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The extraction method for the recovery of DNA from bone samples at the UNT Center for Human Identification (UNTCHI) Missing Persons Laboratory is both time-consuming and laborious. The results of this study show that the AutoMate Express™ can yield both DNA quantity and STR profiles comparable or greater to that of the standard organic extraction method.

The incorporation of this automated technology could significantly reduce the time and streamline the process while increasing the amount of genetic information obtained. Based on the results of this study, the use of the AutoMate Express™ for the extraction of DNA from skeletal remains could be very advantageous to UNTCHI DNA analysts.

EFFECTS OF pH ON BINDING OF DNA USING THE PREPFILER BTA™
FORENSIC DNA EXTRACTION KIT ON THE AUTOMATE
EXPRESS™ DNA EXTRACTION SYSTEM

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THESIS

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CHAPTER I

INTRODUCTION

The analysis of polymorphic regions of DNA has been used in forensic casework since the mid-1980s (1). A common method for DNA analysis targets highly variable genetic loci referred to as short tandem repeats (STR) markers. STR typing kits can target and amplify up to 15 of these highly polymorphic regions for analysis providing the ability to discriminate between individuals.

DNA analysis from unidentified human remains (UHR) involves many steps, including: the extraction of DNA from bone samples; quantification of the extracts to determine the amount of DNA present; normalization of the sample DNA concentration; amplification of the targeted STR markers; capillary electrophoresis for the separation of the amplified targets; and the analysis of the resulting profiles. The extraction and optimized recovery of DNA is paramount in this process. A minimum quantity and quality of DNA is required for amplification to obtain the most informative genetic profiles.

There have been many improvements since the first forensic applications of DNA analysis from common evidentiary samples, such as blood and semen. Many of these improvements have greatly increased the ability to obtain quality genetic profiles. Despite these improvements, the extraction of DNA from bone samples still remains a challenge. Depending upon their age and environmental exposure, DNA from bone samples can

be limited as well as highly degraded. In addition, known PCR inhibitors can co-purify with the extracted DNA. All of these factors can negatively impact the PCR amplification and the quality of the DNA profiles generated.

Typically with missing person's cases, only skeletal remains are available as an evidentiary source of DNA. The number of unsolved missing persons cases has increased greatly over time. On any given day, there are between 85,000 and 100,000 active missing persons cases in the United States (2), with 87,000 active cases reported in the National Crime Information Center in December 2012 (3). Furthermore, the 2004 Medical Examiners and Coroners Bureau of Justice Statistics Report stated that in a typical year medical examiners and coroners offices nationally handle approximately 4,400 unidentified human remains with approximately 1,000 remaining unidentified after one year (4). Due to the large number of missing persons cases, bone extraction methods must be as efficient as possible in order to maximize the recovery of DNA and genetic data obtained.

There are a number of methods currently used to extract DNA from bone. The phenol chloroform isoamyl alcohol (PCIA) organic extraction method is the most common method used for DNA extraction. Prior to extraction, the bone sample must first be pulverized. A SPEX CentriPrep 6750 Freezer/Mill® Grinder (SPEX SamplePrep, Metuchen, NJ) with liquid nitrogen is first employed to pulverize the bone. The result is a fine to course powder from which the DNA can be extracted. Following pulverization, current procedures for the recovery of DNA include a demineralizing lysis step. A study by Loreille *et al.* has shown that complete demineralization improves yields of DNA (5). Bone mainly consists of hydroxyapatite, an inorganic mineral containing: calcium phosphate, calcium carbonate, calcium fluoride, calcium hydroxide, and citrate. These minerals can prevent the release of DNA by blocking access of

extraction reagents to the DNA. The demineralizing step allows the release of DNA and therefore improves DNA yield (5). During demineralization, the bone powder is incubated in an extraction buffer containing detergents, proteinases, and ethylenediaminetetraacetic acid (EDTA). The detergent and proteinases act together to disrupt the cell membrane and digest proteins. EDTA is added to the lysis buffer to dissolve the bone structure and also inactivate DNases by chelating cations that are necessary for DNase function. Following demineralization, a PCIA mixture is added to the samples to separate the DNA from other cellular components, such as proteins and lipids. DNA is more soluble in the aqueous portion of the mixture whereas proteins, lipids, and other cellular components are more soluble in the organic portion. The aqueous phase, containing the DNA, is then removed for further analysis. While PCIA is a cost-effective method for the isolation of DNA from bone, it is both laborious and time-consuming, as the initial lysis/demineralization step can take up to a day to complete. The organic reagents are not only hazardous to the analyst but require multiple manual tube transfers increasing the likelihood for both sample loss and contamination.

Bench-top automated extraction systems are common in the forensic community. Automated systems are advantageous as they can reduce cost and user error, potentially minimize contamination and sample loss, and ultimately shorten extraction time. Examples of automated systems are the Maxwell®16 (Promega Corporation, Madison Wisconsin); the EZ1® Advanced XL (Qiagen, Germantown, Maryland); and the AutoMate Express™ (Life Technologies, Carlsbad, California). All three systems utilize a similar extraction methodology. Polymer-coated magnetic beads, in the presence of a chaotropic salt bind the DNA, followed by several washing steps to remove inhibitors, and the DNA is then eluted in a low salt solution. These platforms were tested and compared using a variety of sample types including bone and

teeth. Across the broad range of sample types, none of the platforms outperformed the others in terms of DNA yield. The AutoMate Express™ and EZ1® Advanced XL showed similar performance when extracting from bone and tooth samples with comparable DNA recovery and genetic profiles (6).

The AutoMate Express™ uses the PrepFiler® or PrepFiler® BTA Forensic DNA Extraction Kits (Life Technologies). Both kits use polymer coated magnetic beads in the presence of a chaotropic salt to bind the DNA and separate it from other materials in solution. The PrepFiler® BTA Forensic Extraction Kit is specifically used when extracting DNA from challenging samples, such as bone, teeth, and adhesive forensic sample types (chewing gum, cigarette butts, tape lift samples). When using bone samples, the bone powder is initially incubated for two hours using the PrepFiler® BTA Lysis solution, which consists of PrepFiler® BTA Lysis Buffer (7) with added dithiothreitol (DTT) and Proteinase K. These reagents lyse the cells, chelate ions from solution, and digest proteins. Following lysis, the sample is centrifuged and the supernatant containing the DNA is transferred to a sample tube. The sample tubes, elution tube, tips, and PrepFiler® cartridges are inserted into the AutoMate Express™ according to the manufacturers configurations. The cartridges contain the PrepFiler® BTA beads, a chaotropic salt (guanidine thiocyanate), sarkosyl, and a chelating salt that act to bind the DNA and chelate ions (8). The PrepFiler® chemistry first binds the DNA through the presence of the chaotropic salt forming a salt bridge between the negatively charged DNA and the polymer-coated magnetic bead. Maximum binding of DNA occurs under conditions of high salt and low pH allowing the salt bridges to form between the DNA and the particle (11). Following binding to the PrepFiler® beads, wash buffers are used to remove degraded proteins and other inhibitors. The wash buffers do not disrupt the salt bridge between the DNA and the particle, allowing the

DNA to remain bound during the wash steps. The DNA is then eluted using the PrepFiler® Elution Buffer, which appears to be similar to TE⁻⁴ buffer (9). While the DNA binds to the column under high salt conditions, DNA can be eluted using buffers with low salt concentration. A graphical depiction of the PrepFiler® cartridge and its compartments is shown in Figure 1.

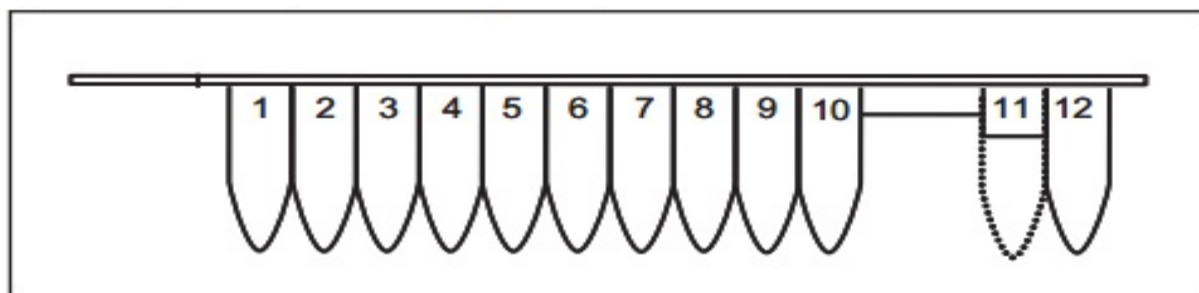


Figure 1: PrepFiler® Cartridge. Tube 1 contains a lysis buffer. Tube 2 contains the magnetic particle suspension. Tube 3 contains the binding solution. Tubes 4 through 6 contain wash buffers. Tube 7 contains the elution buffer. Tube 12 is a heated chamber for elution. The remaining tubes are not utilized with the BTA Protocol. (Photo from Life Technologies)

UNTCHI has traditionally used an extensive demineralization process followed by the organic extraction method. The quantity of DNA recovered using the PrepFiler® chemistry and AutoMate Express™ have shown to be similar to the organic extraction method (10). However, we hypothesize that the amount of DNA obtained using the AutoMate Express™ System can be optimized which could result in a greater yield than the manufacturer's recommended protocol. We believe there is inefficient binding of DNA to the magnetic particle due to the pH level of the sample after incubation with PrepFiler® BTA Lysis Buffer. The reagents in the PrepFiler® BTA Forensic DNA Extraction Kit have a pH of 8.1 (7); however, other studies have been shown that when the pH of the reagents are below 8, DNA yield increases with the silica based extraction method. Lowering the pH changes the surface protonation state of the silica and increases the DNA binding (11). This problem was identified on the EZ1® Advanced XL system with the EZ1® DNA Investigator Kit (Qiagen) by the North Louisiana Criminalistics Laboratory (12).

They increased DNA yield through the addition of 3 M sodium acetate (NaOAc), pH 5.2, to increase DNA binding to the column. The use of 3M NaOAc both decreased the pH of the lysis solution and also potentially increased the salt concentration. NaOAc was added after incubation of the bone sample and before purification. The results showed that DNA yield was increased by the addition of NaOAc. When no NaOAc was added DNA recovery was approximately 0.03 ng/200 mg bone powder. Total DNA yield increased to 0.15 ng/200 mg of bone powder when 10µl of 3M NaOAc was added and further increased to 0.55 ng/200 mg of bone powder with 20 µl of 3M NaOAc. There was no increase in DNA yield beyond the addition of 20 µl of 3M NaOAc. This study showed the pH dependence of DNA absorption to the silica membrane and determined that if the pH of the solution is above 7.5, DNA recovery was decreased (13). This study did not mention an optimized pH at which binding occurred, but only that the addition of 3M NaOAc, pH 5.2 to lower the pH increased DNA yield.

The PrepFiler® BTA Forensic DNA Extraction Kit and the EZ1® DNA Investigator Kit utilize similar chemistries for extraction of DNA. Following lysis, both platforms use polymer-coated magnetic based DNA purification to separate and purify the DNA. Binding to the polymer beads in the presence of a chaotropic salt separates DNA from the lysis solution followed by washing and eluting of the purified DNA. The PrepFiler® BTA Forensic DNA Extraction Kit contains the chaotropic salt guanidine thiocyanate where the EZ1® DNA Investigator® Kit contains a salt solution with guanidine thiocyanate and guanidinium chloride. Where it is known that the EZ1® DNA Investigator Kit contains silica in the polymer coating, the components of the PrepFiler® BTA Kit are proprietary so it is unknown if silica is present in the polymer coating.

Based on recent studies (13), we believe there is a potential to increase DNA yield from bone samples using the AutoMate Express™ with the PrepFiler® BTA Forensic DNA Extraction Kit. There are significant advantages in optimizing this system for bone samples. Extraction is performed in an automated manner after loading of the sample tube, cartridges, and elution tube. This method has fewer tube transfers and is less likely for sample switching or contamination. The AutoMate Express™ can process 13 samples in about 30 minutes and the overall extraction time (including lysis and incubation) decreases from multiple working days via the organic method to 2.5 hours. The organic extraction method currently employed at UNTCHI Missing Persons Laboratory can take up to two and a half working days to complete. The demineralization process requires a 12-18 hour incubation period, followed by multiple reagent additions; several tube transfers, and a number of centrifugation steps, making it both time-consuming and laborious for the analyst. However, the PrepFiler® BTA lysis step requires a 2-hour incubation period and the bone lysate is loaded into the PrepFiler® columns, bound, washed, and eluted in 30 minutes. The AutoMate Express™ extraction system carries out the same steps in approximately two and a half hours, saving more than two days in processing time to recover the DNA. Figures 2 and 3 contrast the two extraction methods.

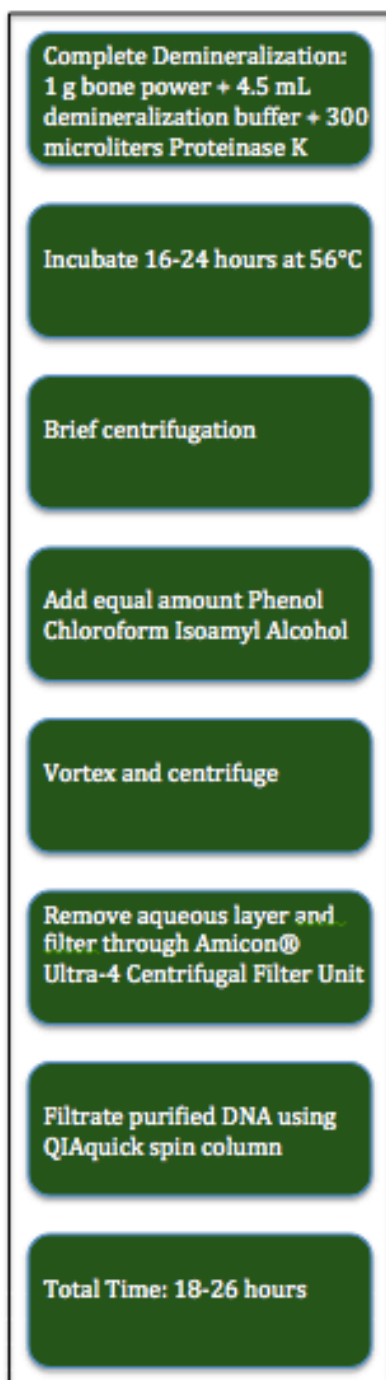


Figure 2: UNTCHI Organic Extraction Protocol. Outline of the standard operating procedure used in UNTCHI Missing Persons Laboratory for the organic extraction method.

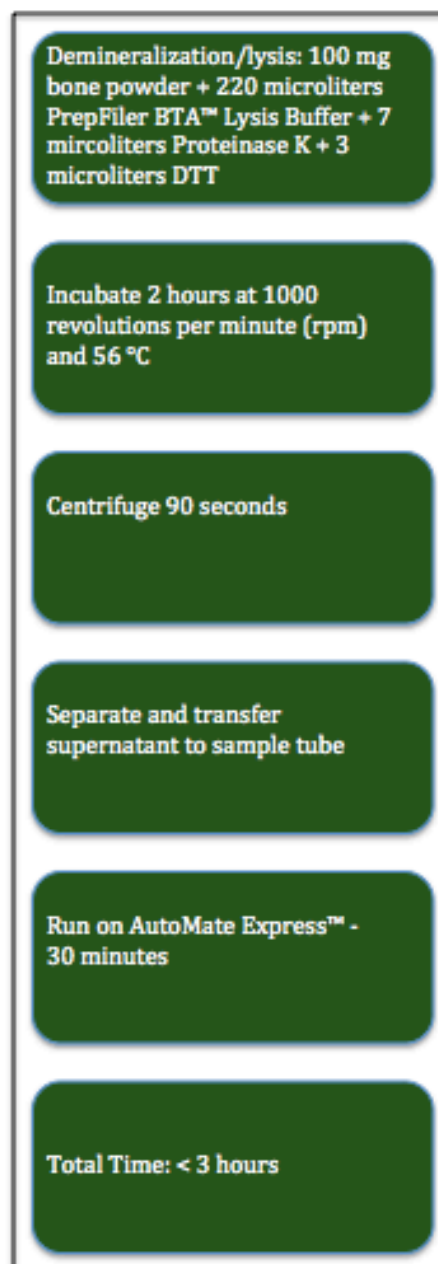


Figure 3: AutoMate Express™ Protocol. Outline of the AutoMate Express™ procedure based on the manufacturers recommendation.

The incorporation of the new extraction chemistries, combined with the automated extraction systems, could increase DNA yield from bone samples and decrease turn around time by streamlining the process. For these reasons, optimizing this method would be very advantageous to UNTCHI DNA analysts for the extraction of DNA from bone solution.

CHAPTER II

MATERIALS AND METHODS

Bone Sample Selection

Three cadaver bone samples from the UNTHSC Willed Body Program were selected to initially determine the recovery of DNA with the AutoMate Express™ and PrepFiler BTA™ chemistry. In addition, three casework bone samples previously processed by the UNTCHI Missing Persons Laboratory were selected based upon the quality of the genetic profiles obtained. The DNA yield and the quality of the profiles were compared to the results of this study. Table 1 outlines the samples used in this study.

Sample Name	Weight	Sample Type	Bone Type
Sample 1 - Left Tibia	100 mg/sample	Cadaver Bone	Tibia
Sample 2 - Left Humerus	100 mg/sample	Cadaver Bone	Humerus
Sample 3 - Right Humerus	100 mg/sample	Cadaver Bone	Humerus
Sample 4 - UNTCHI-0073	100 mg/sample	Casework Bone	Femur
Sample 5 - UNTCHI-0079	100 mg/sample	Casework Bone	Femur
Sample 6 - UNTCHI-0080	100 mg/sample	Casework Bone	Femur

Table 1: Bone Samples Used in This Study. Samples were provided from the UNTHSC Willed Body Program and the UNTCHI Missing Persons Laboratory. Provided is the sample name, weight, sample type and bone type for each of the bones used in this study.

Bone Sample Preparation

Bone sample preparation was performed following UNTCHI's protocol, "Preparation of Skeletal Remains and Teeth for DNA Extraction". All work was done inside a negative airflow sanding station to prevent loss of sample and contamination. Prior to use, all required equipment were cleaned and UV irradiated following UNTCHI's protocol, "Irradiation of Reagents and Supplies in the Ultraviolet Crosslinker" to remove any DNA or contaminants from the tools. The part of the bone to be cut (about three by two inches) was sanded with a Dremel® (Racine, WI) tool and sanding cone or barrel and wiped with 5% Tergazyme™ (ALCONOX, White Plains, NY) to remove any remaining dust. The sanded area was cut using a Dremel® tool with a cutting disk. The cutting area and tools were bleached between samples. Consumable supplies (sanding cones, barrels, disks) were sterile and single use. Each cut sample was placed into a separately labeled 15 or 50 mL conical tube and 50% bleach was added to each conical tube to cover the bone sample and allowed to soak for 5 minutes. The waste was decanted and the same amount of water was added to each tube followed by shaking and decanting of water. This water wash step was repeated until the water was clean and there was no smell of bleach (approximately 3-6 washes). Lastly, an equal amount of 100% ethanol was added to each tube and then poured off to clean the sample. The sample was removed from its tube and allowed to dry for 30 minutes. After drying the sample was directly added to a labeled 15 or 50 mL conical tube until pulverization was performed.

Cyrogenic Grinding using SPEX 6750 Freezer/Mill

Pulverization of the bone samples was done following UNTCHI's protocol, "Preparation of Skeletal Remains and Teeth for DNA Extraction". Prior to use, all of the required equipment

(including plastic cylinders, metal cap ends, and impactor' s) were cleaned and UV irradiated following UNTCHIs protocol, "Irradiation of Reagents and Supplies in the Ultraviolet Crosslinker" to remove any DNA or contaminants. The cut bone fragments were added to individual cylinders with a maximum of 4 g per cylinder. The cylinders were assembled by inserting the impactor and sealing the cylinder with end caps. Liquid nitrogen was added to the reservoir of the SPEX 6750 Freezer/ Mill ® up to the fill line. After a chill down period of approximately seven minutes, additional liquid nitrogen was added to the fill line if necessary before processing the sample. The cylinder with the fragmented sample was inserted into the freezer mill and allowed to cool for approximately 5 minutes. The sample was ground for approximately 7 minutes and then inspected to ensure proper pulverization. If further pulverization was necessary, the sample was reinserted and subjected to additional grinding. After adequate grinding, the sample was set aside and allowed to return to room temperature (approximately 90 minutes). The end cap of the cylinder and the impactor were removed and the bone powder was weighed and stored in labeled 15 mL or 50 mL conical tubes.

Automated Extraction using AutoMate Express

The manufacturer's procedure for extraction using the AutoMate Express™ was followed for extraction from bone samples. A total of 100 mg of bone powder was used for each sample. The powdered bone sample was transferred into a PrepFiler® Bone and Tooth Lysate Tube (Life Technologies). The PrepFiler® BTA lysis solution was made containing 220 µL PrepFiler® BTA Lysis Buffer; 3 µL freshly prepared 1 M DTT; and 7 µL of Proteinase K (20 mg/ml). The buffer was added to the lysate tube containing the powdered bone sample, the tube was vortexed and centrifuged, and placed in a thermal shaker to incubate at 56°C and 1,100 revolutions per

minute (rpm) for a minimum of 2 hours. The tube was then centrifuged for 90 seconds at 10,000 x g to pellet any remaining bone powder and the lysate was transferred to a new PrepFiler® Sample Tube. If the amount of lysate in the sample tube came out to be less than 230 µL, additional PrepFiler® BTA Lysis Buffer was added to the Bone and Tooth Lysate Tube to bring the lysate volume up to 230 µL. The sample was vortexed and centrifuged again, and the lysate was transferred to the corresponding PrepFiler® Sample Tube. The lysate was now ready for extraction using the AutoMate Express™.

The Automate Express™ instrument was set up by inserting the tip and tube rack, sample tubes, elution tubes, and cartridges according to the manufacturer's configurations. Up to 12 samples and one reagent blank were run at one time (one in each cartridge). After loading the samples, the AutoMate instrument carried out the extraction and purification in an automated manner. The sample was mixed with polymer coated magnetic beads and other reagents for subsequent binding to the magnetic beads; washed to remove PCR inhibitors; and dried to remove ethanol used during the washing steps. The purified DNA was eluted in 50 µL PrepFiler® BTA elution buffer. The final elution tube contained the purified DNA in a volume of 50 µL and was then stored at 4°C.

This extraction method was carried out adding different amounts of 3M NaOAc, pH 5.2 to examine both yield and quality of genetic profiles with respect to pH of the lysis solution. 3 M NaOAc, pH 5.2 was added following the lysis step and prior to the extraction with the AutoMate Express™. The pHs tested in duplicate were 7.9 (original pH after lysis step), 7.0, and 6.0. Previously, the North Louisiana Crime Laboratory had added 3M NaOAc, pH 5.2, (BioVision, Milpitas, CA) to adjust the pH of the binding solutions. The modification to the Qiagen's EZ1 protocol had resulted in an increased DNA yield after the addition of NaOAc following

digestion and prior to purification. 3M NaOAc (pH 5.2) was added to the lysis solution after incubation, and the pH was determined using a Waterproof Double Junction pHTestr® 10 (Oaklon® Instruments, Vernon Hills, IL) with a Micro pH electrode (Fischer Scientific, Pittsburgh, PA) meter prior to use on the AutoMate Express™. Two experiments were performed:

Experiment 1: Samples from three cadaver bones from UNTHSC Willard Body Program were extracted in duplicate at each pH observed (6.0, 7.0, 7.9) for a total of 6 samples from each bone. Each sample was quantified using Quantifiler® Duo in duplicate following extraction.

Experiment 2: Samples from three casework bones previously analyzed by UNTCHI Missing Persons Laboratory were extracted in duplicate at each pH observed (6.0, 7.0, 7.9) for a total of 6 samples from each bone. Each sample was quantified using Quantifiler® Duo in duplicate following extraction.

The pH of the PrepFiler® BTA lysis buffer was adjusted after incubation using 3M NaOAc, pH5.2. Preliminary testing showed that adding 10 and 25 µL of NaOAc lowered the pH to 7.0 and 6.0, respectively.

DNA Quantification

The DNA extracts were quantified following UNTCHI's protocol, "Human DNA Quantification using Applied Biosystems Quantifiler® Kit" using Quantifiler® Duo. Quantification was performed using a 7500 Real-Time PCR System (Life Technologies). The quantification results were compared against a standard dilution series to determine the relative quantity of amplifiable DNA used for STR amplification.

STR Amplification

Nuclear DNA was amplified on a GeneAmp® PCR System 9700 (Life Technologies) using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) following UNTHSC's protocol, "STR Amplification". The thermal cycling conditions followed were: 1) 95°C for 11 minutes 2) 28 or 29 cycles alternating 94°C for 20 seconds and 59°C for 3 minutes 3) 60°C for 10 minutes and 4) a 4°C hold indefinitely. When possible, the target concentration of input DNA for STR amplification was 0.5 to 1.0 ng of DNA. A maximum of 10 µL of template DNA was added to the 25 µL reaction volume.

Capillary Electrophoresis and Data Analysis

The resulting PCR products obtained from amplification were electrophoresed using a 3500 Genetic Analyzer (Life Technologies). The data was analyzed using GeneMapper® *ID-X* software (Life Technologies). Interpretation guidelines from the UNTHSC Center Forensic Excellence validation of the AmpFLSTR® Identifiler® (Life Technologies) PCR Amplification Kit on the 3500 Genetic Analyzer were used. The minimum detection threshold used was 100 relative fluorescence units (RFUs) for heterozygous loci. Additionally, homozygous alleles were only reported if the peak height was greater than or equal to 200 RFUs.

CHAPTER III

RESULTS

DNA Extraction Using the AutoMate Express™

The extraction of DNA from evidentiary samples is a crucial step in the generation of genetic profiles. Bone extraction methods must be very effective in order to maximize the recovery of DNA for subsequent amplification and analysis. Recent studies indicated that when the pH is below 8.0 the binding of DNA is further optimized, in conjunction with silica-based extraction methods from bone samples.

The purpose of this study was to evaluate a modified extraction method utilizing the PrepFiler® BTA Forensic DNA Extraction Kit in conjunction with the AutoMate Express™ Forensic DNA Extraction System. In order to determine the effect of lowering the pH on DNA binding, the pH of the lysis buffer was adjusted using 3M NaOAc (pH 5.2) following incubation of the samples and prior to extraction. The bone samples used in this study were extracted in duplicate at each pH observed (pH 7.9, pH 7.0, and pH 6.0). When 10 and 25 µL of 3M NaOAc (pH 5.2) was added to the lysis solution, the pH was lowered to 7.0 and 6.0, respectively.

This study also aimed to determine if the DNA extracted using this modified protocol would yield quantities of DNA and genetic profiles that were equivalent to or greater than the results

obtained using the current organic extraction method performed at the UNCHI Missing Persons Laboratory.

DNA Recovery Under Different Binding pH of Lysis Solution

Total DNA quantity was determined using the Applied Biosystems Quantifiler® Duo Kit by taking the average of the quantification results for each sample. The internal positive control (IPC) cycle thresholds (Ct) were evaluated to determine if inhibition might be present. Figures 4 and 5 show the Ct results for the cadaver and casework samples. The IPC cycle thresholds were consistent across the samples with both the cadaver and casework samples indicating the assay worked properly and there was no inhibition indicated. The cycle thresholds seen in the casework samples were also indicative of the yields obtained. As expected, a lower cycle threshold correlated with higher DNA yield whereas higher cycle thresholds correlated with lower DNA yields within these samples.

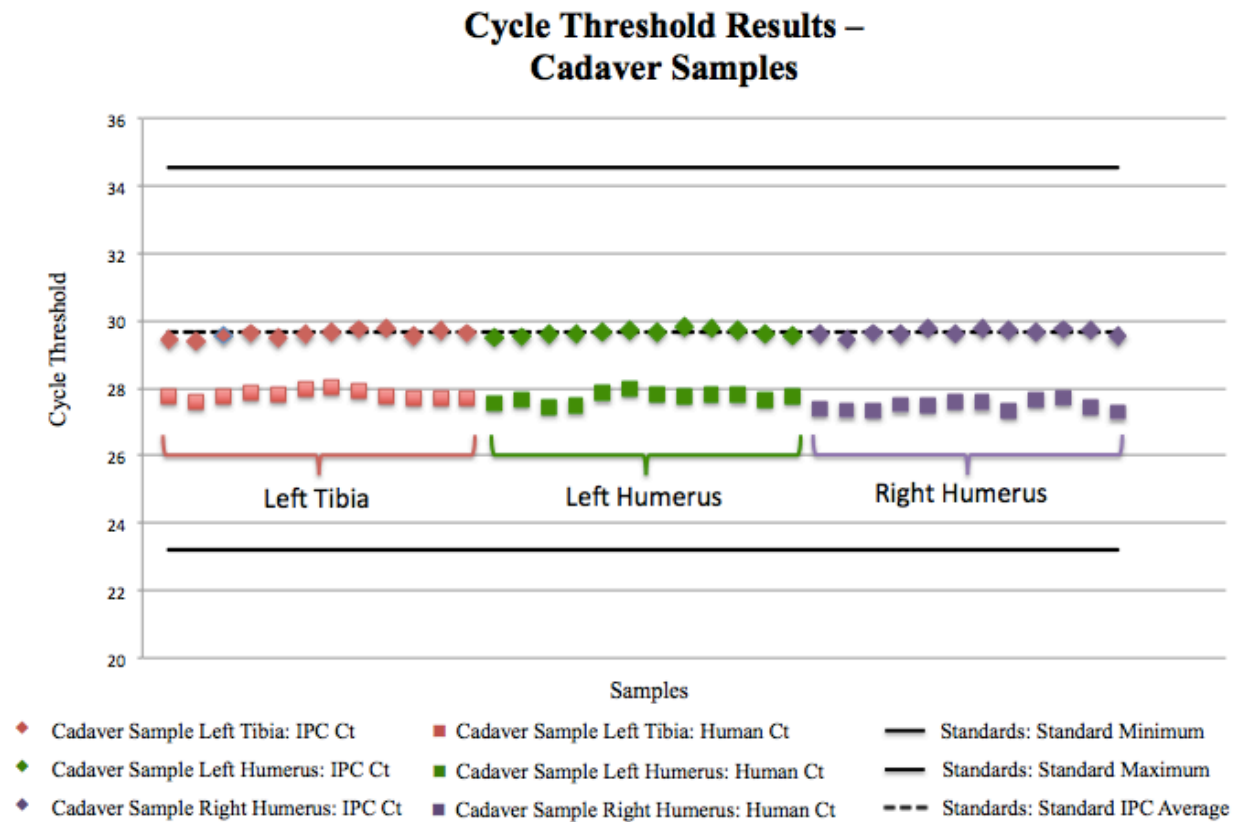


Figure 4. Cycle Threshold Results: Cadaver Samples. A graphical representation of the cycle threshold results for the cadaver samples.

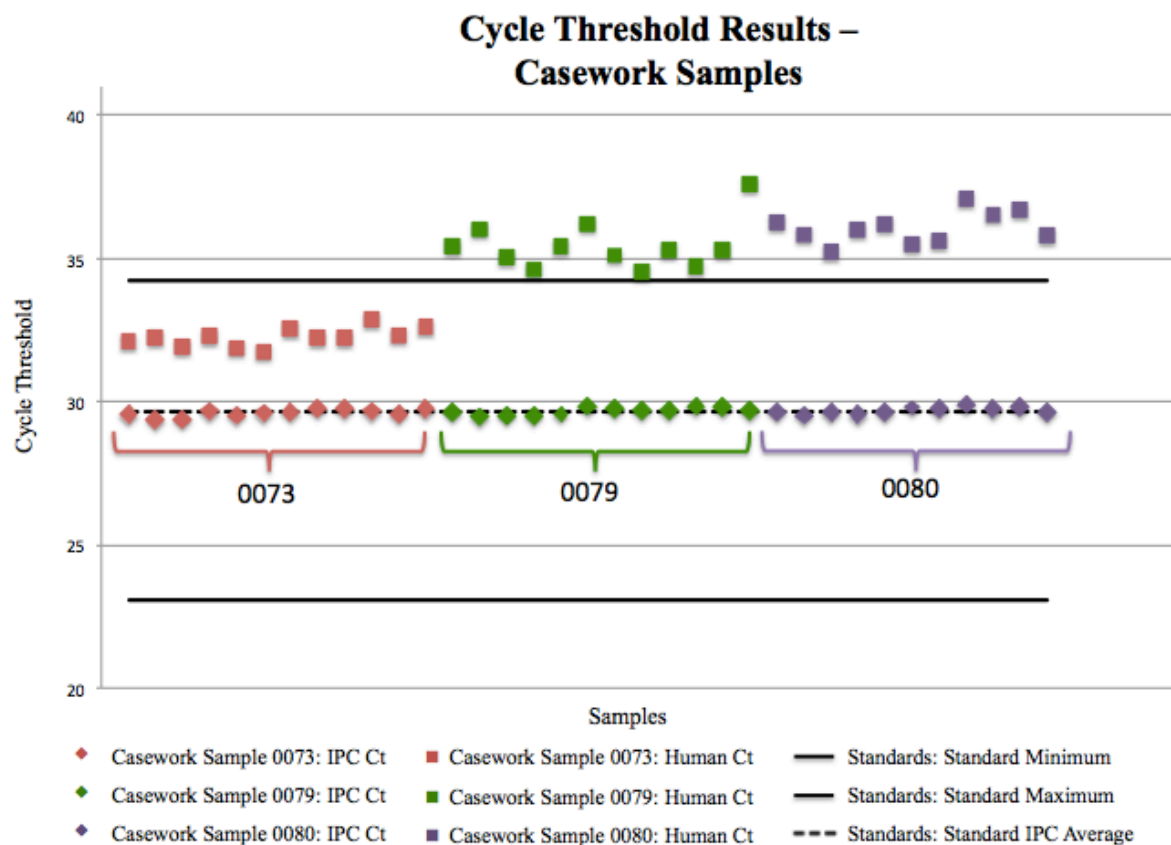


Figure 5. Cycle Threshold Results: Casework Samples. A graphical representation of the cycle threshold results for the casework samples.

Table 2 shows the results of total DNA recovered from each bone sample under the varying pH's. Four of the six samples showed a general decrease in DNA yield with the addition of 3M NaOAc. One of the samples (Left Tibia) showed an initial decrease in DNA yield (pH 7.0); however, the yield increased with the further addition of 3M NaOAc, pH 5.2. One sample (UNTCHI- 0073) showed a slight increase in DNA yield (pH 7.0), however, the DNA yield was significantly lower at pH 6.0.

Sample	AutoMate Express™ SOP	pH 7.0	pH 6.0
	Average Quantity of DNA (ng/100 mg bone powder)	Average Quantity of DNA (ng/100 mg bone powder)	Average Quantity of DNA (ng/100 mg bone powder)
Left Tibia	115.5	101.0	115.8
Left Humerus	133.3	106.8	114.3
Right Humerus	146.3	136.8	134.5
UNTCHI-0073	4.700	4.900	3.670
UNTCHI-0079	0.555	0.555	0.478
UNTCHI-0080	0.365	0.353	0.230

Table 2. Total DNA Recovered Versus pH of Binding Solution. Total DNA recovered is represented as an average of the quantification values for each sample. Yield is represented per 100 mg of bone powder.

Figures 6 and 7 show a graphical representation of the results under the varying pH's for the bones from UNTHSC Willied Body Program and from UNTCHI Missing Persons Laboratory.

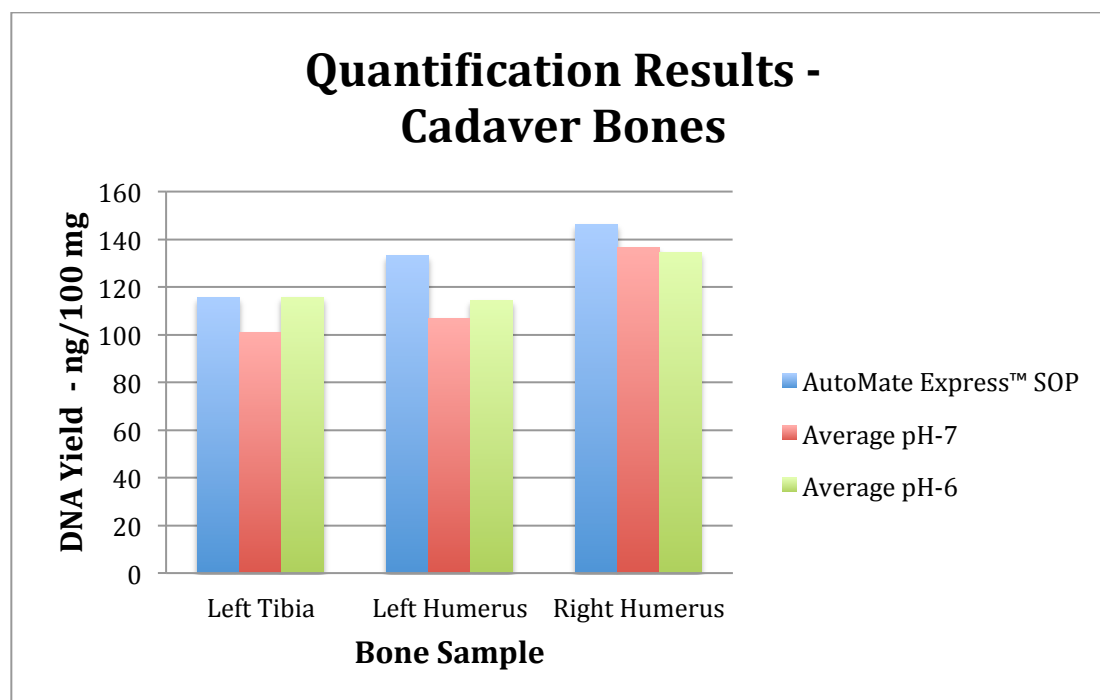


Figure 6. Total DNA Recovered Versus pH of Binding Solution: Cadaver Samples. A graphical representation of the quantification values for the cadaver bones. Yield is represented per 100 mg of bone powder.

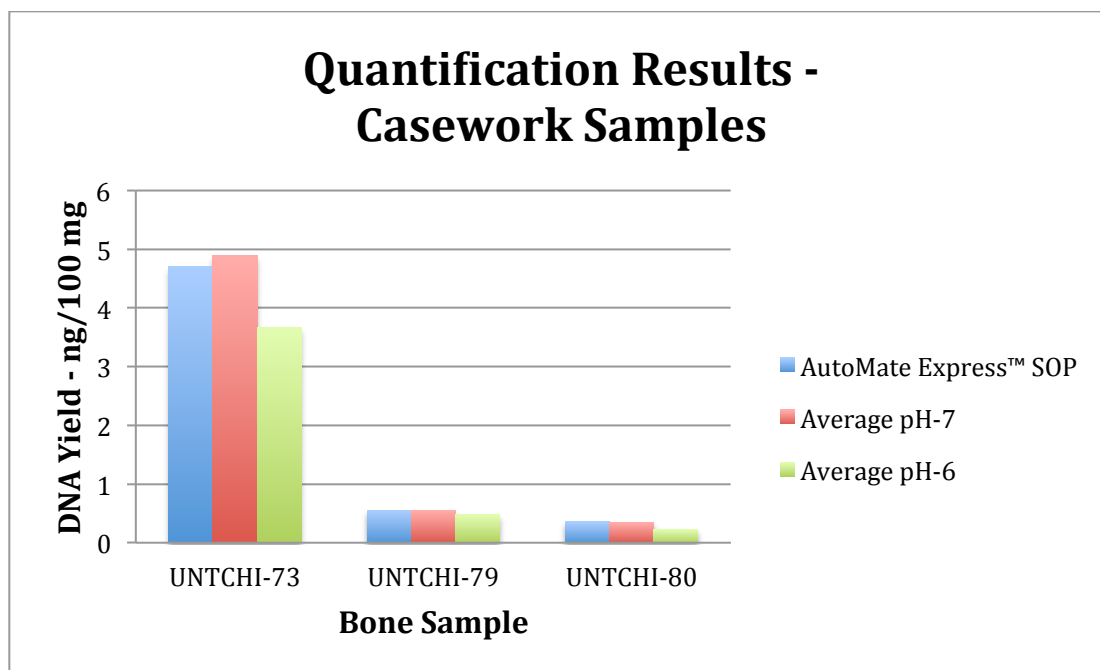


Figure 7. Total DNA Recovered Versus pH of Binding Solution: Casework Samples. A graphical representation of the quantification values for the casework samples. Yield is represented per 100 mg of bone powder.

STR Data Quality Under Different Binding pH of Lysis Solution

The DNA extracts that gave the highest quantification results for each pH observed from each bone were amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles. The samples were electrophoresed using a 3500 Genetic Analyzer and analyzed using GeneMapper® *ID-X* software. The samples were analyzed using the guidelines from the UNTHSC Center for Forensic Excellence validation of the AmpFLSTR® Identifiler® Direct PCR Amplification Kit on the 3500 Genetic Analyzer. The minimum detection threshold used was 100 relative fluorescence units (rfu) for heterozygous loci. Additionally, homozygous alleles were only reported if the peak height was greater than or equal to 200 RFUs. Table 3 shows the number of reportable loci. Three samples (Left Humerus, Right Humerus, and UNTCHI-0073) showed the same number of reportable loci regardless of the addition of 3M NaOAc, pH 5.2 to

the binding solution. Three samples (Left Tibia, UNTCHI-0079, and UNTCHI-0080) showed a decrease in the number of reportable loci with the addition of 3M NaOAc, pH 5.2. Figures 8 and 9 depict a graphical representation of the number of reportable loci for each of the bone samples under the varying pH's of the binding solution.

Sample	Number of Reportable Loci		
	AutoMate Express™ SOP	pH 7.0	pH 6.0
Left Tibia	15	15	13
Left Humerus	15	15	15
Right Humerus	15	15	15
UNTCHI-0073	15	15	15
UNTCHI-0079	12	9	12
UNTCHI-0080	13	12	12

Table 3. Number of Reportable Loci Versus pH of Binding Solution. The number of reportable loci was determined following the guidelines set forth by the UNTHSC Center for Forensic Excellence.

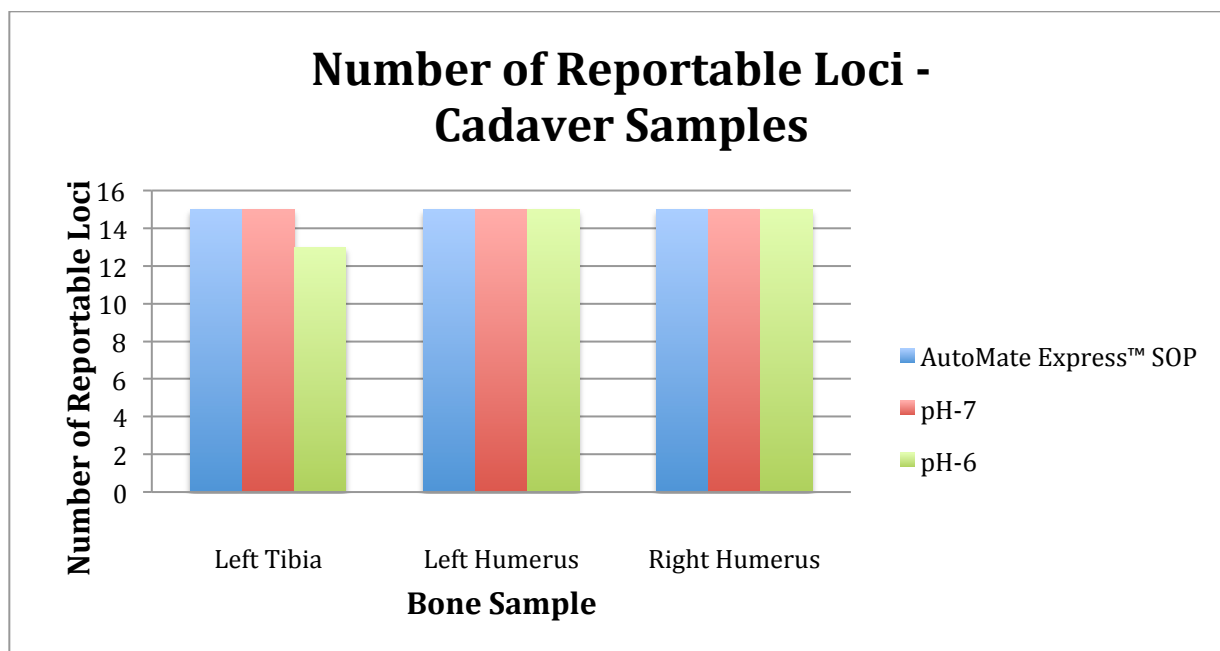


Figure 8. Number of Reportable Loci Versus pH of Binding Solution: Cadaver Samples. A graphical representation of the number of reportable loci as a result of the pH of the binding solution for the cadaver samples. The number of reportable alleles were determined following the guidelines set forth by the UNTHSC Center for Forensic Excellence.

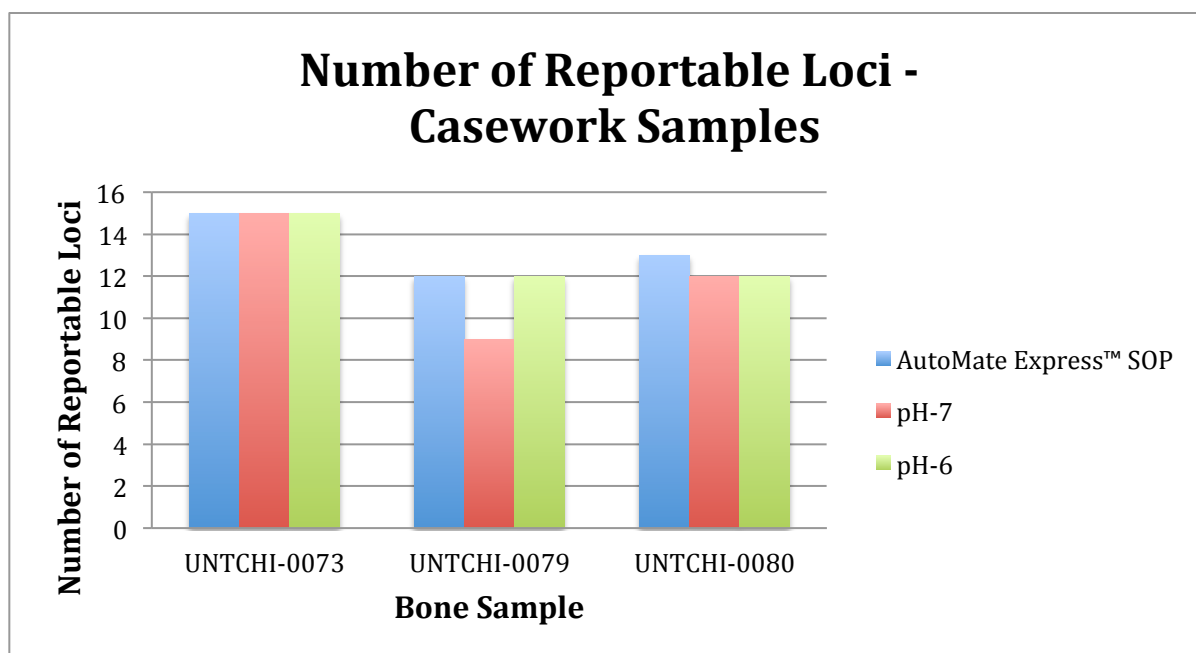


Figure 9. Number of Reportable Loci Versus pH of Binding Solution: Casework Samples. A graphical representation of the number of reportable loci as a result of the pH of the binding solution for the casework samples. The number of reportable loci were determined following the guidelines set forth by the UNTHSC Center for Forensic Excellence.

The effect of lowering pH on peak heights was also evaluated. Figures 10-15 show a graphical representation of the change in peak heights, measured in RFUs, with the addition of 3M NaOAc, pH 5.2. When looking at the average trend lines of the peak heights, three samples (Left Tibia, Left Humerus, and Casework sample 0080) showed the greatest peak heights when following the AutoMate Express™ standard operating procedure and no 3M NaOAc, pH 5.2 was added (Fig 12, 13, and 17). The Right Humerus (Fig 14) showed the lowest average peak heights when following the AutoMate Express™ standard operating procedure and had comparable results when both 10 and 25 µL of 3M NaOAc were added. Casework sample 0073 (Fig 15) showed comparable peak heights following both the standard operating procedure and when 10 µL of 3M NaOAc was added. The addition of 25 µL of 3M NaOAc to case work sample 0073 resulted in a decrease of peak heights. Casework sample 0079 (Fig 16) showed the greatest average peak heights following the standard operating procedure when the allele size was 100-200 base pairs but showed the greatest average peak heights when 25 µL of 3M NaOAc, pH 5.2 was added when the allele size was greater than 200 base pairs. Casework sample 0079 had the lowest peak heights when 10 µL of 3M NaOAc, pH 5.2 was added.

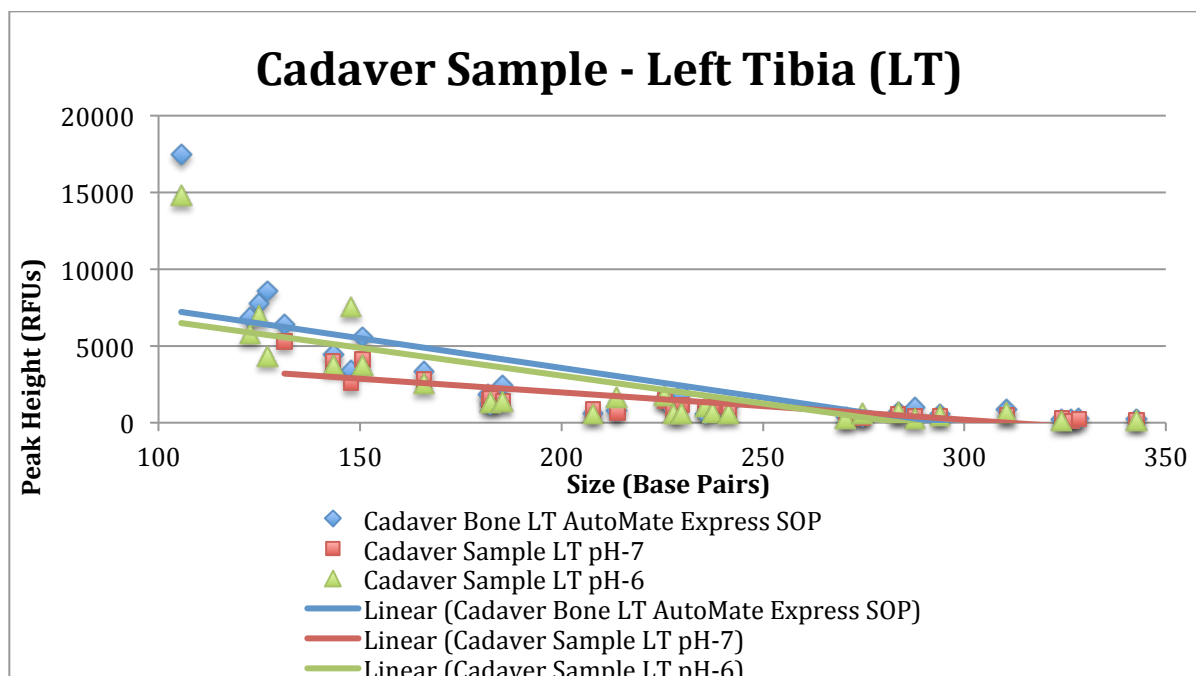


Figure 10. Peak Heights: Left Tibia. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

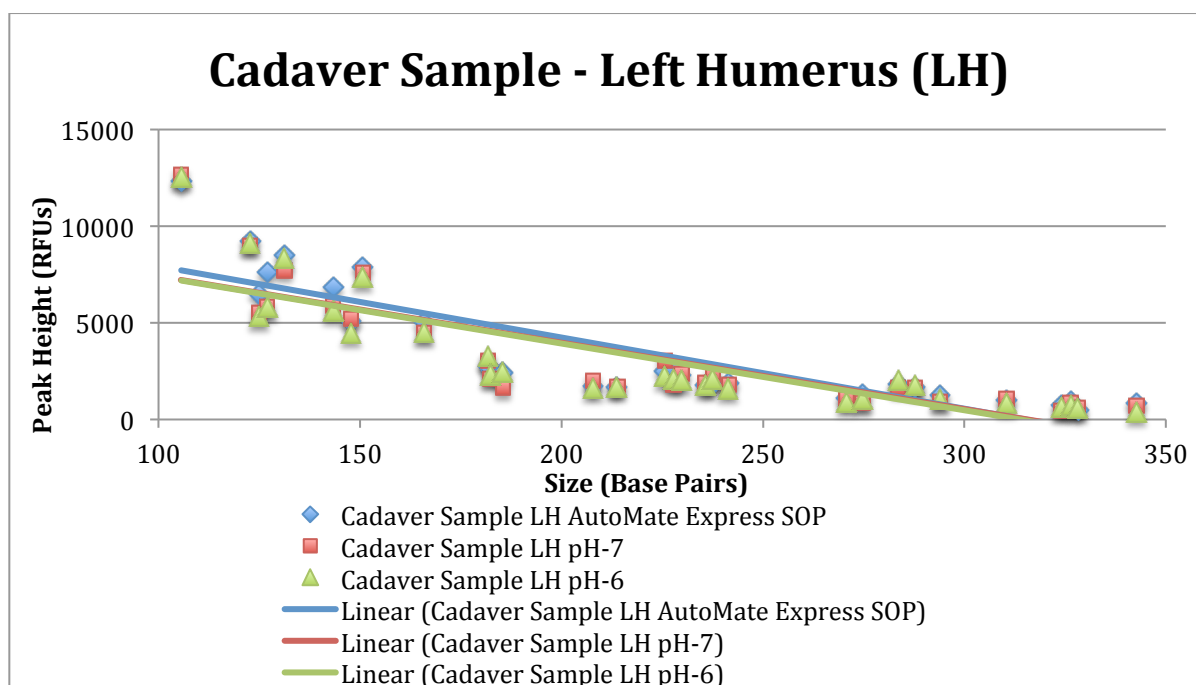


Figure 11. Peak Heights: Left Humerus. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

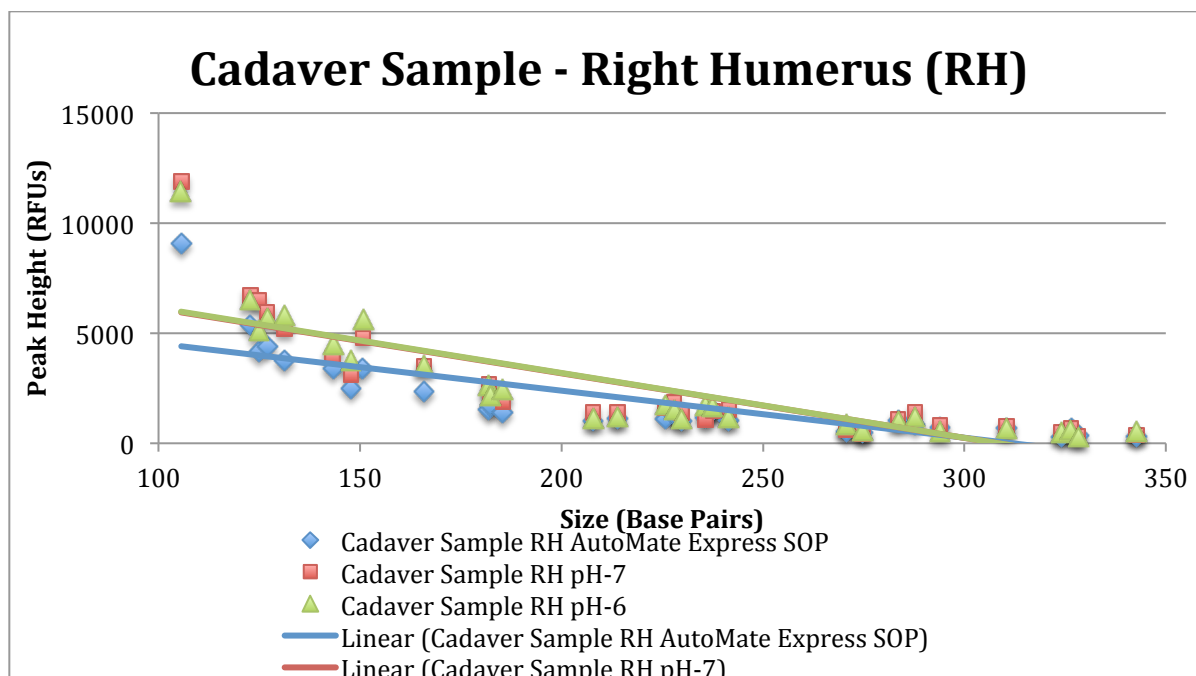


Figure 12. Right Humerus: Left Tibia. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

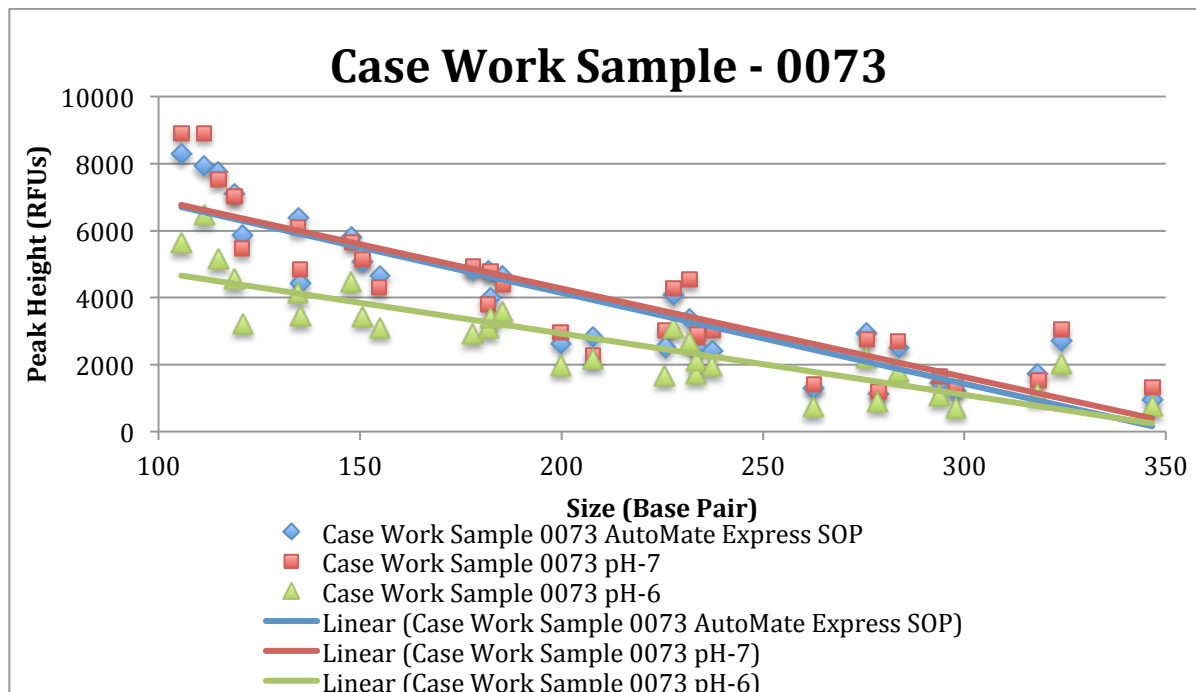


Figure 13. Peak Heights: Casework Sample 0073. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

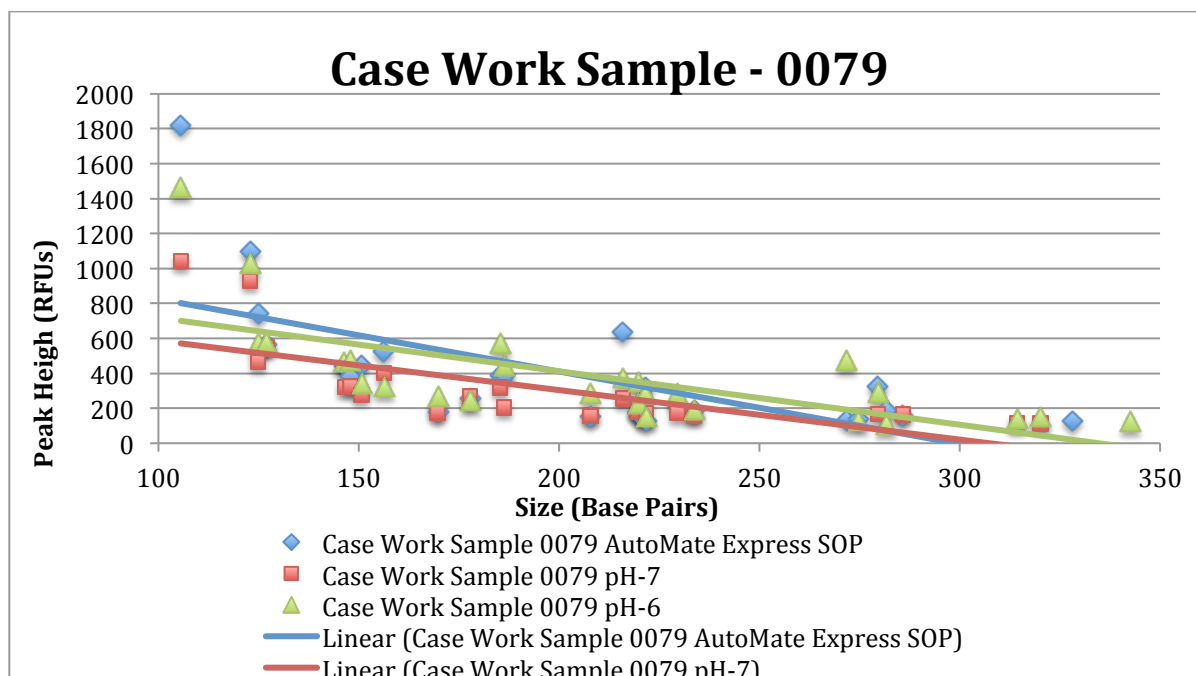


Figure 14. Peak Heights: Casework Sample 0079. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

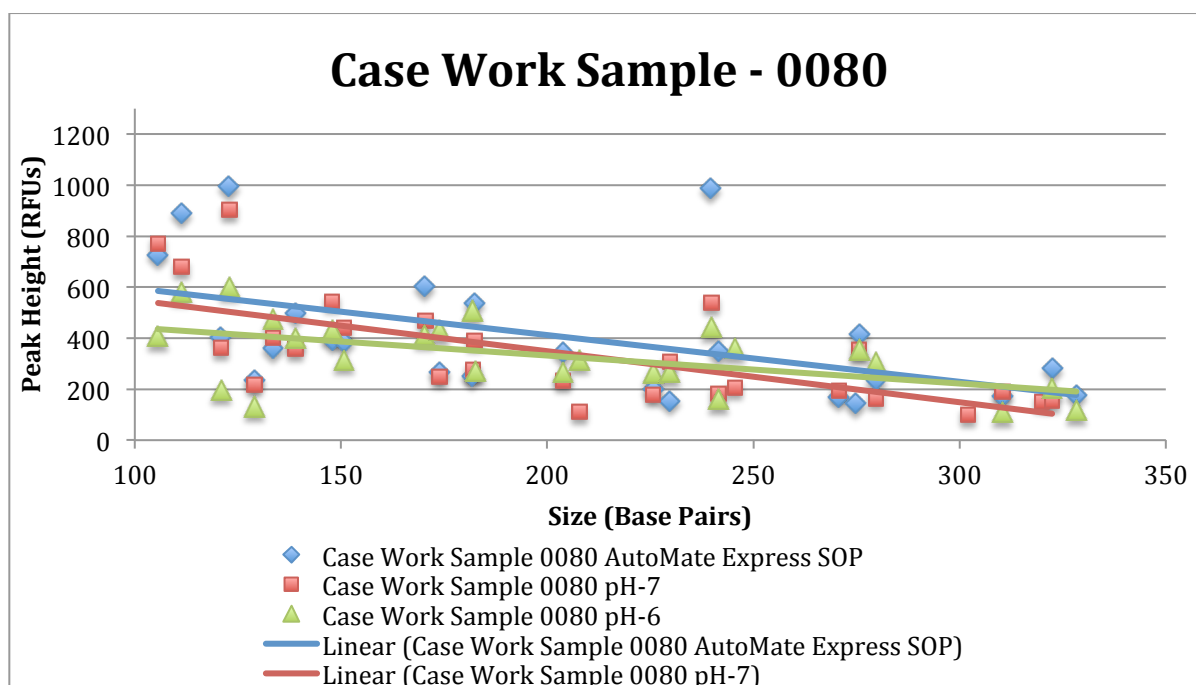


Figure 15. Peak Heights: Casework Sample 0080. A graphical representation of the affect of lowering pH using 3M NaOAc, pH 5.2 on peak heights.

Throughout the samples the greatest peak heights were seen when following the AutoMate Express™ manufacturers recommended procedure. Although there was a declining trend seen with the addition of 3M NaOAc it was not a substantial difference. Figures 16, 17, and 18 show the electropherograms for casework sample 0080. These are representative of the trends seen in all samples.

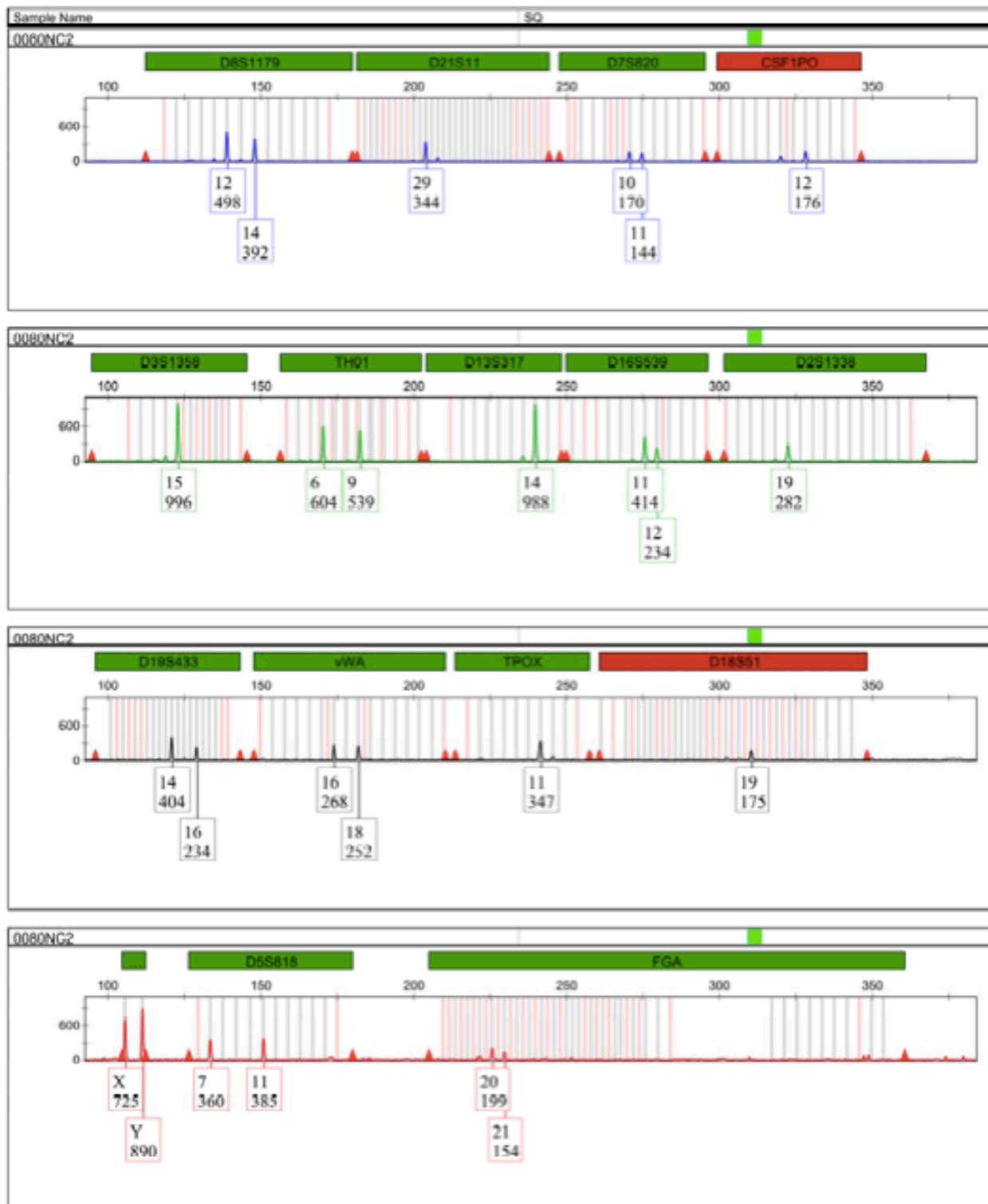


Figure 16. Casework Sample 0080 Electropherogram (Manufacturers Recommended Procedure). Electropherogram for casework sample 0080 with no added 3M NaOAc, pH 5.2.

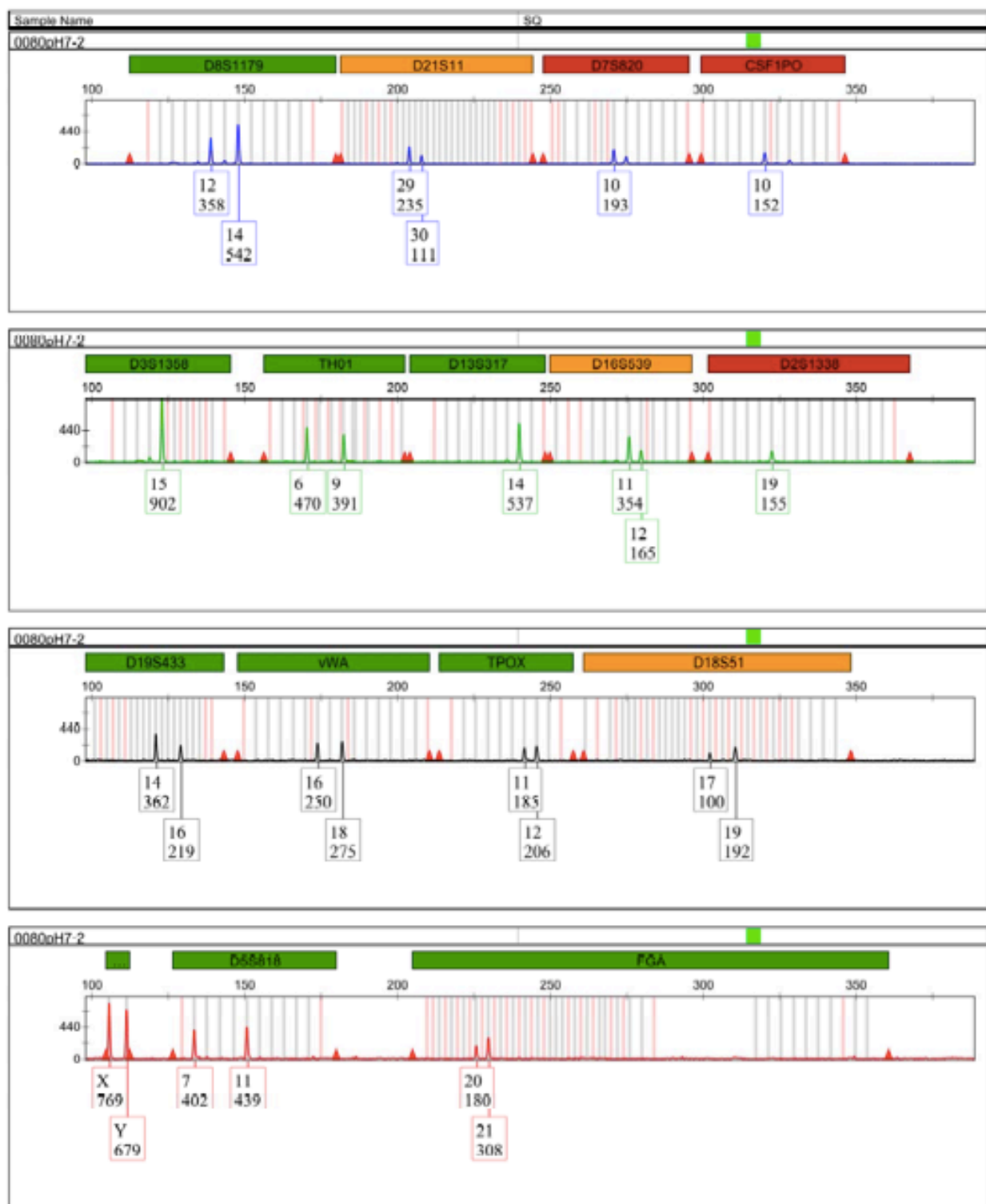


Figure 17. Casework Sample 0080 Electropherogram (pH 7.0). Electropherogram for casework sample 0080 with 10 μ L of 3M NaOAc, pH 5.2 added.

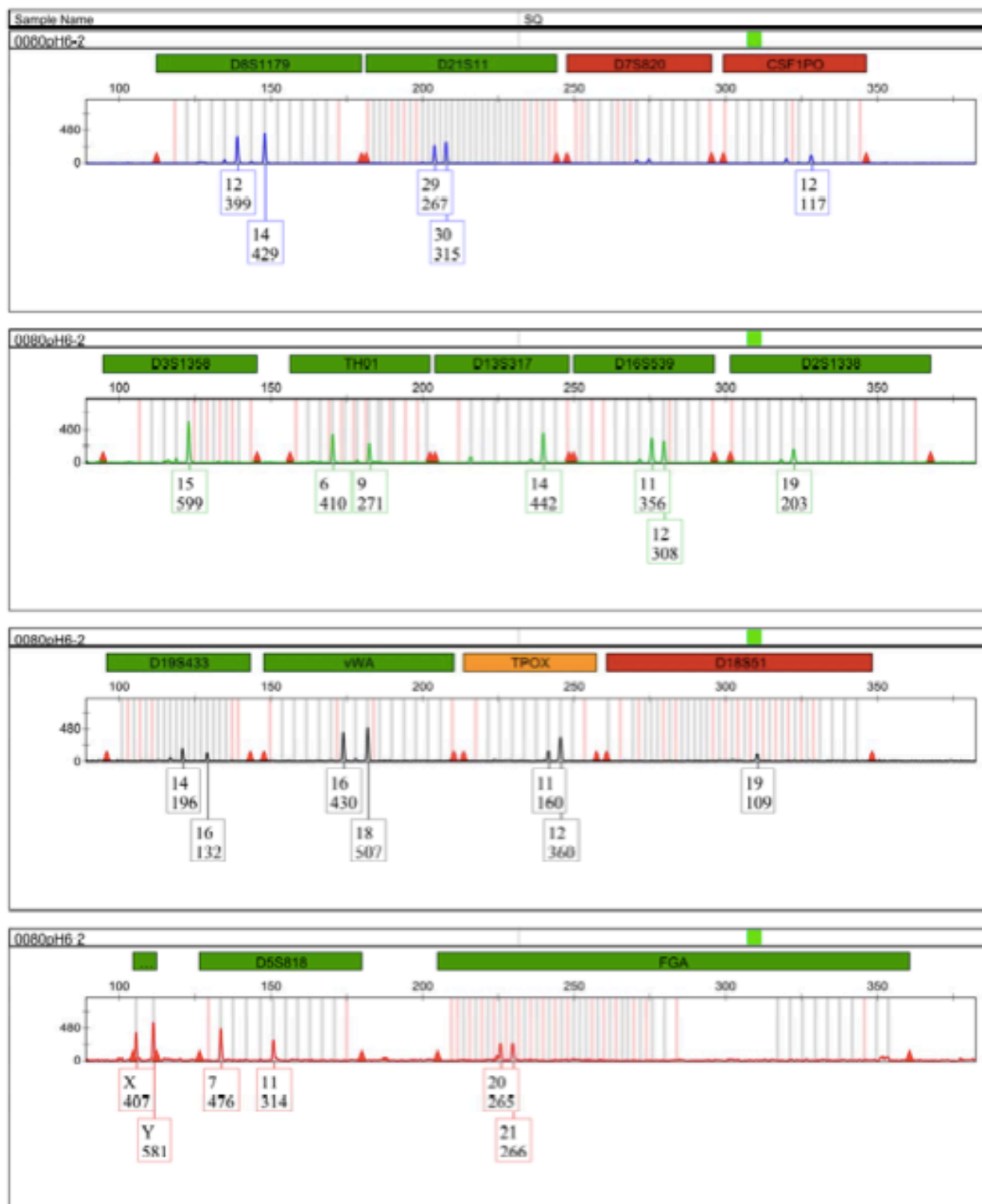


Figure 18. Casework Sample 0080 Electropherogram (pH 6.0). Electropherogram for casework sample 0080 with 25 μ L of 3M NaOAc, pH 5.2 added.

Comparison to Data Generated from UNTCHI Missing Persons Laboratory

We compared the results from casework samples 0073, 0079, and 0080 to those from UNTCHI Missing Persons Laboratory in order to determine if the quantity of recovered DNA and the quality of the STR profiles were comparable or better than those determined using the organic extraction method. UNTCHI Missing Persons Laboratory provided quantification data and STR data using AmpFLSTR® Identifiler® Plus for comparison with results from this study using the AutoMate Express™ Forensic DNA Extraction System.

Comparison of Total DNA Recovery between the AutoMate Express™ versus the Organic Extraction Method

The quantification results obtained using the AutoMate Express™ was compared to those following the standard organic extraction method (OEM) in the UNTCHI Missing Persons Laboratory. The AutoMate Express™ protocol used 100 mg of bone powder during extraction versus 1 g of bone powder used in organic extraction method. Table 4 compares the quantification results between the two extraction methods. Casework sample 0073 showed a four-fold increase in total DNA recovery when extracting DNA using the AutoMate Express™ regardless of the pH of the lysis solution. Casework samples 0079 and 0080 had approximately twice the DNA yield when extractions were done using the AutoMate Express™ as compared to the organic extraction method. Figure 18 depicts a graphical representation of DNA yield for each of the bone samples when comparing the results from the AutoMate Express™ to those obtained using the standard organic extraction method.

Sample	AutoMate Express™ SOP	pH 7.0	pH 6.0	Standard OEM
	Quantification Results			
UNTCHI-0073	4.70 ng/100 mg	4.90 ng/100 mg	3.65 ng/100 mg	1.05 ng/100 mg
UNTCHI-0079	0.55 ng/100 mg	0.55 ng/100 mg	0.45 ng/100 mg	0.24 ng/100 mg
UNTCHI-0080	0.35 ng/100 mg	0.35 ng/100 mg	0.25 ng/100 mg	0.19 ng/100 mg

Table 4. Comparison of Total DNA Recovery Between the Differing Extraction Methods. Compares the total DNA recovery between the AutoMate Express™ under the varying pH's of the lysis solution to yield using the standard organic extraction method. Yield is represented per 100 mg of bone powder.

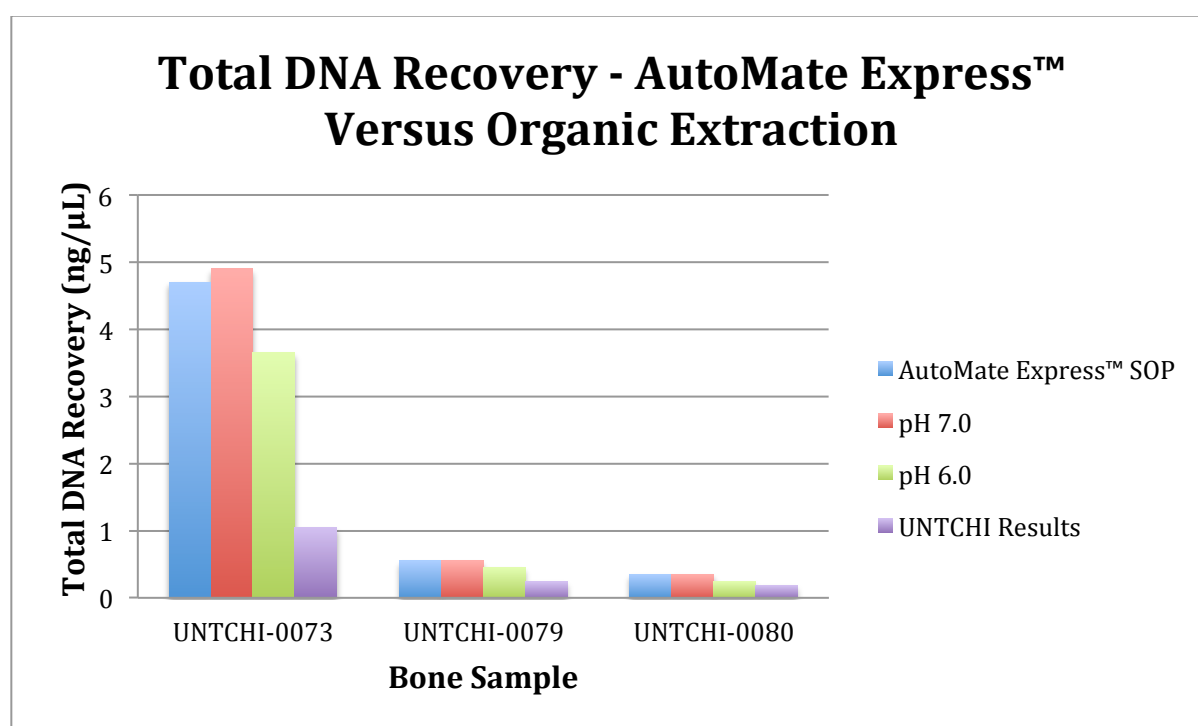


Figure 19. Comparison of Total DNA Recovery Between the Differing Extraction Methods. Graphical representation of the DNA recovery between the AutoMate Express™ under the varying pH's of the binding solution to yield using the standard organic extraction method. Yield is represented per 100 mg of bone powder.

Comparison of STR profiles between DNA extracted on the AutoMate Express™ versus the Organic Extraction Method

STR profiles from DNA extracted using the AutoMate Express™ were compared to those extracted using the conventional organic extraction method. Both samples were amplified using

the AmpFLSTR® Identifiler® Plus PCR Amplification Kit. DNA extracts from the AutoMate Express™ were amplified at 28 cycles whereas those extracted using the conventional organic method were amplified at 29 cycles. Table 5 shows number of reportable loci when comparing DNA extracted using the AutoMate Express™ to DNA extracted using the standard organic extraction procedure. Casework sample 0073 resulted in a full profile using both the standard organic extraction method and the AutoMate Express™. Casework samples 0079 and 0080 showed three and six additional reportable loci, respectively, when DNA was extracted following the AutoMate Express™ standard operating procedure. Figure 19 depicts a graphical representation of the number of reportable loci for each of the bone samples under the varying pH's of the binding solution using the AutoMate Express™ and when using the standard organic extraction method.

Sample	AutoMate Express™ SOP	pH 7.0	pH 6.0	UNTCHI Standard OEM
Number of Reportable Loci				
UNTCHI-0073	15	15	15	15
UNTCHI-0079	12	9	12	9
UNTCHI-0080	13	12	12	7

Table 5. Number of Reportable Loci Comparing AutoMate Express™ and Organic Extraction. Shows the number of reportable loci when DNA was extracted with the AutoMate Express™ versus the UNTCHI standard organic extraction method.

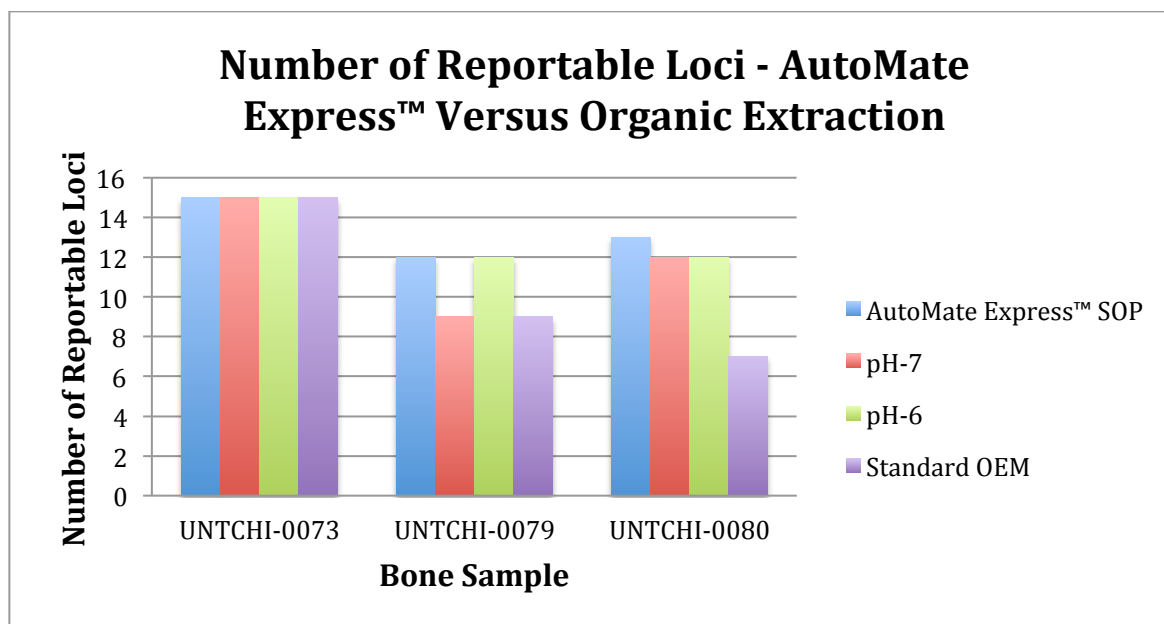


Figure 20. Number of Reportable Loci Comparing AutoMate Express™ and Organic Extraction. A graphical representation of the reportable number of loci when comparing samples extracted using the AutoMate Express™ to those extracted using the UNTCHI standard organic extraction method.

CHAPTER IV

CONCLUSIONS

There are certain forensic cases, such as mass disasters and missing person's cases, in which only skeletal remains are available as a source of identification. In these instances, DNA analysis is critical in identification of remains. Often fresh bone samples can yield sufficient amounts of DNA for analysis; other samples can be compromised or degraded as a result of age or environmental conditions. These samples can be problematic due to the limited amount of DNA, the quality of DNA recovered, as well as the presence of PCR inhibitors that can co-purify with the extracted DNA. All of these factors can negatively impact amplification and DNA typing of bone samples.

The number of unsolved missing persons cases has greatly increased over time. On a daily basis, there are between 85,000 and 100,000 active missing persons cases in the United States, with 87,000 active missing persons cases reported by the National Crime Information Center in 2012. Furthermore, the 2004 Medical Examiners and Coroners Bureau of Justice Statistics Report stated that in a typical year medical examiners and coroners offices nationally handle approximately 4,400 unidentified human remains with approximately 1,000 remaining unidentified after one year.

Due to the quantity of missing persons cases and the number of samples that require DNA analysis, bone extraction methods must be as effective as possible in order to maximize the

recovery of DNA and the amount of genetic data obtained. The current methods employed at the UNTCHI Missing Persons Laboratory to extract DNA from skeletal remains are both time consuming and laborious. The current method utilized is a manual extraction, which could result in sample loss and contamination. Both of these factors can negatively impact the results obtained from DNA typing.

The aim of this project was to test new extraction chemistries using the PrepFiler® BTA Forensic DNA Extraction Kit with the AutoMate Express™ Forensic DNA Extraction System for the extraction of DNA from bone samples. Enhancing the recovery of DNA from bone powder, using optimized automated extraction chemistry, could help resolve more missing persons cases by maximizing the amount of genetic information obtained. Furthermore, the incorporation of an automated extraction method will decrease turn-around time through a shorter lysis/incubation period as compared to the manual organic extraction method currently employed at UNTCHI Missing Persons Laboratory. An automated extraction would streamline the process and could therefore increase the number of samples a laboratory can process.

DNA Recovery Under Different Binding pH of Lysis Solution

Three cadaver bone samples from the UNTHSC Willed Body Program and three casework samples from the UNTCHI Missing Persons Laboratory were extracted in duplicate and binding under each pH was determined. The binding under varying pH's was analyzed in order to determine if adjusting the pH of the binding solution following incubation and prior to extraction could increase the quantity of DNA recovered or the quality of the genetic profiles obtained. The amount of DNA recovered was greater when the pH was not adjusted in four of the six samples tested. Two samples (Left Tibia and UNTCHI-0073) showed an increase in DNA yield when the pH was lowered. The Left Tibia showed a minor increase when comparing pH 7.9 (no change) to

pH 6.0. UNTCHI sample 0073 showed a minor increase when the pH was lowered to 7.0 but there was a decrease in yield when further lowered to pH 6.0. Adjusting the pH of the binding solution did not show an increased DNA yield. In the majority of the samples there was a decrease in the amount of DNA recovered with only minor increases shown in the other samples. The quantification results did not support that lowering the pH of the binding solution would result in an increased DNA yield.

STR Data Quality Under Different Binding pH of Binding Solution

The bone extracts which yielded the greatest amount of DNA for each bone at the different pHs observed were amplified to determine if varying the pH of the bone binding solution affected the resulting genetic profiles. The effect of the addition of 3M NaOAc, pH 5.2 on the number of reportable alleles, the number of reportable loci, and the peak height was evaluated.

Two of the samples (Left Tibia and UNTCHI-0080) showed a decrease in the number of reportable alleles as the pH of the binding solution was decreased. Two samples (Left Tibia and Casework Sample 0080) showed a decrease in the number of reportable alleles with the addition of 3M NaOAc, pH 5.2. Three samples (Left Humerus, Right Humerus, and Casework Sample 0073) showed the same number of reportable alleles regardless of the pH of the binding solution. Casework Sample 0079 showed a decrease in the number of reportable alleles when 10 μ L of 3M NaOAc, pH 5.2 was added but an increase when 25 μ L of NaOAc, pH 5.2 was added.

When looking at the number of reportable loci, pH did not have an effect on the Right and Left Humerus from the UNTHSC Willard Body Program Samples, with both producing a full profile regardless of the pH of the binding solution. The Left Tibia showed a decrease in the number of reportable loci when the pH of the binding solution was lowered to 6.0. UNTCHI sample 0073 produced a full profile regardless of the pH of the binding solution. UNTCHI sample 0079

showed a decrease in the number of reportable loci when the pH was lowered to 7.0. UNTCHI sample 0080 showed a decrease in the number of reportable loci with decreasing pH of the binding solution.

The effect of lowering pH on peak height, measured in RFUs, was also evaluated. When comparing the average trend lines of the peak heights three samples (Left Tibia, Left Humerus, and Casework Sample 0080) showed the greatest peak heights following the AutoMate Express™ standard operating procedure. The Right Humerus showed comparable results when 10 and 25 µL of 3M NaOAc, pH 5.2 was added but had the lowest peak heights following the standard operating procedure. Casework sample 0073 showed comparable results following the standard operating procedure and when 10 µL of NaOAc, pH 5.2 was added, producing the greatest peak heights under these conditions. Casework Sample 0079 showed the best results following the standard operating procedure and when 25 µL of NaOAc, pH 5.2 was added.

The results from the DNA amplification did not show a consistent or positive increase in the quality of the STR profiles nor the amount of genetic data obtained. Therefore the amplification results did not support lowering the pH of the binding solution.

Comparison to Data Generated from UNTCHI Missing Persons Laboratory

The aim of this study was to optimize the DNA extraction using the PrepFiler® BTA Forensic DNA Extraction Kit with the AutoMate Express™ for the extraction of DNA from bone samples. The results of the casework bones (0073, 0079, and 0080) from this study were compared with those from UNTCHI Missing Persons Laboratory. The quantity of DNA and the quality of the STR data obtained using the AutoMate Express™ were compared to the results generated when the samples were extracted using the standard organic extraction method.

Comparison of Total DNA Recovery between the AutoMate Express™ versus the Organic Extraction Method

Total DNA recovery was compared between the two extraction methods. Yields were compared per 100 mg of bone powder. The AutoMate Express™ resulted in higher DNA yield than the standard organic extraction method. The total yield was shown to be four-fold higher when extracting with the AutoMate Express™ for Casework Sample 0073. Casework Samples 0079 and 0080 showed approximately double the DNA yield when extracting with the AutoMate Express™ as compared to the standard organic extraction method.

Comparison of STR profiles between DNA extracted on the AutoMate Express™ versus the Organic Extraction Method

The STR profiles generated with DNA extracted with the AutoMate Express™ showed to be comparable to those determined when extracting DNA with using the standard organic extraction method. UNTCHI sample 0073 generated a full profile regardless of the extraction method (AutoMate Express™ versus organic extraction method) and pH of the binding solution when using the AutoMate Express™. UNTCHI samples 0079 and 0080 showed an increased number of reportable loci with DNA extracted with the AutoMate Express™ as compared to DNA extracted using the standard organic extraction method.

The results from the PCR amplification did not show a consistent or positive increase in the quality of the STR profiles nor the amount of genetic data obtained when the pH of the samples was adjusted during extraction with the AutoMate Express™. Therefore the results did not support lowering the pH of the binding solution. However the STR profiles generated when extracting DNA using the AutoMate Express™ showed to be comparable to, and greater than, those generated when following the standard organic extraction procedure.

Overall Conclusions

The data generated from this study showed that pH is not a significant factor when recovering DNA from bone samples with the PrepFiler® BTA Forensic DNA Extraction Kit on the AutoMate Express™ Forensic DNA Extraction System. Lowering the pH of the binding solution did not have a significant effect on DNA yield, which did not support the hypothesis that a lower pH could increase DNA binding to the column. The STR data generated also did not justify lowering the pH of the binding solution.

When comparing these data to that provided by UNTCHI Missing Persons Laboratory, the AutoMate Express™ generated results comparable or better than those obtained using the standard organic extraction method. The DNA recovered from 100 mg of bone powder using the AutoMate Express™ resulted in greater DNA yield compared to the standard organic extraction method. The STR profiles generated from DNA obtained using the AutoMate Express™ were comparable to those generated using the organic extraction method. In some bone samples, the DNA extracted using the AutoMate Express™ yielded a higher number of reportable loci than those extracted with the UNTCHI Missing Persons Laboratory standard organic extraction method. The results of this study showed that extracting DNA from bone samples using the PrepFiler® BTA chemistry with the AutoMate Express™ could increase the DNA yield and could potentially increase the quality of the genetic profiles. Furthermore, incorporation of this automated extraction procedure into UNTCHI Missing Persons Laboratory could decrease turn-around time, streamline the DNA extraction process, and increase the number of samples that could be processed. The automated nature of this method also decreases the risk of potential contamination and sample switching as compared to the manual method. Based on the results of this study, the AutoMate Express™ could be highly advantageous to the UNTCHI DNA analysts

for the extraction of DNA from bone samples.

APPENDIX

Abbreviations

- BTA: bone, teeth, adhesive, adhesive containing samples
- DNA: deoxyribonucleic acid
- DTT: dithiothreitol
- EDTA: ethylenediaminetetraacetic acid
- mL: milliliter
- NaOAc: sodium acetate
- OEM: organic extraction method
- PCIA: phenol choloform isoamyl alcohol
- PCR: Polymerase Chain Reaction
- RFU: relative fluorescence units
- SOP: standard operating procedure
- STR: short tandem repeats
- UHR: unidentified human remains
- UNTCH: University of North Texas Health Science Center for Human Identification
- UV: ultraviolet
- μ L: microliter

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