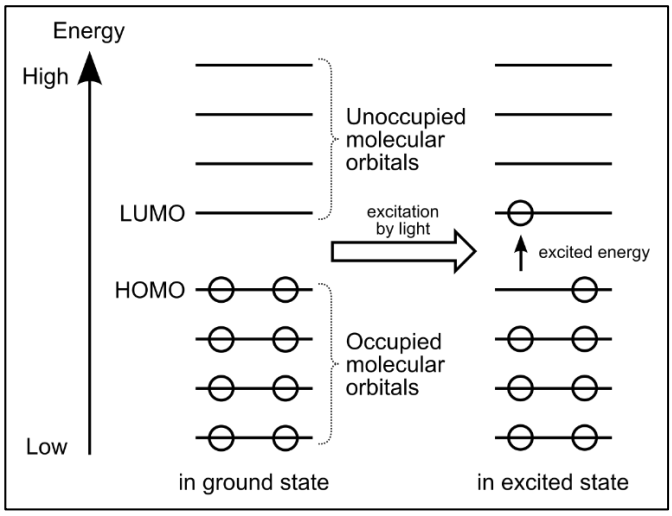

Figure 5. The excitation of an electron from the HOMO to the LUMO[24].

The energy transitions that are most common in the UV-Vis range are either π- π* or n- π*, with the asterisk designating an excited state, or anti-bonding orbital. π orbitals arise from double bonds while an n orbital arises from non-bonding electrons. There are also σ- σ* and n- σ* orbitals, but they are higher in energy and thus are less useful[23].

The absorbance of a solution can be calculated using the equation $A=\log_{10}(I_0 / I)$ where A represents absorption, I_0 represents the light intensity at a given wavelength, and I represents the transmitted light intensity. The absorption of the solution is also directly proportional to the concentration; thus UV/Vis spectroscopy also allows for the quantification of the concentration of an analyte using Beer-Lambert's law ($A = \varepsilon bC$). The A represents the measured absorption, b

represents the optical path length, C represents the concentration of the absorbing species, and ε is molar absorptivity/extinction coefficient, which is unique to each material[23].

UV/Vis spectroscopy is performed with a UV/Vis spectrophotometer. Its basic parts include a light source, sample compartment, diffraction grating in a monochromator, and a detector. The light source emits light, which travels through the monochromator to separate the light into different wavelengths and directs the light into a sample-filled cuvette. The light passes through the cuvette into the detector where the data is analyzed[25]. The resulting spectrum is generated by comparing the difference in intensity between the incident light and the light received by the detector.

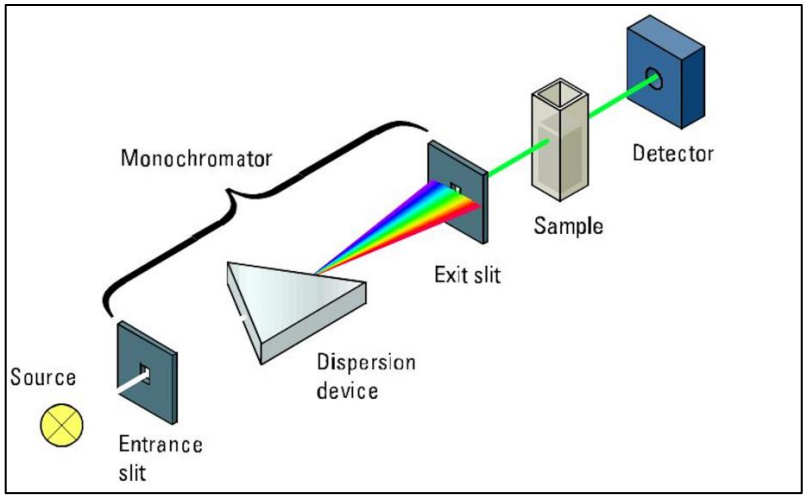

Figure 6. Single beam spectrophotometer diagram[23].

1.7 DNA Profiling

DNA profiling is a process that allows for the characterization of an individual's genetic composition. After its invention in 1984 by Sir Alec Jeffreys, forensic scientists are able to use DNA profiling methods to identify individuals, and to look at familial relationships[26]. Two primary modern methods have been developed to identify individuals using nuclear DNA: the

older technique, restriction length fragment polymorphism (RFLP), and the newer technique, short tandem repeats (STR)[27]. An additional technique, mtDNA typing, can reveal a shared maternal relationship between individuals through the analysis of mitochondrial DNA.

The first method using RFLPs is an older technique that requires a large amount of intact DNA (25 ng). With this criterion, performing DNA profiling using RFLPs may be challenging due to the degradation of DNA over time[27]. RFLP determines genetic characterization by digesting DNA with a restriction enzyme. The restriction enzyme digests the DNA strand at specified recognition sites creating polymorphic fragments. The polymorphic fragments are visualized using gel electrophoresis, which separates the fragments based on size. The genetic characteristics of an individual can be compared to another individual by visualizing and comparing characteristics[28].

The second method utilizes STRs of DNA to determine the identity of individuals. In comparison to RFLPs, the identification of an individual using STRs only requires ~1 ng of DNA. STRs are a group of 2 to 5 base pairs that repeat in succession within the genome. For example, the sequence TAACG can be repeated in succession creating the STR 'TAACGTAACGTAACGTAACG.' In the human genome, everyone has similar STRs, but the length of the STR can differ. One individual might have 5 and 8 repeats of a certain STR, while another individual might have 3 and 7 repeats of the same STR. Depending on a specific STR locus, the number of repeats can range between 3 and 21 repeats. While the length of the repeats between individuals differs, the sequences that flank both sides of the STR are known and are the same for every individual. With the use of PCR, the region between the flanking sequences can be amplified to accurately determine the length of the STR. Since there is a finite number of repeats in a STR at a specific locus, an individual can have the same number of repeats as

another individual at that given locus. However, there are thousands of STR locations within the genome that can be used for genetic profiling increasing the probability of having different flanking sequences between individuals[27].

The US Federal Bureau of Investigation maintains the national DNA database, Combined DNA Index System (CODIS), that holds over $1x10^7$ core STR markers. In the US, there must be a match at 20 core STR loci for the DNA evidence to be admissible in court. With the current system in place, using 20 similar STRs has the ability to identify a unique individual out of $3.10x10^{21}$ individuals[27]. Thus, the accuracy of STRs in DNA profiling is unrivaled in discriminating between individuals' DNA.

As previously stated, mtDNA cannot be used to identify specific individuals, but it can be used to identify individuals sharing a common maternal ancestor. Through examination of the HRV1, HRV2, and coding region (CR) of mtDNA, an individual's most recent common maternal ancestor can be determined. There are three tests based on the number of regions being studied: low resolution (HRV1 only), high resolution (HRV1 and HRV2), and the full genome sequence (HRV1 + HRV2 + CR). Since DNA does not give a specific time period of the last common maternal ancestor, statistics are needed to determine a range. If an identical match on the low-resolution test has been determined, then there is a 50% probability that there was a common maternal ancestor within the past fifty-two generations. If an identical match on the high-resolution test have been determined, then there is a 50% probability that there was a common maternal ancestor within the past twenty-eight generations. If an identical match has been determined on the full genome sequence test, then there is a 50% probability that there was a common maternal ancestor within the past five generations. Since mtDNA has thousands of

copies of its genome per cell, it can survive unfavorable conditions and for long periods of time, thus is beneficial in determining distant ancestry from damaged remains[29].

2. Materials and Methods

2.1 Previous Destructive Method Validation

Before conducting research on a nondestructive method, DNA was extracted and amplified from five unknown male teeth and five unknown female teeth using a destructive procedure to test the efficacy of a commercial mtDNA extraction kit. The teeth were prepared by cleaning the surface with 0.5 M EDTA solution and placed under UV light for 30 minutes to eliminate contamination. The equipment used (coffee grinder and hammer) were washed twice with 10% diluted bleach and placed in a Purifier Filtered PCR Enclosure to prevent contamination. The teeth were prepared by grinding the tooth into a powder using a coffee grinder. The teeth were placed into a fume hood one at a time on a clean Kimwipe and fragmented using a hammer with minimal force. The fragmented pieces of the tooth were placed into a coffee grinder and pulsed until the tooth reached a fine powder consistency. The samples were then placed in 50 mL Eppendorf tubes. Between each sample, the coffee grinder was cleaned following the procedure listed above then placed in the PCR enclosure for 30 minutes. The Mitochondrial DNA Polymorphism in Human Evolution Kit (mtDNAP) designed and sold by Carolina Biological Supply Company was used for the purification and amplification of the teeth samples. The only items used in the kit included a mtDNA primer/loading dye mix, pBR322 marker, Chelex 100 resin, and PCR tubes containing ready-to-go beads. After the supernatant was mixed with the mtDNA primer/loading dye mix and pBR3222 plasmid, the supernatant was transferred and mixed into the PCR tubes containing ready-to-go beads. The beads contain stabilizers, BSA, dATP, dCTP, dGTP, dTTP, ~2.5 units of DNA polymerase, and

reaction buffer. Each tooth was placed in separate 1.5 mL centrifuge tubes. 1 mL of 0.9% saline solution was added to the tube and the sample was centrifuged for 2 minutes at 13,000 × g. 950 µL of saline solution was extracted from the mixture, then 100 µL of 10% Chelex from the mtDNA Carolina Kit was added to the solution. The centrifuge tubes were placed on a heat block as it heated up to 100 ºC. Once it reached 100 ºC, the samples were left for 10 minutes as the block continued to increase in temperature. After the heating step, the tubes were opened to avoid pressure build-up then closed. The tubes were vortexed for 15 seconds and then centrifuged for 2 minutes at 13,000 x g. Once centrifuged, 30 µL of the clear supernatant was extracted from each tube and placed in different labeled PCR tubes containing ready-to-go beads.

To prevent chromosomal DNA contamination, a specific primer for mtDNA was used for PCR. The sample was then run through 40 cycles of PCR. The PCR cycles consisted of a 3-minute activation step at 94ºC, followed by the denaturing step at 94ºC for 30 seconds, the annealing step at 51ºC for 60 seconds, and the elongation step at 74ºC for 45 seconds. Afterwards, the samples were analyzed using gel electrophoresis on 0.7% agarose gel using a TAE buffer solution with the addition of ethidium bromide.

2.2 Materials

The materials and methods used in the procedures were developed by Dr. Michael Hofreiter. Each solution should be prepared with high performance liquid chromatography (HPLC) grade water, or with water that has a similar purity[12].

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1. Extraction and Binding Buffer:

- The extraction and binding buffer used consists of 1.5 M guanidinium thiocyanate, 50 mM Tris-HCl, pH 8.0, 25 mM NaCl, 1.3% Triton-X100, 20 mM EDTA, and 50 mM DTT. The guanidinium thiocyanate is a chaotropic salt that functions to lyse the cells of the dentine and cementum in addition with Triton-X100. Guanidinium thiocyanate also functions as a protein denaturant and nucleic acid protector by preventing DNase enzyme activity in addition with EDTA, a metal chelator. Tris-HCl helps stabilize the solution at a pH of 8.0, and with NaCl, helps DNA partition into the aqueous phase of the solution. DTT functions as a "deprotecting" agent of thiolated DNA and reduces the formation of disulfide bonds.

2. Silica Suspension:

    - The silica suspension was created by weighing 4.8 g of silicon dioxide and adding ddH_2O to obtain a final volume of 40 mL. The solution was then vortexed until the silicon dioxide became homogenized within the liquid. The solution was allowed to settle for 1 hour, then the upper 39 mL was transferred into a new tube and allowed to settle for 4 hours. After the 4 hours, the upper 35 mL was discarded, leaving 4 mL of the silica suspension. Finally, 48 μL of 30% HCl was added. The solution was vortexed and stored at room temperature in the dark. When silica, DNA, water and a sufficient concentration of a chaotropic salt are in a solution, along with a buffer to control the pH, DNA adsorption to silica occurs through three different competing forces: 1) dehydration, 2) weak electrostatic repulsion forces, and 3) hydrogen bond formation[31]. The complete mechanism of DNA adsorption to silica is still not fully understood.

3. Washing Buffer 1:
    - Washing buffer 1 consists of 5 M guanidinium isothiocyanate and 0.3 M sodium acetate (pH 5.2). Washing buffer 1 was stored at room temperature in the dark. Sodium acetate functions to increase ionic strength allowing for DNA to precipitate out of the solution by neutralizing the charges of the sugar-phosphate backbone of DNA.
4. Washing Buffer 2:
    - Washing buffer 2 consists of 50% ethanol, 125 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA. Washing buffer 2 was stored at room temperature. Ethanol also functions to allow DNA to precipitate out of the solution by removing the solvation shell that surrounds it, which promotes the aggregation of DNA.
5. Elution Buffer:
    - The elution buffer consists of 10 mM Tris-HCl and 1 mM EDTA (pH 8.0).

Five anonymous teeth samples stored in a jar with an unknown liquid were obtained from local dentists and the Mitochondrial DNA Polymorphism in Human Evolution Kit (mtDNAP) kit designed by Carolina Biological Supply Company was used to attempt to extract the DNA from the supernatant.

2.3 Nondestructive Procedure: Methods

Five anonymous teeth samples (canines, incisors, and molars) were obtained from local dentists and were washed in 10% diluted bleach and placed under UV light for thirty minutes to eliminate contamination. Afterwards, each tooth was placed in a separate 50 mL Eppendorf tube,

and 10 mL of the GuSCN extraction buffer was placed in each tube. The ten separate samples were placed on a rotary machine for 3-5 days under constant agitation in the dark.

After tooth preparation, one tooth out of the five samples used the full method presented. The tooth was removed from the tube, and the supernatant was centrifuged at 12,000 × *g* for two minutes. The supernatant was transferred to a new tube, leaving behind a pellet of any particles from the solution. Afterwards, 100 μL of the silica suspension was added into the supernatant, then the supernatant was placed under constant agitation for three hours in the dark. The supernatant was then centrifuged at 5,000 × *g* for two minutes. The supernatant was then removed, and the silica was resuspended with 1 mL of washing buffer 1. The sample was then centrifuged at 5,000 × *g* for two minutes, the supernatant was discarded, and the silica was resuspended with washing buffer 2. The sample was then centrifuged again at 5,000 × *g* for two minutes, the supernatant was discarded, and the silica was resuspended with 1 mL of washing buffer 1. Afterwards, the sample was centrifuged at 16,000 × *g* for two minutes, and any remaining supernatant was discarded. The silica was allowed to air dry for 15 minutes by leaving the tubes open. After the silica had dried, 50 μL of elution buffer was added to the silica and carefully resuspended with the pipette tip. The samples were then incubated for 10 minutes with a closed lid. The sample was then centrifuged at 16,000 × *g* for two minutes, and the supernatant was transferred into 0.5 mL tubes.

The method listed above was unsuccessful, so additional teeth were examined after stopping the method at each step to test for DNA contamination and to evaluate individual buffer solution efficacy. Evaluations consisted of using the extraction buffer only, the extraction buffer with washing buffer 1, the extraction buffer with washing buffer 2, the extraction buffer with washing buffers 1 and 2, and the extraction buffer with the elution buffer. Each new sample,

representing a different stopping point in the method, was amplified with PCR and analyzed with gel electrophoresis. The silica suspension was added to each step to isolate and purify DNA.

The mtDNAP kit was used to extract the DNA from the supernatant from all five different trials. To prevent chromosomal DNA contamination, a specific primer for mtDNA was used for PCR. The sample was then run through 40 cycles of PCR. The PCR cycles consisted of a 3-minute activation step at 94 ºC, followed by the denaturing step at 94 ºC for 30 seconds, the annealing step at 51 ºC for 60 seconds, and the elongation step at 74 ºC for 45 seconds. Afterwards, the samples were analyzed using gel electrophoresis using 1X TAE buffer with the addition of ethidium bromide.

2.4 Current Destructive Method Control

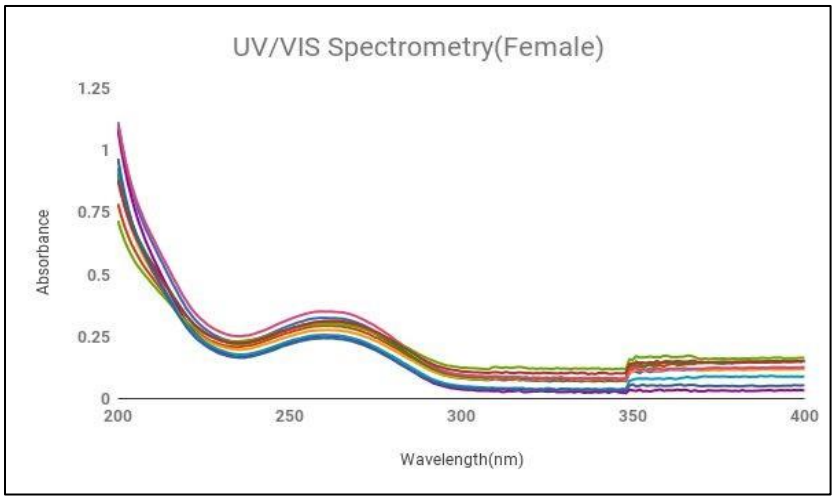
Since previous trials of the nondestructive method did not yield any visible DNA, two destructive procedures, as well as a cheek swab were used as a positive control. One anonymous tooth sample was obtained from local dentists and washed with 10% diluted bleach and placed under UV light to eliminate contamination. The destructive procedure that aimed to test the efficacy of commercially available mtDNA extraction kit was used to prepare the teeth for DNA extraction.

After the tooth sample was prepared destructively, aliquots were taken for both the nondestructive procedure and the same destructive procedure, as well as cheek swabs, to test the efficacy of commercial mtDNA extraction kits. To prevent chromosomal DNA contamination, a specific primer for mtDNA was used for PCR. The sample was then run through 40 cycles of PCR. The PCR cycles consisted of a 3-minute activation step at 94 ºC, followed by the denaturing step at 94 ºC for 30 seconds, the annealing step at 51 ºC for 60 seconds, and the

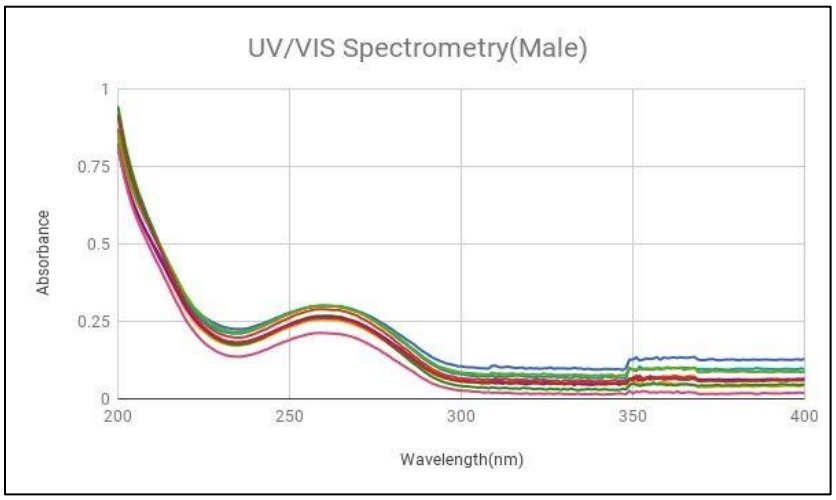
elongation step at 74 ºC for 45 seconds. After amplification, gel electrophoresis was performed on samples from all control methods using 3% agarose gel along with a 1X TAE buffer solution with ethidium bromide.

3. Results

Graph 1 and **Graph 2** show the expected DNA UV/Vis absorption bands at 260nm for the 10 samples tested in the destructive procedure. **Graph 1** shows five unknown female teeth samples with duplicates, accounting for ten total points on the graph. The same is repeated on **Graph 2** using five unknown male teeth samples with duplicates. **Figure 7** displays the gel electrophoresis results from using the mtDNA Carolina kit (destructive method). The appearance of DNA bands in lanes 2-11 that corresponds to the ~383 base pair bands in lanes 1 and 12 (DNA ladders) suggests that the destructive method was successful in all 10 samples. The results from **Graphs 1** and **2,** and **Figure 7,** show the expected results for the nondestructive method.



Graph 1. UV/Vis absorption results of the destructive procedure using mtDNA Carolina kit.



Graph 2. UV/Vis absorption results of the destructive procedure using mtDNA Carolina kit.

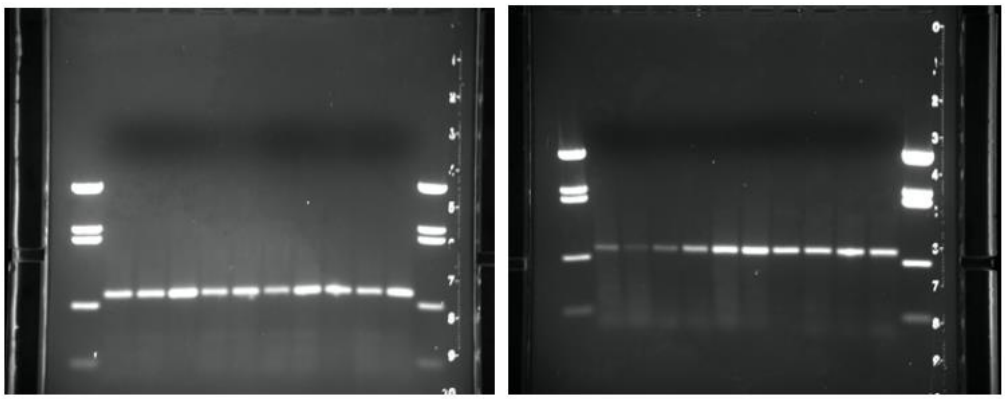
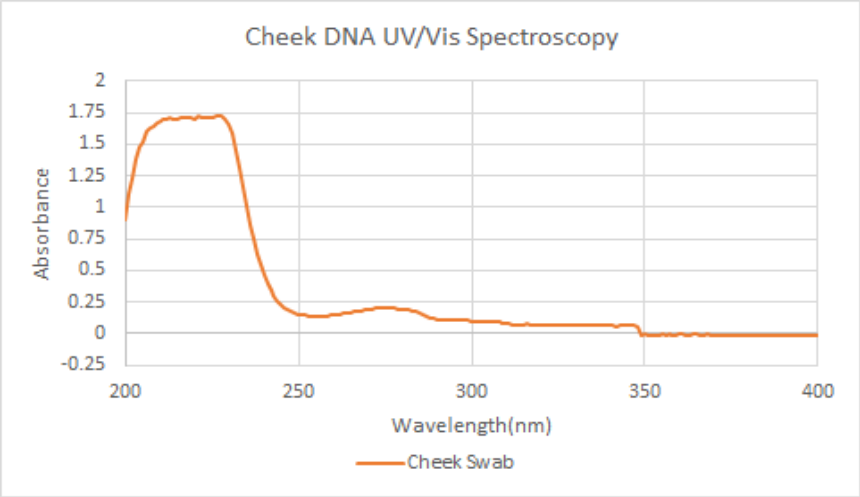


Figure 7. Agarose gel results of duplicate teeth samples using mtDNA Carolina kit.

The production of amplicons in the PCR thermal cycler were confirmed with UV/Vis spectroscopy as seen in **Graph 3**. A slight peak is visible at 280 nm, whereas a peak at 260 nm indicates the presence of DNA. The successful extraction and amplification of DNA from the cheek swabs can be seen in **Figure 8** which shows the gel electrophoresis data. In **Figure 8**, lane 1 contains the DNA ladder. Lanes 2-6 represent the following: lane 2- full method, lane 3- extraction buffer only, lane 4- extraction buffer with washing buffer 1, lane 5- extraction buffer with washing buffer 1 and 2, lane 6- extraction buffer, washing buffer 1 and 2, and elution

buffer. The same procedure was followed for lanes 7-11. The samples were prepared in this fashion to test for DNA contamination in the buffer solutions. Lane 12 contains the results from a cheek swab positive control that yielded a visible DNA band that corresponds to the DNA ladder peak of ~383 base pairs.



Graph 3. UV/Vis absorption results of a cheek swab using mtDNA Carolina kit.

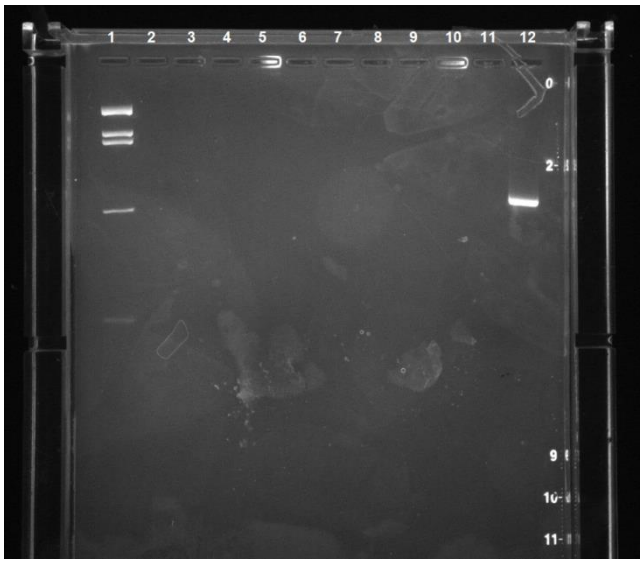


Figure 8. Agarose gel results using nondestructive extraction and a destructive extraction for the cheek sample. Well 1 contains the control ladder. Wells 2-11 contains samples using the nondestructive method. Well 12 contains DNA from a cheek swab.

4. Discussion and Conclusion

When examining **Graph 3**, peaks at 230 nm and ~280 nm are visible. A peak at 230 nm is visible due to salts such as GuSCN, EDTA, non-ionic detergents such as Triton X-100, proteins, and phenol. The presence of a peak at 260 nm in a UV/Vis spectrum suggests that DNA is present. The cheek DNA data in **Graph 3** reveals a peak at 282 nm which could indicate protein contamination. When searching for answers on possible explanations for the occurrence of the bathochromic shift (red shift), we examined the source of the cheek swab. The volunteer that donated the cheek cells had recently consumed a protein shake which could have possibly resulted in the increased amount of protein in the sample. The ratio of the absorbance at 260nm/280nm resulted in a value of 0.75 which is representative of a DNA sample contaminated with protein. A pure DNA sample would have a 260/280nm ratio of ~1.8[32].

UV/Vis spectroscopy and gel electrophoresis results for the nondestructive and destructive procedures provided little useful data. In **Figure 8**, lanes 2-11 contained samples using a nondestructive method, but no visible DNA bands were present. The gel electrophoresis results of the destructive method also provided minimal useful data, even when using the same method that provided important results in **Figure 7**. Figure 8 displays a visible DNA band from a cheek swab positive control at ~383 base pairs in Lane 12. The agreement of this band at ~383 base pairs with that of the DNA ladder, suggests the extraction and amplification procedures were successful for the cheek swab control. **Figure 8**, fluorescent signals can be seen in wells 5 and 10 where the sample appeared to not migrate up or down the agarose gel. These signals could be the result of the presence of ethanol somehow playing a role in neutralizing the positively charged fluorescent ethidium bromide molecule. Free ethidium bromide exists as a positive ion in aqueous solutions and migrates toward the negative electrode during the gel

electrophoresis process as expected in lanes 2-4, 6-9, and 11. Once the ethidium bromide is neutralized, it is not attracted to either of the electrodes and remains in the well where it fluoresces under UV light as seen in wells 5 and 10.

The failure of the nondestructive extraction method may have resulted from the teeth themselves. When the data in **Graph 1** and **2** and **Figure 7** was obtained, the teeth samples were stored in dry containers. The teeth that were used in the nondestructive study were donated to the laboratory in a jar with an unknown liquid. Over time, the liquid in which the teeth were stored could have degraded the DNA. This could explain why the destructive method was successful in earlier experimental runs, but neither the nondestructive nor the destructive runs produced measurable DNA in the runs that were performed months later.

Due to Covid-19, we conducted research on a shortened timeframe and were unable to troubleshoot the errors encountered in the procedure. Both the nondestructive and destructive procedures should be conducted again with a new set of teeth samples that are stored in a dry container to prevent degradation of the DNA within the teeth. The DNA obtained from the cheek cells should be purified by removing the protein with a phenol-chloroform mixture prior to DNA-precipitation. In conclusion, future research is needed to investigate nondestructive procedures of DNA extraction.

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