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I completed a validation study at the Armed Forces DNA Identification Laboratory. It was an evaluation of freezer mill pulverization of teeth for use in nuclear casework. The teeth were cleaned using a bleach and sonication method and then ground using a freezer mill. DNA extraction was achieved by using silica based capture techniques. The DNA was quantified, amplified and evaluated using fragment analysis.

Several different amounts of input tooth powder were attempted to increase the DNA yield. Qiagen MinElute® produced higher DNA yields then Promega's DNA IQ™ system for the small sample size tested. After reviewing the results it was concluded that the DNA was most likely highly degraded and therefore did not produce complete genetic profiles for the samples tested.

EVALUATION OF SPEX6750 FREEZER MILL PULVERIZATION OF TEETH FOR USE IN NUCLEAR CASEWORK AT THE ARMED FORCES DNA IDENTIFICATION LABORATORY

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VALIDATION STUDY OF FREEZER MILL PULVERIZATION OF TEETH FOR USE IN NULCEAR CASEWORK AT THE ARMED FORCES DNA IDENTIFICATION LABORATORY

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CHAPTER I

INTRODUCTION

Deoxyribonucleic acid (DNA) has revolutionized the field of forensic science. It is possible to analyze DNA from many different types of samples and obtain the DNA with different extraction techniques (1-5). Samples analyzed for casework include a wide range of biological substances, and testing can be completed in a shorter amount of time and with fewer hazards for the analyst (1,6-10). DNA must first be extracted from the sample, quantified to determine the amount in the sample, amplified by the polymerase chain reaction (PCR) to make many copies of the interested segments and the short tandem repeats (STRs) analyzed using fragment analysis. DNA is analyzed at specific locations (loci) within the genome and a genetic profile is generated based on the number of STRs detected on each chromosome pair. Typically evidentiary samples are compared to known or reference samples to determine if both samples could have originated from the same source.

The teeth are the hardest substances in the body and in many cases are all that remains of an individual at a burial site. Tooth samples have often been analyzed in ancient DNA cases due to the protection the tooth itself gives to the DNA contained within, but these samples are used for current analysis as well (1-3,5,10-17). It has been reported that DNA can be extracted from teeth that have been buried in soil (16). Many techniques for extracting DNA from teeth require maintaining the morphology of the

tooth to be used for other forms of identification such as comparison to dental records (11,15-16). If the morphology does not have to be maintained, then the entire tooth can be consumed to obtain the DNA.

Multiple methods have been developed to extract DNA such as organic chemicals or silica based beads or membranes. Silica-based extraction techniques offer an easy, safe and efficient way to capture the DNA in a solution (1,4,18-21). The DNA is captured based on pH dependent binding to the silica matrix and repeated washings to remove impurities. The DNA is concentrated by eluting into a desired volume of buffer or water. The silica can be different mediums such as a magnetic resin bead, as in Promega Corporation's DNA IQ™ or attached to a spin column in the Qiagen MinElute® PCR Purification Kit. The use of silica provides a sample that is free of impurities and PCR inhibitors and the eluted sample can be directly added to a PCR reaction (19,21).

I completed an internship at the Armed Forces DNA Identification Laboratory (AFDIL) in Rockville, Maryland. The mission of the laboratory is to provide scientific consultation, research and educational services around the world in forensic science. AFDIL is also responsible for the collection, accession and storage of reference samples for the United States military and other authorized personnel. AFDIL services the Department of Defense and other agencies (www.afip.org). There are four divisions within the laboratory; nuclear, mitochondrial, automation and research. Within the Nuclear Division, the Quality Control and Validation team is responsible for control of the reagents and instruments and to be sure that the techniques and instruments used for casework are properly validated.

The Armed Forces Medical Examiners often recover fresh and intact teeth from human remains; consequently, the Medical Examiner may select and submit teeth for DNA analysis that can be destroyed and consumed in their entirety during the analysis process. Currently, teeth are processed using a method where drilling out the pulp cavity of the tooth maintains the integrity of the tooth's morphology, which is followed by DNA purification using an organic extraction protocol. This validation study evaluates an alternative method for the recovery of nuclear DNA from teeth pulverized using the SPEX Freezer Mill.

Most of the extraction methods used in this evaluation have already been validated within AFDIL for other sample types, such as bone. The validation study I completed applies these techniques to pulverized teeth so that another sample type can be used to provide genetic profiles. The methods that can be changed the most are the cleaning method, incubation in demineralization buffer and extraction with DNA IQ™. The other downstream events such as amplification, electrophoresis and fragment analysis are more constant.

CHAPTER II

BACKGROUND

Teeth

Teeth can be used in forensic investigations by comparing the physical appearance to dental records and/or by extracting DNA from the tooth. The DNA profiles generated from the tooth are then compared to family and known reference samples. Since teeth are the hardest substances found in a human body they can withstand a large amount of stress and still maintain their physical appearance (16). The hard tissue, dentin and enamel, also offers protection to the DNA contained in a tooth, refer to Figure 1 (15). Much of the genetic research based on teeth employs mitochondrial DNA (mtDNA) sequence analyses, as opposed to nuclear DNA and STR-based testing methods (1,2,13).

Teeth contain acellular tissues and nucleated cells (11). There are several published techniques to extract the DNA contained within a tooth, most of these techniques are employed to maintain the morphology of the tooth for other analysis techniques (1,5,10,11,13-17). Conventional endodontic access, vertical split, horizontal split or crushing the entire tooth when the physical morphology does not have to be maintained can be used to extract the DNA (15). All of these techniques, besides complete crushing, attempt to access the dental pulp chamber (refer to Figure 1) which, based on gross volume, is the best source of DNA in a tooth (15). However, the pulp

chamber size and root canal diameter normally decreases with age along with the cellularity of the pulp tissue limiting the amount of DNA present in those tissues (15).

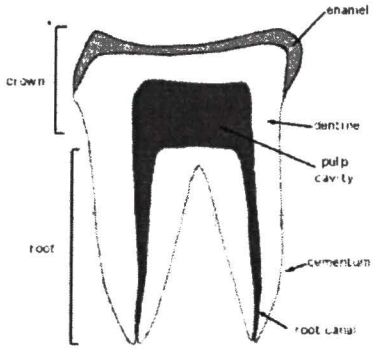

Figure 1 – The structure of individual teeth showing the pulp cavity (22)

There is great variation in the amount of DNA among different tooth samples. Sweet et al. (16) sampled 20 teeth with a mean weight of tooth powder recovered from each sample of 1.77g and the average yield of DNA from each molar was 30.9 μg (range: 0.5 μg - 97.5 μg) and the average amount of genomic DNA recovered from these samples was 18.4 μg DNA/g (16). Smith et al. (15) sampled 10 pairs of teeth from individuals with two different methods, horizontal sampling and crushing the entire tooth, and reported 4.0 μg and 18.1 μg, respectively of total DNA recovered. More DNA was recovered from crushing the whole tooth, but the DNA indicated signs of more shearing than sampling just the pulp (15). In all cases, enough DNA existed from the tooth powder for successful PCR-based testing. Sweet, et al. (16), used older techniques no longer in practice in casework laboratories. The DNA quantification was performed using quant blot, slot blot hybridization or the Warburg-Christian equation from ultra-

violet (UV) absorption values (15,16). Classic organic DNA extraction methods were employed for these studies and if fragment analysis was completed it was achieved by polyacrylamide gel electrophoresis and not based on the 13 core STR loci used currently (15,16).

Cleaning Method – Sodium Hypochlorite - Bleach

A thorough cleaning method is necessary to remove all extraneous DNA, other contaminating human sources and bacterial DNA. Other unwanted residue present on the tooth must also be removed. One possible cleaning method is the use of bleach (Sodium Hypochlorite) (12). This method is cost effective and often less laborious than surface removal of flesh from either teeth or bones (12). Bleach may cause DNA degradation by oxidative damage causing base modifications or the production of chlorinated base products and complete breakdown occurs when DNA is exposed to high concentrations of bleach (12). Kemp and Smith (12), reported that exposure to 3.0% (w/v) sodium hypochlorite for at least 15 minutes is necessary for removal of surface contamination from bones and that the endogenous DNA is quite stable and typically unaffected (12). Steadman et al (23) reported that bleach was the worst cleaning method that they evaluated, due to poor DNA amplification. They tested bones that were subject to the bleach for long periods of time (33 days) with the intent of flesh removal (23). The cleaning method used in the validation study I completed was developed by Jennie McMahon in the mtDNA unit at AFDIL. Soaking the teeth in bleach with sonication

followed by wiping with bleach and ethanol has showed to remove contaminating DNA from the surface of teeth

Cryogenic Freezer Mill

Most cases that examine DNA results from teeth must maintain the morphology of the tooth to compare to existing dental records to help assist in identification (13,15). The process requires drilling or cracking the tooth to access the DNA contained within and then gluing the tooth back together. In some cases, the morphology does not have to be maintained and the tooth can be consumed in its entirety. The tooth can be manually crushed using a hammer or blender or with a freezer mill. The most common method used in forensic laboratories for processing teeth for DNA testing employs the freezer mill to pulverize the sample. The use of a cryogenic freezer mill allows for the crushing of the tooth while it is immersed in liquid nitrogen achieving a temperature of approximately -200°C making the tooth very brittle (24). A magnetically driven impactor is used to crush the sample against the two stainless steel end plugs achieving a finely ground powder as shown in the following Figure (24). A benefit of using the freezer mill over other techniques is that the sample is contained with the sample tube throughout the entire grinding process.

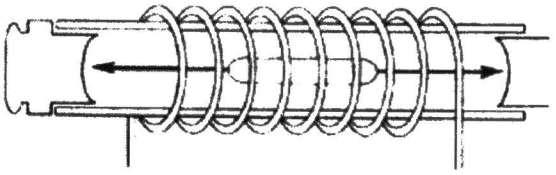

Figure 2 – Spex freezer mill sample tube - showing movement of the impactor that is magnetically driven and crushes the sample against the two end plugs (24)

Demineralization Buffer

Demineralization buffer allows for the extraction of DNA from samples such as bone and teeth by breaking down the mineral matrix. The DNA within teeth and bone is protected by areas of mineralization that offer physical barriers and must be broken down in order to access the DNA (25). Demineralization buffer incubates the tooth/bone powder in ethylene diamine tetra-acetic acid (EDTA), which demineralizes the bone and inactivates DNAses. Most extraction techniques require the collection of the supernatant or multiple wash steps in EDTA (25). Both of these prior techniques lead to the loss of DNA through either undissolved tooth powder or suspended DNA being discarded in the wash steps (25).

The use of demineralization buffer for full physical dissolution of the bone sample was used and reported at AFDIL (25). Complete demineralization was achieved by making the EDTA a component of the lysis buffer. The use of demineralization buffer retains and concentrates all the reagent volume, for complete DNA recovery. All of the DNA contained in a sample can be collected with incubation and subsequent extraction of the demineralization buffer. However, AFDIL's demineralization buffer was tested

only on bone samples using organic extraction and not on tooth samples. It was also reported that 15ml of 0.5M EDTA and an overnight incubation was needed to completely dissolve 1 g of bone powder (25). They stated that use of the demineralization buffer resulted in higher quality DNA being recovered (25). The high quality DNA was likely from more DNA being recovered but possibly also from complete demineralization leading to access of endogenous DNA of higher quality found within greatly protected areas of the bone (25). Teeth are the hardest parts of the human body and this is due in a large part to the enamel which will not breakdown in the demineralization buffer. Even with complete demineralization of the tooth powder some powder will remain due to the undissolved enamel in solution (Odile Loreille personal communication 2008).

Amicon® Ultra-4 Centrifugal Filter Devices

Many extraction techniques result in high volumes and the DNA must be concentrated. Amicon® Ultra-4 Centrifugal Filter Devices can be used to concentrate DNA samples. The filter is set in a vertical design, as shown in Figure 3, using Millipore Ultracel® regenerated cellulose low binding membrane (26). The filters are non-sterile but can be subject to UV light and used to concentrate DNA for nuclear STR analysis. Up to 4 ml of liquid can be added at a time to the filter cup and spun to concentrate the sample which is then collected using a pipette from the filter cup. There are several different products based on the Nominal Molecular Weight Limit (NMWL) of the device, the two used in this evaluation were Amicon® Ultra 30K device – 30,000 NMWL (Ultra-4 30) and Amicon® Ultra 100K device – 100,000 NMWL (Ultra-4 100) (26). Filters with

a larger NMWL could be used for fresh DNA samples that are not degraded and therefore are high molecular weight. Samples that are degraded with smaller DNA fragment sizes should be used with a small filter sizes to retain the DNA present in the sample.

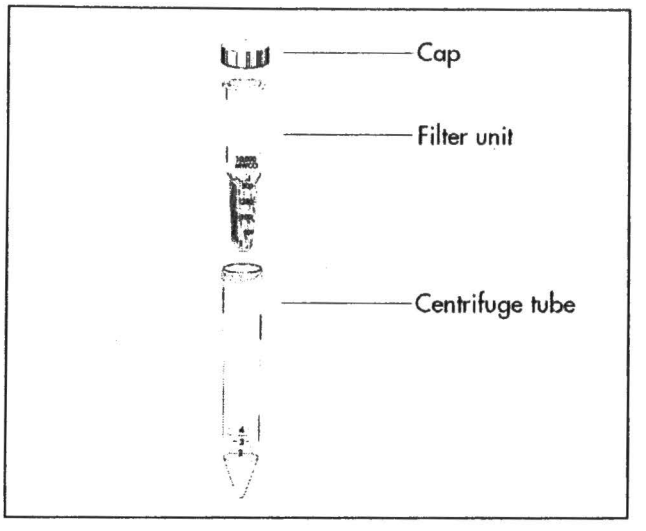

Figure 3 - Amicon® Ultra-4 Centrifugal Filter Devices (26)

DNA Extraction – Silica Based Capture Techniques

Organic DNA extraction methods have long been the main extraction technique for most sample types. Most of the procedures published on DNA extraction from teeth have used organic extraction methods (3, 13,15,16). Organic methods use phenol, chloroform and isoamly alcohol but these chemicals, phenol and chloroform, are hazardous to the analyst and require proper disposal. The extraction is time consuming, often requiring further purification and concentration of the DNA. Chelex® 100 Resin is also used for DNA purification and is a more rapid technique but does not remove PCR inhibitors. Silica-based capture techniques, such as the Promega's DNA IQ™ system

and Qiagen MinElute®, use silica either as beads or as a membrane, respectively, to bind the DNA and remove it from solution (18,19,21,27).

Promega's DNA IQ™ System was designed around a magnetic particle capture technique for small sample sizes where paramagnetic particles facilitate the removal of DNA from a solution (21). Guanidinium HCL dehydrates the DNA and exposes the phosphate groups which bind the DNA reversibly to the beads (18). There are several major benefits to using DNA IQ™ such as, it being a rapid technique, a simple protocol, removes PCR inhibitors, is environmentally friendly and is automatable (21). DNA isolated using this system is not human specific and will therefore also capture contaminating DNA so a thorough cleaning method is necessary. Both mitochondrial and genomic DNA present in the sample will be captured by the beads. The system is designed to bind at a maximum 1 and 4 ng/µl of DNA. The saturation amount of the beads provides a consistent amount of DNA extracted from the system. However, if more DNA is present some will be lost in the lysis buffer and wash steps which can be overcome by adding more resin to the lysis buffer to allow for the excess DNA to bind.

The DNA IQ™ method involves adding two times the amount of lysis buffer to the demineralization buffer for maximum binding efficiency to the resin (18). The resin requires a pH value of approximately 7.3 for optimal binding. The addition of two times the amount of lysis buffer to demineralization buffer lowers the pH to closer to the optimal level. Once the resin is added, the tubes can be placed in a magnetic stand where the DNA bound to the beads collect to the side of the tube and the remaining lysis buffer can be removed and discarded. After several wash steps, in which the beads are

resuspended, the elution buffer is added. The elution buffer rehydrates the phosphate backbone and the DNA is released from the beads (18). The beads can then be discarded (18). Vortexing the solution between each step allows for the resuspension of the beads and a more complete removal of inhibitors. The movement of the beads during vortexing breaks the non-specific binding of the inhibitors to the DNA and the inhibitors can then be removed with the wash buffer.

Promega Corporation and forensic laboratories tested several different tissue and sample types using the DNA IQ™ System. In the Small Sample Casework Protocol, a list is provided of the sample types tested, and any comments regarding the sample (27). Bone was included in the listed evaluations but teeth were not (27). AFDIL had previously validated a protocol using the DNA IQ™ System with bone but did not include teeth in their testing.

Qiagen MinElute® PCR Purification Kit uses a silica membrane to extract DNA from a sample using spin-column technology (19). The DNA adsorbs to the membrane, in the presence of high concentrations of salt while the PCR inhibitors pass through during centrifugation (19). As much as 5 µg of DNA can bind to the membrane. The buffers provided with the kit allow for easy color indication to determine if the pH and salt concentration are correct for binding of the DNA. The salt concentration must be high enough to permit binding which occurs due to chaotropic salts altering the structure of water (19). The membrane used allows for elution volumes as low as 9 µl, for high yields of concentrated DNA samples (19).

CHAPTER III

SPECIFIC AIMS

The project I completed was an internal validation study within the Quality Control and Validation team of the Nuclear Division at AFDIL. The validation study concentrated on the implementation of freezer mill pulverization of teeth for use in nuclear DNA casework. The specific aims of the study were to:

1. Evaluate a bleach sonication cleaning method for the removal of extraneous DNA from tooth samples;
2. Evaluate the SPEX SamplePrep, LLC. 6750 Freezer Mill pulverization of teeth for DNA extraction and to;
3. Evaluate two silica-based DNA extraction techniques for use on tooth samples for nuclear DNA casework.

CHAPTER IV

MATERIALS AND METHODS

Biological Samples

Teeth were donated by a local dentist and were stored at 4°C before analysis. Treatment of the teeth before being received at AFDIL was not recorded or known. The teeth were stored in a plastic bag that appeared to be saturated, possibly with alcohol, on one side and then placed in another plastic zip lock bag. The dentist was instructed to not store the teeth in anything other than alcohol. Teeth were selected for analysis based on basic appearance and if they appeared to be complete with no major broken roots. The size of each tooth was recorded. The pre-ground weight of each tooth used was recorded based on weighing an empty 15ml conical tube and subtracting the value from the weight of the tube and tooth. An Ultra Lum, Inc Crosslinker 1500 series was used for all UV treatments. All measurements were taken with the tooth inside a 15ml conical tube that had been subjected to UV light (3.0 J/cm^2). The teeth used for the study were selected based on the weight Most of the larger teeth were used so that the samples could be replicate tested. Anything unusual about a tooth was noted during the cleaning, cracking or grinding procedures.

Cleaning Procedure for Intact Teeth

Tooth cleaning was carried out inside a laminar flow hood except for the sonication which was performed on the bench top. Teeth that were damaged proceeded to step 2 of the procedure outlined below and were not immersed in bleach.

1. The specimen was initially cleaned by placing the tooth into a 50ml conical tube containing 25ml of freshly made 10% commercial bleach. The tube was capped securely and placed in an ultrasonic water bath for 5 minutes.
2. The tooth was wiped thoroughly with a 10% bleach-moistened sterile 4X4 gauze pad (or Kimwipe), followed immediately by wiping with a 4X4 gauze pad moistened with 95% ethanol. Teeth were allowed to dry in a weight boat under the hood for 5 minutes.
3. The dried tooth was weighted the pre-ground weight was recorded.
4. Teeth were stored in a 15ml conical tube that had been subjected to UV light (3.0 J/cm^2) at calibrated times for maximum exposure.

Cryogenic Grinding

The individual teeth were ground in a cryogenic freezer mill, SPEX SamplePrep, LLC. 6750. The freezer mill sample tubes, 6751C4 polycarbonate center cylinders, end plugs and impactor were subject to UV light at 254nm for 45 minutes. If the tooth could not fit in the sample tube with free movement of the impactor, the tooth was cracked before grinding. Teeth were cracked in a mortar bowl with a metal chisel that had all been rinsed with bleach and water and subject to UV light (minimum amount of 3.0

J/cm^2). The tooth was placed into the sample tube and loaded into the magnetic assembly of the freezer mill; the freezer mill tank was filled with liquid nitrogen to the indicated line. The freezer mill was closed and set for a 15 minute pre-cool and 5 minutes of grinding. The rate was set to 15 which is the highest setting on the machine. After grinding the sample tube was removed and allowed to warm for 30 minutes at room temperature before pouring back into the original 15ml conical tube. The freezer mill was allowed to warm two hours if multiple teeth were ground in the same day. Although the use of reagent blanks is a standard, required procedure, a reagent blank was not prepared from the cylinder components of freezer mill pulverization in these tests.

The amount of tooth powder obtained was determined by weighing the tube containing powder on an analytical balance. Most measurements were taken on an analytical scale that only provided measurements to the tenths. The scale was tared between each measurement. The measurements for the Mass Studies were taken on a scale that provided values to the hundredths. Some powder was lost at each transfer since it is very fine and would stick to all surfaces it came into contact with. To prevent contamination between pouring the tooth powder of each sample all of the surfaces of the hood and gloves were washed with bleach.

DNA IQ™ Extraction of DNA from Fresh Bone

The protocol previously validated at AFDIL using DNA IQ™ for fresh bone that was ground in a blender was used as a starting point for this evaluation of tooth samples ground in a freezer mill. Fresh Bone Protocol will be used to represent this protocol

throughout the rest of this validation study. Prior to working on the tooth samples I was directed to extract 6 samples of previously ground bone powder as a training exercise. The protocol was worked with 0.2g of bone powder and completed through fragment analysis. All tests using DNA IQ™ for DNA extraction from teeth followed this protocol from step 3 onward. When bone incubation buffer was prepared the ratio of demineralization buffer to Proteinase K and 1M DTT was kept constant. A reagent blank was initiated for the extraction procedure each time DNA was extracted from the tooth powder.

Fresh Bone Protocol

1. Bone Incubation Buffer (400µl Demineralization Buffer, 40µl Proteinase K (Promega 18 mg/ml) and 40µl 1M DTT) was prepared.
2. 400µl of Bone Incubation Buffer was added to each 1.7 ml tube containing approximately 0.2 g of bone powder. A Reagent Blank was initiated; samples were mixed by vortexing and incubated at 56°C for a minimum of 2.5 hours, and samples were vortexed every 45 minutes for 60 seconds.
3. Lysis Buffer/DTT solution (990µl of Lysis Buffer and 10µl 1M DTT) was prepared.
4. The tubes containing bone powder were centrifuged at 3000 RPM in a microcentrifuge for 3 minutes and the lysate was transferred to a sterile labeled 1.7ml tube.

5. 800µl of Lysis Buffer/DTT solution was added to each tube and mixed by pipetting up and down.
6. The DNA IQ™ Resin was vortexed to re-suspend the magnetic beads. 15µl of resin was added to each tube, vortexed for 3-5 seconds, and incubated at room temperature for 5 minutes, vortexed to remix every 2 minutes.
7. Samples were vortexed for 3-5 seconds and placed in Magnesphere magnetic stand, beads were allowed to pellet to the side and the lysate gently removed and discarded into a bleach filled beaker. Disrupting of the beads was avoided.
8. 100µl of Lysis Buffer/DTT was added, vortexed 3-5 seconds, allowing the beads to pellet on the magnetic stand and the wash buffer was removed and discarded.
9. 100µl of 1X Wash Buffer was added, vortexed 3-5 seconds, allowing the beads to pellet on the magnetic stand and the wash buffer was removed and discarded. 1X Wash Buffer was removed and washes were completed two more times for a total of 3 washes.
10. The pellet was allowed to air dry for 5 minutes.
11. 100µl of Elution Buffer was added, vortexed 3-5 seconds, and incubated at 65°C for 5 minutes.
12. Samples were vortexed 3-5 seconds, the beads were allowed to pellet on the magnetic stand, and the eluted DNA was transferred to a sterile labeled 1.7ml tube.

DNA Extraction from Teeth using Fresh Bone Protocol

Since 0.2g of bone powder is used and generates full profiles, as represented by the training samples, the same amount of ground tooth powder was tested. 0.2g of tooth powder was added to 1.5 ml conical tube and 400 µl of bone incubation buffer was added to the powder. The bone incubation buffer was an insufficient amount of liquid to suspend the powder so the tubes were disposed of.

The amount of bone incubation buffer was doubled, 0.2g and 0.1g of tooth powder was added to tubes labeled A and B, respectively. Two different teeth were used throughout the testing. The Fresh Bone Protocol was followed however, the ratio of 2:1 for lysis buffer to bone incubation buffer was altered with the sample volume of 800 µl being added resulting in a ratio of 1:1with the Lysis Buffer.

Mass Study – 0.1g, 0.075g, 0.05g

Complete demineralization was reported with a ratio of 15:1 of bone incubation buffer to sample in AFDIL's earlier studies (25). Different amounts of tooth powder were processed to evaluate effectiveness of demineralization. The volume of tooth powder and bone incubation buffer, as indicated in Table 1, were attempted in 1.7 ml tubes in order to maintain the same equipment as used for the Fresh Bone Protocol. One tooth sample was used to make sure the tooth was not causing the change in amount of recovered DNA.

The bone incubation buffer was prepared (Table 1) and added to the each sample. The 0.1g of tooth powder sample was not completed as the amount of liquid in combination with the bone powder exceeded the capacity of the tube. The excess liquid

present on the outside of the tube was determined to be a contamination issue and therefore not a probable volume for future casework samples.

The samples were incubated overnight in a 56°C heat block with vortexing while I was on site. After incubation, 300 to 400 µl of lysate was transferred to two new tubes and 800 ml of lysis buffer was added to provide a ratio of 2:1. The extraction process was completed as stated in the Fresh Bone Protocol.

Table 1 - Bone Incubation Buffer - Mass Study – 0.1g, 0.075g. 0.05g – A mastermix of bone incubation was made and then indicated volumes were added to the samples.

Sample amount	**Bone Incubation buffer**	**Demineralization Buffer**	**Proteinase K (18mg/ml)**	**1M DTT**
0.1g	1.5 ml	1500 µl	150 µl	150 µl
0.075g	1.125 ml	1125 µl	112.5 µl	112.5 µl
0.05g	0.75 ml	750 µl	75 µl	75 µl

Mass Study – 0.5g

To evaluate if increasing the amount of tooth powder would increase the amount of DNA recovered from the sample, two teeth were extracted in duplicate with an input of 0.5 g of bone powder. Extractions were performed in 15 ml conical tubes used to maintain the ratio required for complete demineralization that had been subject to UV light (3.0 J/cm^2). Each extraction reaction contained 0.5g of tooth powder ,7.5 ml bone incubation buffer, 750 µl Proteinase K(concentration) and 750 µl DTT. The samples were incubated at 56°C overnight on a shaker, at maximum velocity, so that the liquid

had constant movement and access to all of the tooth powder.. After incubation, the samples were concentrated using an Ultra-4 100 that had been subject to UV light (3.0 J/cm^2). Since the teeth were assumed to be fresh and non-degraded the high molecular weight filters were used. As Ultra-4s capacity is approximately 3.5 ml of solution the extract was added in two stages, each centrifuged at 4000Xg for 5 minutes in a fixed rotor centrifuge until approximately 400 µl of sample remained. Care was taken to not transfer any of the residual bone powder into the filter cup. The retentate was transferred into a 1.7 ml microcentrifuge tube that had been subject to UV light (2.0 J/cm^2) and the Fresh Bone Protocol was then followed for the remaining extraction.

Mass Study – 0.5g Qiagen

Qiagen MinElute® PCR Purification Kit was evaluated in place of DNA IQ™ to determine the effectiveness of an alternative silica-based extraction method on tooth DNA recovery. One half gram of tooth powder from the same teeth used in the previous evaluation was added to a 15 ml conical tube that had been subject to UV light (3.0 J/cm^2) with 7.5 ml of demineralization buffer, 100 µl Proteinase K and100 µl 1M DTT. The samples were incubated for 4 hours on a shaking platform at maximum RPM for constant movement of the liquid.

To test the concentration ability of Ultra-4 100s and 30s the samples were concentrated after incubation for the tooth sample that had enough remaining powder to be processed in duplicate. The samples were concentrated to 100 µl and transferred using a pipette. The Qiagen MinElute® protocol was then followed as outlined below.

Qiagen MinElute® PCR Purification Kit

1. 5 volumes (500 µl) of Buffer PBI was added to 1 volume (100 µl) of the sample, (tooth powder, demineralization buffer and Proteinase K), and mixed in a MinElute® column and placed in a provided 2 ml collection tube. The color was verified to be yellow.
2. The tube and column were centrifuged using tabletop microcentrifuge for 1 minute.
3. The flow-through was discared. The MinElute® column was placed back into the sample tube
4. 750 µl Buffer PE was added to the MinElute® column and centrifuged for 1 minute.
5. The flow through was discarded and the MinElute® column was placed back in the same tube. The column was centrifuged using a tabletop microcentrifuge for an additional 1 minute.
6. The MinElute® column was placed in a clean 1.5 ml microcentrifuge tube.
7. 100 µl (maintaining same elution volume as DNA IQ™ protocol) Buffer EB was added to the center of the membrane, the column was incubated at room temperature for 1 minute and then centrifuged for 1 minute using a tabletop. microcentrifuge. Samples were stored at 4°C until amplification.

Quantification

Quantification of DNA samples was completed by AFDIL technicians and analysts. The Quantifiler™ Human DNA Quantification Kit from Applied Biosystems (ABI) was used with q-PCR performed on an ABI Prism® 7000 Sequence Detection system. Printouts of quantification analysis results were provided with the concentration (ng/µl) of DNA for each sample.

Promega PowerPlex® 16 Amplification

The DNA was amplified on GeneAmp PCR system 9700 thermalcycler using Promega's PowerPlex® 16 in duplicate reactions. Dilutions were performed if necessary to achieve a target input amount of DNA at 0.5ng to 1ng. The following amplification protocol was followed.

1. The sterile water and amplification tubes were subjected to UV light (3.0 J/cm^2 and 2.0 J/cm^2 respectively) and all other reagents were allowed to defrost.
2. An amplification master mix was made with sterile water, Promega Gold Star 10x buffer, Promega PowerPlex® 16 10x Primers and Applied Biosystems AmpliTaq Gold. Two more reactions were used in the calculation then needed for samples to allow for loss in pipetting.

Table 2 - PowerPlex® 16 Master Mix

Reagent	Volume (µl)
Sterile Water	9.2
Promega Gold Star 10x buffer	2.5
Promega PowerPlex® 16 10x Primers	2.5
Applied Biosystems AmpliTaq Gold	0.8

3. Reagent blanks were prepared first followed by samples and then the positive and negative control.
4. Water was added first, followed by 15 µl master mix and then the necessary volume of sample. The total volume for all reactions is 25 µl.
5. Samples were centrifuged so all liquid was at the bottom of the tubes.
6. Samples were the amplified for 30 cycles following the manufacturer's recommended cycling parameters and then stored at 4°C until electrophoresis was performed.

Fragment Analysis

Electrophoresis was performed on an ABI Prism® 3130xl Genetic Analyzer and fragment analysis was performed using PowerPlex® 16 in GeneMapper® ID. The capillary array was 36 cm. Samples were injected for 5 seconds. Performance Optimized Polymer (POP) 4 was used for analysis. PowerPlex® 16 Advanced Analysis was the parameter set used. The raw data was saved as unedited and all printed electropharograms were printed and included in the validation notebook. The interpretation threshold was set at 100 Relative Fluorescent Units (RFU).

CHAPTER V

RESULTS AND DISCUSSION

Table 3 summarizes the type, condition and mass of the teeth that were pulverized using the freezer mill in this validation study. The pre-and post-grinding weight was recorded for all of teeth except for the first two which were accidentally omitted. The average tooth weight was 1.89g (2.03g not including tooth 1 or 2) the average tooth powder weight was 1.92g and therefore the average loss was 0.11g.

DNA Extraction from Teeth using Fresh Bone Protocol

The DNA quantification results for tooth 1 and 2 extracted for the Fresh Bone Protocol are in Table 4. The reagent blank had no detectable amounts of contamination from the reagents used. The quantification results indicate low amounts of DNA with the smaller amount of input powder actually having more DNA extracted from the samples which was unexpected. Tooth number 1 gave a partial profile with dropout occurring at loci D21S11, D18S51, Penta E, D13S317, CSF1PO, Penta D, D8S1179, TPOX and FGA in at least one of the samples but dropout was not seen at all of these loci in every sample. Tooth number 2 was from a male individual as indicated by one of the samples at the amelogenin locus. Dropout could not have occurred since males have two different alleles and both were indicated in the electropherogram. The electropherograms (Figure 4) are consistent with DNA that is degraded. Allelic dropout occurred at the larger loci

as predicted for DNA degradation. The RFU levels of many of the alleles are close to not exceeding the call threshold and many are below the 100 RFU interpretation threshold. The electropherogram from Sample TG-1B1 (0.1g) in Figure 4 is an accurate representation of all the samples extracted with this protocol.

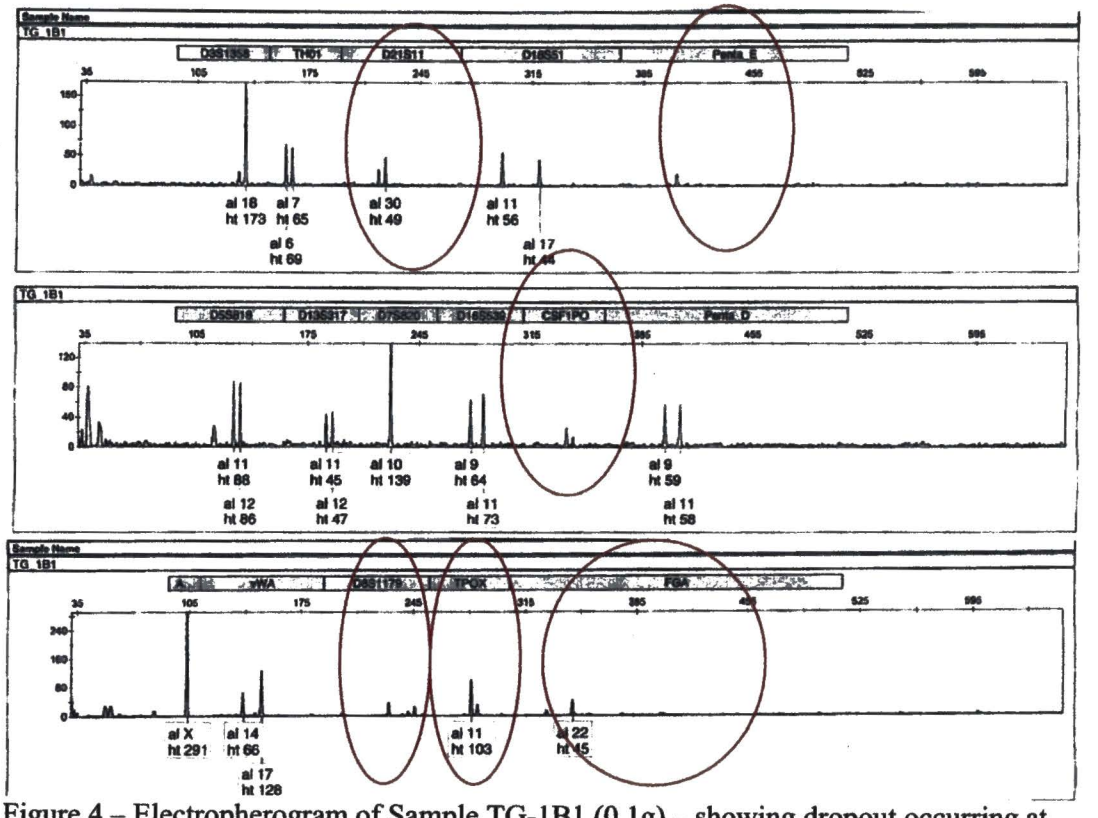

Figure 4 – Electropherogram of Sample TG-1B1 (0.1g) – showing dropout occurring at several loci indicated by red circles and low RFU values for all alleles.

Table 3 – Condition of Teeth

Tooth Number	Tooth Type	Condition of Tooth	Weight of Pre-Ground Tooth	Weight of Tooth Powder
1	Small Molar	No visible cracks – root is not divided seems to be broken off at end but significant portion remaining	1.4g	-
2	Small Molar	No visible cracks – small dark line on chewing surface – two separated roots, complete with no broken ends	1.1g	-
3	Very Small Molar	No visible cracks – tooth in good condition	0.8g	0.7g
4	Medium Sized Molar	No visible cracks – one root was cracked at bottom – tooth had to be cracked/split	1.7g	1.5g
5	Very Small Molar	No visible cracks – One root that does not split – some discoloration in chewing surface, most gone after cleaning	1.3g	1.2g
6	Large Molar	No visible cracks – three roots, one that splits into two – tooth had to be cracked/split - When tooth was cracked a small amount of liquid appeared in crucible	2.6g	2.5g
7	Very Large Molar	No visible cracks – Complete roots, no broken tips – tooth required cracking multiple times	3.4g	3.1g
8	Very Large Molar	No visible cracks – Two roots with complete ends, not broken – Once tooth was cleaned "pinhole" was in bottom of root – tooth had to be cracked/split	2.8g	2.7g
9	Very Large Molar	No visible cracks -chewing surface was very white, possible fillings – small hole is side of chewing surface did not appear to enter central cavity	2.4g	2.4g
10	Very Small Molar	No visible cracks – One root that was not split, no broken ends	1.1g	1.0g
11	Large Molar	No visible cracks – tooth had to be cracked	2.2g	2.2g

Mass Study – 0.1g, 0.075g, 0.05g

The DNA quantification results for tooth 5 extracted for the mass study are listed in Table 4. One large tooth had enough mass to be able to be used for this entire test. The reagent blank had no detectable amounts of contamination from the reagents used. The results are below the lower limit of the quantification standard curve. Most of the 0.05g input amount of tooth powder did not produce detectable amounts of DNA. Amplification was not attempted since the quantification results indicated such a small amount of DNA was present in these samples.

Mass Study – 0.5g

The quantification results for tooth 4 and 7 used for the 0.5g Mass Study are listed in Table 4. The reagent blank had no detectable amounts of contamination from the reagents used. Tooth 7 has consistently higher quantification values representing the variation of amount of DNA previously reported between different tooth samples (15,16). These samples were quantified twice, once prior to this test and again with the Qiagen comparison. The ones reported were quantified simultaneously. Tooth number 7 gave a partial profile with dropout occurring at loci D3S1358, THO1, D21S11, Penta E, D7S820, CSF1PO, Penta D, D8S1179, TPOX and FGA in at least one of the samples but dropout was not seen at all of these loci in every sample. Tooth number 4 had three alleles called in the samples tested. The electropherogram from sample TG-7A2 0.5g is shown in Figure 5 and is an accurate representation of all the samples extracted with this protocol.. The electropherograms are consistent with DNA that is degraded (refer to

Figure 5). Allelic dropout occurred at the larger loci as predicted for DNA degradation. The RFU levels of many of the alleles are close to not exceeding the call threshold and many are below the 100 RFU interpretation threshold.

Mass Study – 0.5g Qiagen

The quantification results for tooth 4 and 7 used for the 0.5g Mass Study using Qiagen MinElute® PCR Cleanup are listed in Table 4. The amount of tooth powder used for each sample is noted in the sample name, sample 4C and 7C were concentrated using Ultra-4 30s and 7D used an Ultra-4 100. The reagent blank had no detectable amounts of contamination from the reagents used. The quantification results are the highest amounts of DNA extracted in any of the evaluations but are still lower than expected. Amplification was attempted with an input amount of DNA at 1.0 ng. Tooth number 7 gave a partial profile with dropout occurring at most of the loci in at least one of the samples. Tooth number 4 had no allele calls but many of the peaks appeared to be just under the 100 RFU threshold.

The amplifications were repeated with a target input amount at 0.5ng to determine if inhibition of the PCR was occurring. Due to time constraints I was unable to perform fragment analysis on the samples after the second amplification. The Ultra-4s generated quantification results contrary to what would be expected since the filter with the lower NMWL had lower results then the filter with the higher NMWL.

Figure 6 shows the amount of DNA extracted from the tooth samples based on how much input powder in each sample. The Qiagen MinElute® PCR Purification Kit

yielded consistently higher amounts of DNA extracted from the tooth powder. The amount of DNA indicated in the quantification results is consistent with the incomplete genetic profiles that were generated. The quantification results are perhaps an overestimation of the DNA present in the sample. Quantification measures the amount of DNA based on smaller DNA fragments then amplification. Amplification using Promega's PowerPlex® 16 will be unsuccessful when only small fragments of DNA are present even though quantification results indicated enough DNA present in the sample for successful amplification.

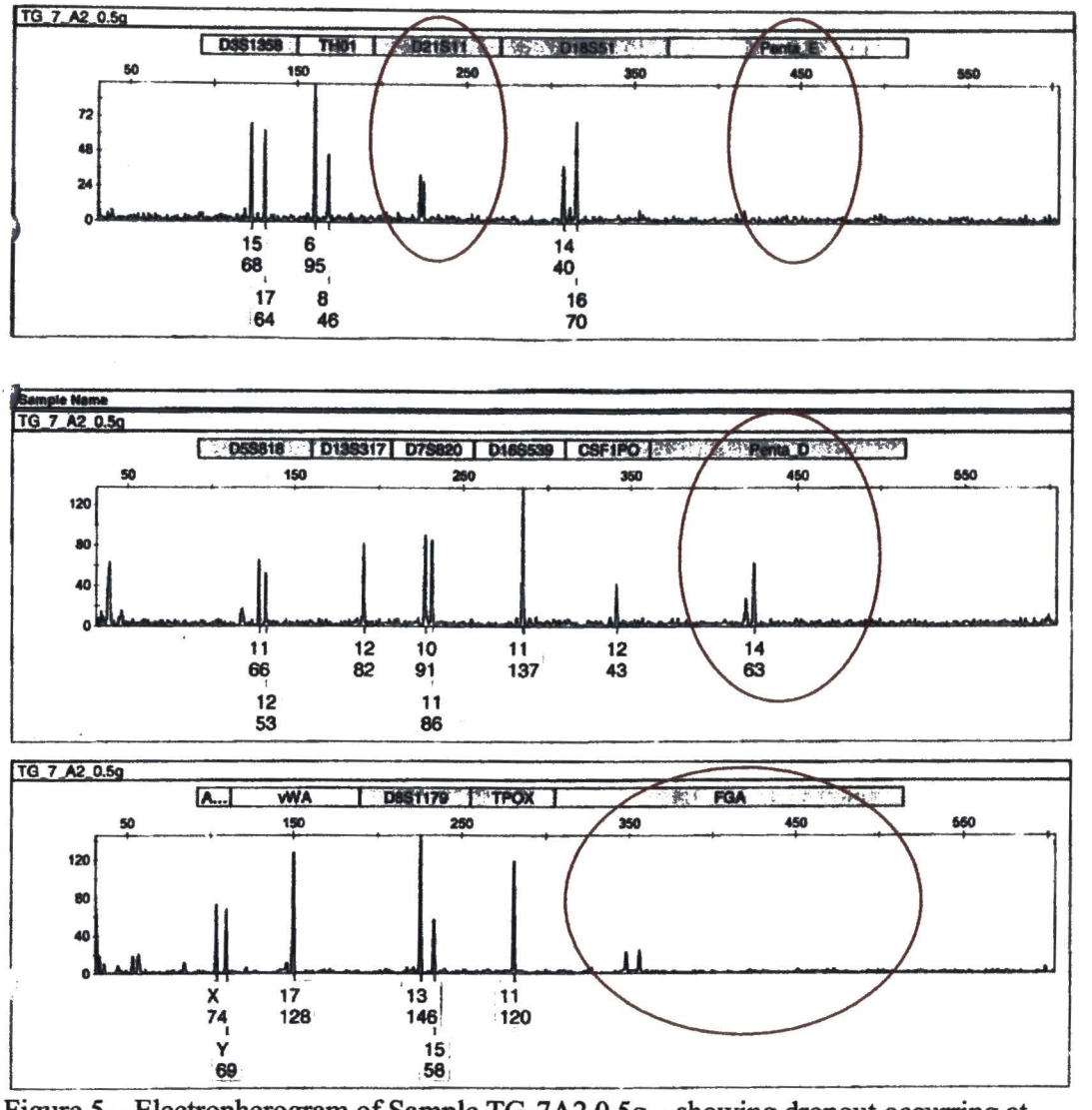

Figure 5 – Electropherogram of Sample TG-7A2 0.5g – showing dropout occurring at several loci indicated by red circles and low RFU values for all alleles.

Table 4 – Overall Quantification Results – The concentration of DNA (ng/µl) and the quantity of DNA in each extracted tooth sample (eluted at 100 µl)

Sample	Concentration (ng/µl)	Amount of DNA (ng)
TG – RB1 KAB	UND	-
TG – 1 A (0.2g)	0.2020	20.20
TG – 1 B (0.1g)	0.2567	25.67
TG – 2 A (0.2g)	UND	-
TG – 2 B (0.1g)	0.0133	1.33
0.075g TG – 5 A1	0.0025	0.25
0.075g TG – 5 A2	0.0053	0.53
0.075g TG – 5 B1	0.0149	1.49
0.075g TG – 5 B2	0.0045	0.45
0.05g TG – 5 A1	0.0052	0.52
0.05g TG – 5 A2	UND	-
0.05g TG – 5 B1	UND	-
0.05g TG - 5 B2	UND	-
RB 1 and RB 2 TG- 5	UND	-
0.5g TG – 4 A	0.0077	0.77
0.5g TG – 4 B	0.0034	0.34
0.5g TG – 7 A	0.0378	3.78
0.5g TG – 7 B	0.0676	6.76
0.5g RB	UND	-
0.5g TG – 4 C Sil	0.1570	15.7
0.5g TG – 7 C Sil	0.1450	14.5
0.5g TG – 7D Sil	0.3950	39.5
0.5g RB Sil	UND	-

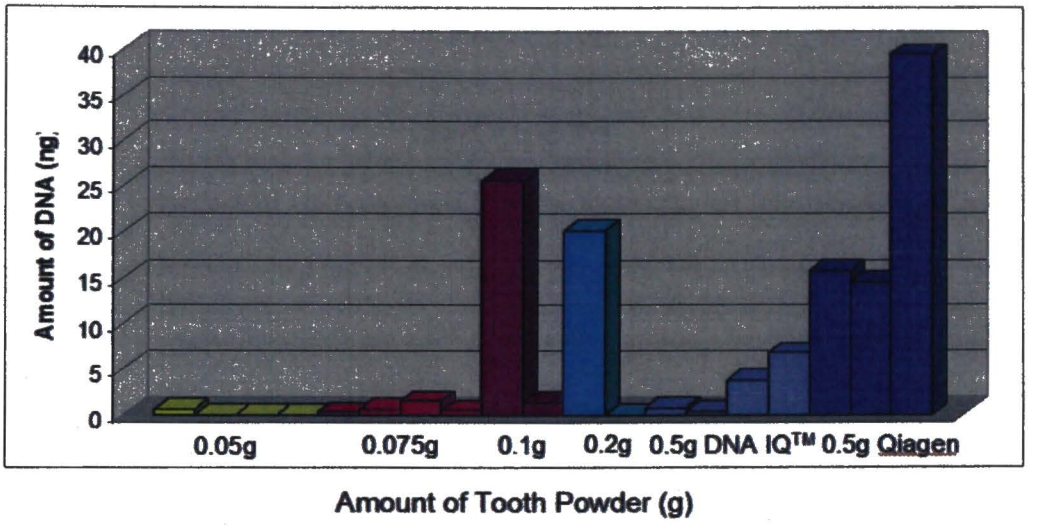

Figure 6.- Overall Quantification Results – The amount of DNA present in the samples as shown by the amount of input tooth powder.

CHAPTER VI

CONCLUSIONS

Due to the lack of DNA obtained from the tooth samples full validation of the procedures performed could not be completed. All of the tested samples produced low quantification results for validating a DNA extraction protocol based on high molecular weight DNA samples. The lack of DNA recovery could be caused by several different factors. The amount of tooth powder used, the submersion in bleach, the loss of DNA during concentration, the extraction procedure not capturing enough DNA, the amount of Proteinase K or the tooth samples being treated in a fashion detrimental to the DNA could all contribute to low amount of DNA recovered.

The amount of input tooth powder was tested in this validation study to determine which volume produced the greatest DNA yield. Further testing of the methods used in this evaluation should be performed with an input of 0.5g of tooth powder. An amount of 0.5g of tooth powder has produced results in other studies and looked promising in this validation study to provide enough DNA to be extracted to generate full genetic profiles (16). At 0.5g the ratio for complete demineralization could be achieved without altering the equipment used for bone samples extracted with the Fresh Bone Protocol since it can be carried out in a 15ml conical tube with concentration in the Ultra-4s.

The Qiagen MinElute® method needs additional evaluation to determine if it consistently yields more DNA than Promega Corporation's DNA IQ™ and would be a better method for use on tooth samples. There could possibly be something present in the

composition of teeth that affects the ability of the DNA to bind to silica. Both methods used in this evaluation were silica capture extraction techniques and research has indicated that teeth yield higher amounts of DNA when extracted with organic DNA extraction techniques. The Ultra-4s also need to be tested further since they produced unexpected results with the larger filter size producing higher quantification results then the smaller filter size.

Although several studies have indicated that submersion in bleach breaks down DNA (12,23). The tooth itself should provide enough protection to the DNA inside the pulp chamber and therefore enough DNA should be present to generate a complete profile. However, due to the low amounts of DNA being recovered in these evaluations the cleaning method must be considered to see if it is degrading the DNA. The tooth could be wiped with bleach and ethanol without submersion and sonication in the bleach. Cleaning the tooth with a bleach moistened wipe should remove most of the exogenous DNA without compromising the sample. Since this validation study looked at STR data and without implementing any low copy number protocol, such as increasing the number of amplification cycles, there should not be an issue with mixed profiles due to the amount of DNA present in the tooth compared to the small amount of contaminating DNA after a thorough cleaning method of the outside of the tooth.

Another option that could have contributed to the low quantification results was the amount of Proteinase K that was used for the extractions with DNA IQ™. High volumes of Proteinase K in solution can actually have reverse effect on it's ability to breakdown proteins (Odile Loreille, personal communication 2008). The enzyme will

actually start breaking down itself and therefore will not be available to help in the breakdown of the tooth powder and release of DNA. Due to this reason we decreased the amount of Proteinase Kadded in the Qiagen MinElute® protocol compared to the Fresh Bone Protocol used in the majority of the validation study.

The final option that must also be considered is the source of the teeth. There have been documented cases of successful DNA extraction and STR analysis from teeth buried in soil but perhaps there was some form of treatment on the teeth sampled in these tests that degraded the DNA. The storage of the teeth in a moist environment could have promoted bacterial growth which degraded the DNA. Teeth from another dentist or with documentation on the treatment of them since removal from the patient could be tested to determine if this protocol does in fact generate full STR profiles from tooth samples. Buccal swabs from the subjects who donated their teeth would also be necessary as reference samples to compare the generated genetic profiles to correctly validate the process.

The limitations of this project noted at the beginning were the teeth that were provided. The genetic reference profiles for these teeth are unknown and therefore I can not be sure that the profile generated is that of the donor. Since the quantification results were low and time was spent testing other methods, teeth samples from a known source were not tested.

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