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The current methodology used by the University of North Texas Center for Human Identification Missing Persons Laboratory (UNTCHI) to recover DNA from skeletal remains is time-consuming, laborious and not readily amenable to automation. The constraints of the current process limit the number of samples that can be analyzed.

The results of this study show that extractions performed with the AutoMate Express™ Forensic DNA Extraction System (Life Technologies, Carlsbad, CA) can produce comparable DNA quantity and quality to the current procedure used by UNTCHI. The utilization of the AutoMate Express™ Forensic DNA Extraction System in our operational laboratories would help streamline the process of DNA extraction from human skeletal remains and potentially provide increased amounts of genetic information.

A SEMI-AUTOMATED METHODOLOGY FOR THE EXTRACTION OF DNA FROM HUMAN SKELETAL REMAINS

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THESIS

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By

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CHAPTER I

INTRODUCTION

The use of DNA analysis in forensic investigations has significantly increased in the last twenty years. DNA profiles from evidence samples recovered at a crime scene are compared to DNA profiles from known individuals in order to determine if the individual can be included or excluded as a potential contributor of the evidentiary item. Many biological samples from a crime scene such as blood, sperm, or saliva can be processed in a fairly simple way. In order to obtain a genetic profile from these samples, cells are lysed to release DNA from the nucleus, and proteins and other cellular debris are degraded and removed to purify the DNA for polymerase chain reaction (PCR) amplification. Automated DNA extraction procedures for these types of samples have been developed and are used by many forensic laboratories to increase their throughput capability. However, the recovery of DNA from bone samples is a more time-consuming and laborious process, often yielding very limited quantities of nuclear DNA.

The choice of bone sample used is critically important to maximize the recovery of DNA from skeletal remains. Studies have been conducted to identify which type of bone is most likely to yield the greatest quantity and quality of DNA. The DNA obtained from skeletal remains is most often recovered from osteocytes protected within the structure of compact bone. The osteocytes are embedded in spaces between layers of bone material characteristic of long bones.

A study found that bones such as the femur, tibia, fibula, and humerus generated the highest success rate when obtaining reportable autosomal DNA profiles. The cranium, radius, ulna, and clavicle were also able to yield sufficient DNA for the identification of skeletal remains (1). Another study examined the success rate of generating 100 base pairs of reportable mitochondrial DNA (mtDNA) sequence, and it found that long bones such as the femur and tibia had the highest success rate. The humerus, radius, ribs, mandible, and pelvis had the next highest success rate in generating reportable mtDNA data. The ulna, metacarpals, metatarsals, fibula, patella, clavicle, and vertebrae were found to be less desirable, and the skull was the least desirable bone to use for mtDNA recovery (2). These studies support the concept that compact bone is most desirable to use for DNA recovery due to the density of the bone material (Figure 1).

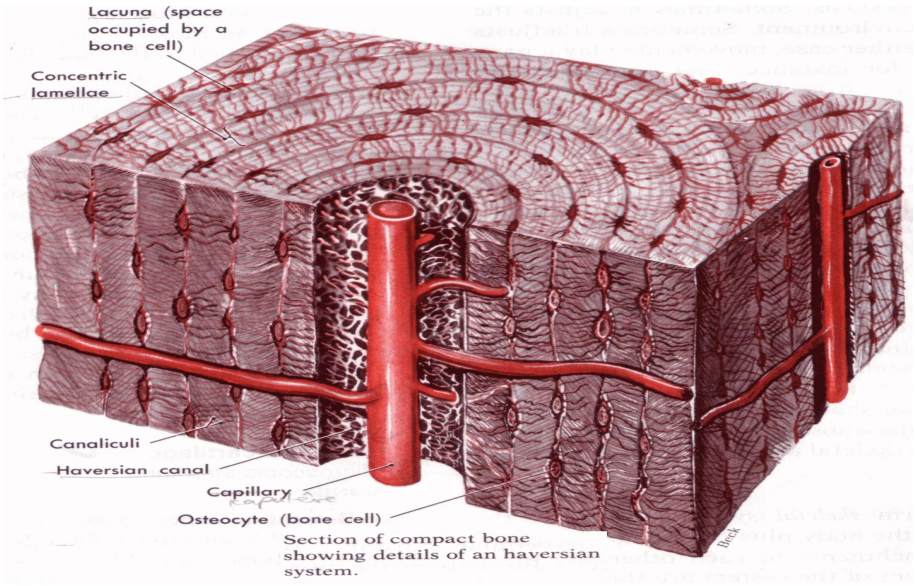


Figure 1. Compact Bone. A pictorial representation of compact bone showing the osteocytes deep within the bone matrix, which contains the DNA (photo from http://www.arthursclipart.com).

A bone sample is first prepared by removing the outer surface using a sanding instrument, and then it is cleaned with bleach and water so that the contamination from

extraneous DNA can be minimized. A small window approximately 2 to 4 cm long is then cut out of the bone while still preserving the intact bone for anthropological analysis (Figure 2). The bone samples are then pulverized with the use of liquid nitrogen and the Spex CertiPrep 6750 Freezer/Mill Grinder (SPEX Sample Prep®, Metuchen, NJ) (Figure 3) (3,4).

Figure 2. Typical Bone Sample. An example of a bone sample with a ruler indicating the size of the fragment cut from the bone to powder and extract DNA for testing (photo from UNTCHI Missing Persons Laboratory).

A SPEX CertiPrep 6750 Freezer/Mill Grinder (Freezer/Mill) with liquid nitrogen is widely used in forensic laboratories to powder the bone which enables the DNA to be more readily extracted from the sample. The addition of liquid nitrogen (-196°C) to the Freezer/Mill instantly freezes the bone sample, making it extremely brittle and easy to break into small pieces and eventually into a fine powder (5,6). This method is used at the University of North Texas Center for Human Identification (UNTCHI) which processes approximately 1,000 bone samples per year. Although very efficient at powdering bone samples, which is needed to optimize the DNA extraction procedure, the use of the Freezer/Mill with liquid nitrogen has some limitations. The necessity of liquid nitrogen adds expense and logistic problems and increases the potential hazards, such as causing burns upon skin contact. The primary tank that holds the liquid

nitrogen in the UNTCHI operational laboratory is very large and not easily transported, yet it must be refilled from an outside vendor every other week. In addition, the metal end caps and impactors for the tubes in which the bones are pulverized are not disposable and must be meticulously cleaned to prevent contamination with extraneous DNA. A system using inexpensive, disposable tubes and no liquid nitrogen would be preferable.

Figure 3. Freezer/Mill Used in Cryogenic Grinding. Picture of the Spex CertiPrep 6750 Freezer/Mill Grinder used in UNTCHI Missing Persons Laboratory to pulverize bones to powder (photo from http://www.spexsampleprep.com).

MP Biomedicals™ (Solon, OH) has developed a device called the FastPrep®-24 (MP Biomedicals™, Solon, OH) that was designed to pulverize resistant or difficult biological samples in sterile, disposable tubes without the need for liquid nitrogen (Figure 4). This instrument would potentially allow for the simultaneous pulverization of up to 12 individual bone samples, in which 1 to 2 g of bone could be added to each sterile single-use 15 mL tube, or 24 individual 200 mg bone samples in 2 mL tubes. Each single-use sample tube is pre-packaged with an optimized mixture of disposable lysing matrix particles. There are several different grinding matrices that can be used to pulverize the bone samples. However, MP Biomedicals

specifically recommended using a mixture of Lysing Matrix M (MP Biomedicals), which is composed of zirconium oxide coated ceramic beads that impact the sample with sharp-ridged edges, and Lysing Matrix A (MP Biomedicals), which is a garnet matrix consisting of industrial-grade garnet shards that attack and shear the bone with sharp ridges. The mixture of the ceramic beads and the garnet shards is hypothesized to enhance the pulverization and powdering of the bone samples. The motion of the FastPrep®-24 instrument allows the bone samples to be impacted simultaneously from multiple directions by the grinding matrices due to the three dimensional vertical angular movement of the instrument (7). Since each bone sample is processed in a single-use disposable sample tube, the need to clean the end caps and impactors is eliminated. If the bones samples can be sufficiently powdered in the FastPrep®-24 device without liquid nitrogen, the overall cost of the extraction process could be reduced. At the time of this study, MP Biomedicals™ had very limited data on the utility of the FastPrep®-24 for powdering human bone samples, and if effective, UNTCHI would consider adopting the FastPrep®-24 as an alternative to the standard pulverization process.

Figure 4. FastPrep-24® Instrument. Picture of MP Biomedicals' FastPrep-24® used in this project to try and pulverize bone fragments into powder (photo from http://www.mpbio.com).

The first specific aim of this project was to determine if the FastPrep-24® instrument can sufficiently pulverize human bone samples in single-use, disposable tubes without the need for

liquid nitrogen. Furthermore, to then demonstrate that the DNA extracted from the bone powder generated with the FastPrep-24® instrument will yield at least equivalent amounts of DNA that can produce autosomal STR profiles and mtDNA sequence data that are comparable to the conventional method of pulverization using a Freezer/Mill and liquid nitrogen.

After pulverization, the recovery of DNA often involved a process in which the bone powder was incubated for varying lengths of time in a lysis buffer containing a proteinase to degrade the protein component of bone. Following protein digestion, the remaining bone powder was centrifuged and the supernatant, presumed to contain the majority of DNA, was removed and an organic extraction procedure performed. However, the residual bone powder was later shown to contain a significant amount of trapped DNA that was not extracted, and therefore, not recovered (8). Bone is composed primarily of an inorganic crystalline mineral, in the form of hydroxyapatite. Approximately 70% of bone consists of hydroxyapatite, which includes calcium phosphate, calcium carbonate, calcium hydroxide and calcium fluoride. It was suggested by multiple researchers that the chemical and physical makeup of bone presented a physical barrier for the complete release of DNA from the bone powder (8,9). As a result, current procedures for the recovery of DNA include a lysis step in which the bone powder is incubated in an extraction buffer containing: detergents, proteinases, and ethylenediaminetetraacetic acid (EDTA). The detergent helps disrupt the cell membrane and then eliminates the secondary and tertiary structure of proteins which facilitates digestion with proteinase. The demineralization occurs through the addition of 0.5M EDTA to the lysis buffer, which results in physical dissolution of the bone powder. In addition, the presence of EDTA will help inactivate DNAases by chelating divalent cations such as Mg^{2+} and Ca^{2+} reducing the potential for further DNA degradation. The volume and the incubation time in the

demineralization/lysis buffer were shown to affect the extent of the demineralization process. Several papers have indicated that prior to the DNA extraction, a full demineralization in which the bone powder is completely dissolved (overnight incubation, on a rocker platform for 12 to15 hours at 56^{o}C in 15 mL of lysis buffer per 500 mg of bone powder) produced the greatest yield of DNA following extraction. The UNTCHI Missing Persons Laboratory utilizes a full demineralization process followed by a conventional organic extraction procedure to isolate DNA. Following the organic extraction, the aqueous layer is then passed through an Amicon® Ultra-4 Centrifugal Filer Unit (Millipore, Billerica, MA) to concentrate the sample. Lastly, an additional purification step is performed using a QIAquick® Spin Column (Qiagen, Germantown, MD), which is a silica based column, to facilitate removal of PCR inhibitors that are co-purified with the DNA during the organic extraction (10). However, this entire procedure is both time-consuming and laborious and requires the use of dangerous and caustic reagents.

Automated DNA extraction instruments have become more common in forensic laboratories in recent years. In addition, liquid handling robots have been shown to not only reduce labor costs but minimize the chance for potential human errors. The larger number of sample manipulations that are involved with an organic extraction increase the potential for sample loss and contamination. The use of these automated extraction instruments could reduce this potential as well as provide analysts with more time to spend on other critical duties such as data interpretation (11). Further, several novel chemistries have been developed that enhance the performance of these automated instruments on both reference samples as well as a variety of common forensic evidentiary sample types. There are many benefits offered by these automated instruments when used in conjunction with these novel chemistries. Common PCR inhibitors found in bone such as humic acid, calcium phosphate, and collagen are more effectively removed

during DNA extraction by these newer methods as compared to the more commonly used phenol chloroform isoamyl alcohol extraction method.

There are currently several types of bench-top automated extraction instruments that have been used by forensic laboratories: the Maxwell 16 (Promega Corporation, Madison Wisconsin); the EZ1® Advanced XL (Qiagen); and the AutoMate Express™ (Life Technologies). These three instruments were previously evaluated at the Institute of Applied Genetics at the UNT Health Science Center on a variety of forensic samples including bones and teeth. Although each of these instruments were effective at recovering DNA from the different sample types, the EZ1® Advanced XL and the AutoMate Express™ showed the greatest potential for extracting high quality DNA from both the bone and tooth samples. These two instruments yielded similar amounts of DNA and nearly equivalent genetic profiles. However, the AutoMate Express™ was found to be the easiest to use in conjunction with PrepFiler Express BTA™ Lysis Buffer (Life Technologies) (11) (Figure 5).

Figure 5. AutoMate Express™ Instrument. The automated extraction instrument used in this project to determine the efficiency of extracting human skeletal remains (photo from Life Technologies).

The PrepFiler Express BTA™ Lysis Buffer (BTA Buffer) was specifically designed to improve the quantity and quality of DNA recovery from **B**one, **T**eeth, and **A**dhesive forensic samples (12). The BTA Buffer, when used in conjunction with the Prepfiler Express™

cartridges (Life Technologies) on the AutoMate Express™, results in the formation of a unique complex between DNA from the lysate and the polymer-coated magnetic particles included in the Prepfiler Express™ cartridges. The complex formation between the DNA and the polymer present on the surface of the magnetic particles is accomplished in the presence of a high salt solution, a detergent, and isopropanol (Figure 6). The wash solution is formulated so that the bond formed between the DNA and the magnetic particles remains intact during the wash steps. This enables other compounds such as PCR inhibitors to be effectively removed from the DNA. Finally, the complex is reduced in the elution buffer, releasing the purified DNA without the need for any further analyst intervention (13). Life Technologies has suggested that a 2 hour incubation of the bone powder in the BTA Buffer is in optimal amount of time for lysis prior to loading the lysate into the Prepfiler Express™ sample tube (Life Technologies) and running the AutoMate Express™.

After the appropriate bone sample is selected, the bone is cleaned, cut, and pulverized into a powder. The current process used by the UNTCHI Missing Persons Laboratory to extract and purify the DNA from the bone powder takes at least 2 days to complete. It is possible that the extraction and purification procedure utilizing the BTA Buffer with the Prepfiler Express™ cartridges and the AutoMate Express™ instrument, once the bone is cleaned, cut, and pulverized into a powder, could be completed in a total of 2.5 hours. Furthermore, DNA extracted utilizing the current UNTCHI organic extraction procedure requires 4 tube transfers while using the PrepFiler Express BTA™ Lysis Buffer with the AutoMate Express™ instrument only requires a single tube transfer. Using the AutoMate Express™ instrument and the PrepFiler Express BTA™ Lysis Buffer could greatly reduce the sample processing time and significantly reduce

the potential for both accidental sample loss and contamination from extraneous DNA as a result of fewer sample tube transfers.

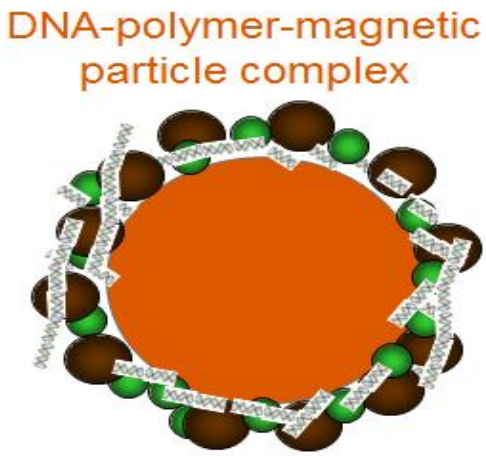


Figure 6. DNA Binding to PrepFiler™ Magnetic Particles. Mechanism demonstrating how DNA is hypothesized to bind to the magnetic particles found in the PrepFiler™ Cartridges, used with the AutoMate Express™. The DNA is shown binding to the polymer-coated surface of the magnetic particle. PrepFiler™ is a multi-component surface chemistry (photo from Life Technologies).

The second specific aim of this project was to determine if the PrepFiler Express BTA™ Lysis Buffer and the AutoMate Express™ instrument could be used effectively for the extraction of DNA from bone samples. Further, to demonstrate that the DNA extracted using the AutoMate Express™ Forensic DNA Extraction System would yield at least equivalent amounts of DNA that would produce STR profiles and mtDNA sequence data that are comparable to or better than the current organic extraction method. **The goal** is to eliminate the time-consuming, laborious, and hazardous organic extraction procedure and replace it with a newer, automated method that will dramatically reduce the processing time.

CHAPTER II

MATERIALS AND METHODS

Bone Sample Selection

Nine bone samples from the UNTCHI Missing Persons Laboratory were selected based on the estimated time since death for the comparative studies conducted. These bone samples had previously been cleaned, cut and powdered by the Unidentified Human Remains (UHR) team using a Freezer/Mill and liquid nitrogen (14). DNA from each of these samples was obtained using UNTCHI's standard bone extraction procedure (10). Varying results for STR and mtDNA data were obtained for each of these bone samples. Three different groups of bones were selected based on the estimated time since death of the individual: Group 1 (samples 1-3) include bones from decedents presumed to be dead greater than 25 years (which would be considered older bones that may be more brittle); Group 2 (samples 4-6) include bones from decedents presumed to be dead between 5 and 15 years (which would be considered mid-range or average bones); and Group 3 (samples 7-9) include bones from decedents presumed to be dead for less than 5 years (which would be considered relatively fresh remains) (Table 1).

	Sample Number	Age (years)	Weight (grams)	Bone Type	Comments
Group 1	1	26-28	1.7	Femur	Skeletonized remains were found 2/1987, found in woods, slightly covered with leaves, estimated that death occurred 1-3 years prior, SE US state
	2	30	1.7	Femur	Remains had been submerged in a river for 1-4 weeks, partially decomposed, found 6/1982, remains stored in a steel container as of 7-1982, exhumed sometime in 2011, NW US state
	3	33	1.6	Femur	Initially buried in 1979 in a body bag (body discovered along a highway 7-1979), exhumed 7-2012, SE US state
Group 2	4	9	1	Humerus	Remains found 1-2004 submerged in ocean water, time since death estimated at several months to several years, SE US state
	5	7-9	0.8	Rib	Remains found 7-2006 on ground within tall grass in a wildlife refuge, estimated time on refuge 6 months-2 years, SW US state
	6	8	2.8	Femur	Found 5-2010, scattered along ground in a desert, possibly been there 6-7 years, W US state
Group 3	7	4	1.3	Femur	Remains still have some intact wet tissue, found 8-2008 in a sugar cane field in moderate decomposition, SW US state (this bone was not bleached after cutting, only rinsed with water and EtOH)
	8	1	1.9	Femur	Partially charred skeletonized remains found 3-2012, in shallow pit on a ranch, SW US state
	9	1	1.5	Femur	Remains exhumed from a shallow makeshift grave in 2-2012, SW US state

Table 1. Bone Samples Used in This Study. Samples were provided by the UNTCHI Missing Persons Laboratory to use for the comparisons performed in this study. Sample number, approximate time since death (age in years), the weight of the bone provided (g), the type of bone sample, and the case description are provided.

Bone Sample Preparation

All of the tools that were used for preparing the bone samples were UV-crosslinked for at least 30 minutes prior to use. An area of bone approximately 2 inches by 3 inches was cleaned with a 5% Tergazyme (Alconox, White Plains, NY) solution inside a negative air flow sanding station. A Dremel tool (Dremel, Mount Prospect, IL) was used to sand the outer surface area previously cleaned, and then used to cut the bone into thin sections. Only one sample at a time was prepared with the Dremel tool, and the cutting area was cleaned thoroughly between samples. The bone fragments were cut to a size small enough to fit into the Spex 6750 Freezer/Mill polycarbonate tubes. The bone fragments from each sample were first placed in individual 50 mL conical tubes to which a 50% bleach solution was added to cover the bone sample. The samples were gently agitated and allowed to soak for approximately 5 minutes, after which the bleach solution was carefully poured off into a container. An equivalent amount of distilled water was then added and decanted. The water washes were repeated several times until the smell of bleach could no longer be detected (approximately 3 washes). Lastly, the sample was covered with 100% ethanol which was also decanted. The bone fragments were removed from each 50 mL tube and transferred into a weigh boat and set aside to dry for approximately 20-30 minutes.

Cryogenic Grinding Using SPEX 6750 Freezer/Mill

All of the tubes and metal end caps and impactors were UV-crosslinked for at least 30 minutes before use. The bone fragments were then placed into individual polycarbonate tubes, a metal impactor was added to the tube, and the end caps were secured (Figure 7). The reservoir of the Freezer/Mill was filled with liquid nitrogen and there was an initial chill period lasting approximately 7 minutes. Following the chill period, additional liquid nitrogen was added to the

Freezer/Mill to fill it. The sample tube was inserted into the Freezer/Mill and allowed to cool for approximately 5 minutes before grinding. The sample was pulverized for 7 minutes and the tube was then visually inspected to insure that the fragments were completely powdered. If additional pulverization was required, the sample cylinder was reinserted and additional grinding performed. After grinding was completed, the sample cylinder was set aside and allowed to warm to room temperature (approximately 30 minutes) (Figure 8). The end caps were removed and the bone powder was weighed and stored in individual 15 mL conical tubes at -20°C (14).

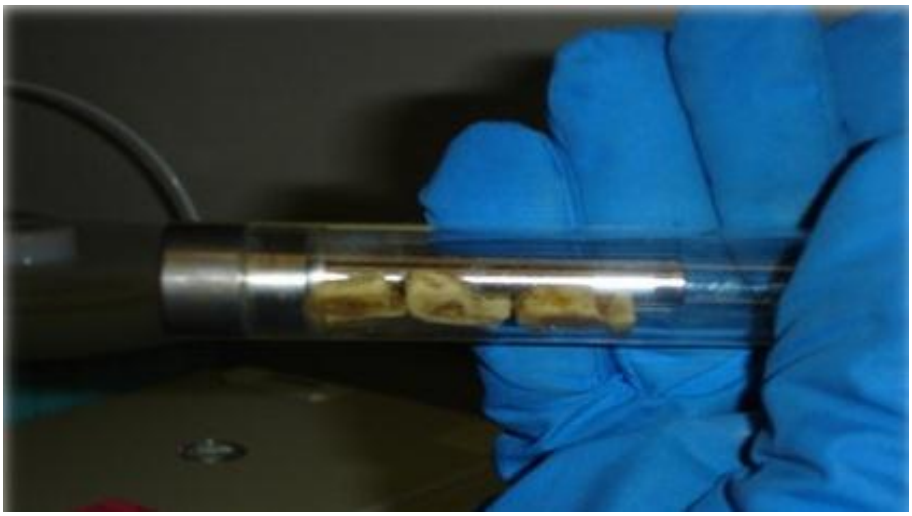


Figure 7. Bone Sample in SPEX 6750 Freezer/Mill Polycarbonate tube. Several small bone fragments are shown in a polycarbonate tube with the metal impactor, with the end caps in place to close the tube at each end (photo from the UNTCHI Missing Persons Laboratory).

Conventional Organic Extraction

The UNTCHI Missing Persons Laboratory followed a conventional organic extraction to isolate DNA from skeletal remains. Prior to the organic extraction a complete demineralization of the bone matrix was performed. One gram (1 g) of bone powder was added to a 15 mL conical tube, followed by 4.5 mL of a demineralization buffer (5 g of sodium N-laurylsarcosinate in 500 mL of 0.5M EDTA) and 300µL of Proteinase K (20 mg/mL). The

samples were incubated overnight at 56°C on an orbital shaker. The following day, the sample tubes were briefly centrifuged to remove condensation and any residual extract from the tube and lid. An equal volume of phenol chloroform isoamyl alcohol (25:24:1) was added to the extract. The sample tubes were vortexed and centrifuged to separate the aqueous layer from the organic layer. The aqueous layer was removed and filtered through an an Amicon® Ultra-4 Centrifugal Filer Unit. Lastly, the filtrate containing the extract was further purified using a QIAquick spin column to facilitate the removal of additional PCR inhibitors.

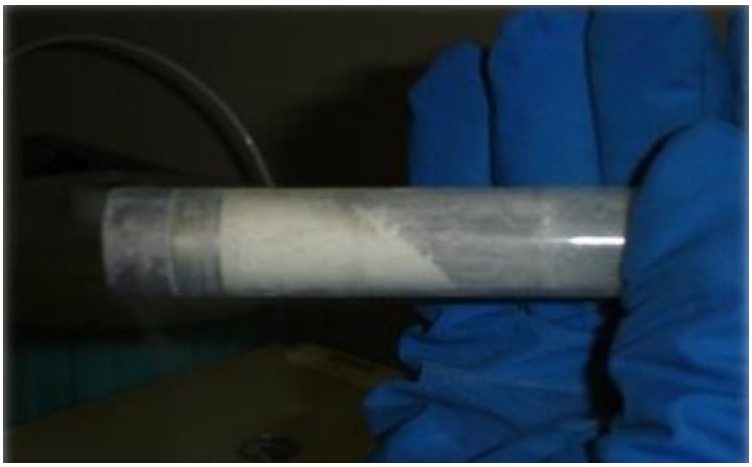


Figure 8. Powdered Bone Sample. Following grinding in the Freezer/Mill, the bone fragments were reduced to a fine powder that can be weighed out and the DNA extracted (photo from the UNTCHI Missing Persons Laboratory).

FastPrep®-24 Pulverization

The FastPrep®-24 was tested in an attempt to develop an alternative method for powdering bone samples. A bone sample from each of the three groups was tested in order to determine if the bone powder generated would produce genetic data that was similar to or better than the genetic data produced from powder generated using the Freezer/Mill and liquid nitrogen. Various combinations of instrument settings (grinding matrices, speed, and time) were tested to determine the optimal settings for pulverizing human bone. Initially, small pieces of

human bone were placed into individual 2 mL tubes to establish the optimum settings on the FastPrep®-24 to generate bone powder. Although none of the various settings on the FastPrep®-24 produced a significant amount of powder, the combination that appeared to be the most effective was a mixture of Lysing Matrices A and M.

Garnet shards from Lysing Matrix A and a total of 10 zirconium oxide coated ceramic beads (¼ inch diameter) from Lysing Matrix M were transferred to a 15 mL polypropylene conical tube. Each bone fragment tested was inserted with an equal amount of beads on either side of it. The samples were processed through four cycles, with each cycle set at a speed of 6.0 m/s for 40 seconds.

In an attempt to enhance the ability of the FastPrep-24® to powder the bone fragments, liquid nitrogen was used to freeze the bone to -196°. A bone sample from each of the three groups was tested using the garnet shards and zirconium oxide coated beads with the addition of liquid nitrogen to each individual sample tube. A similar fragment from each of the bone samples was tested under the same conditions without the addition of liquid nitrogen. Four cycles of 6.0 m/s for 40 seconds each were conducted with and without liquid nitrogen. Each bone sample was weighed before and after grinding to determine the amount of bone fragment that remained intact and did not powder.

Automated Extraction Using the AutoMate Express™

The manufacturer's recommended procedure for the AutoMate Express™ was followed for extraction of bone samples. The PrepFiler Express BTA™ Lysis solution was made fresh using 220 µL of PrepFiler Express BTA™ Lysis Buffer, 3 µL of 1M DTT, and 7 µL of Proteinase K (20 mg/mL) and added to each tube containing 150 mg of bone powder. Once 230 µL of the

PrepFiler Express BTA™ Lysis solution was added, each tube was tightly closed, vortexed, centrifuged briefly, and placed in a Multi-Therm™ shaker (Benchmark Scientific, Inc., South Plainfield, NJ) at 56°C and 1,100 rpm for 2 hours. After the 2 hour incubation, the tubes were centrifuged for 90 seconds at 10,000 x g to pellet any residual bone powder. The lysate was then carefully removed and transferred to a PrepFiler™ sample tube. In some of the samples, 230 µL of lysate was not recovered and additional PrepFiler Express BTA™ Lysis Buffer was added to bring the volume up to a total of 230 µL prior to loading into the instrument. The AutoMate Express™ instrument was set up by inserting up to 13 cartridges into the cartridge rack. A PrepFiler™ Cartridge is shown below in Figure 9.

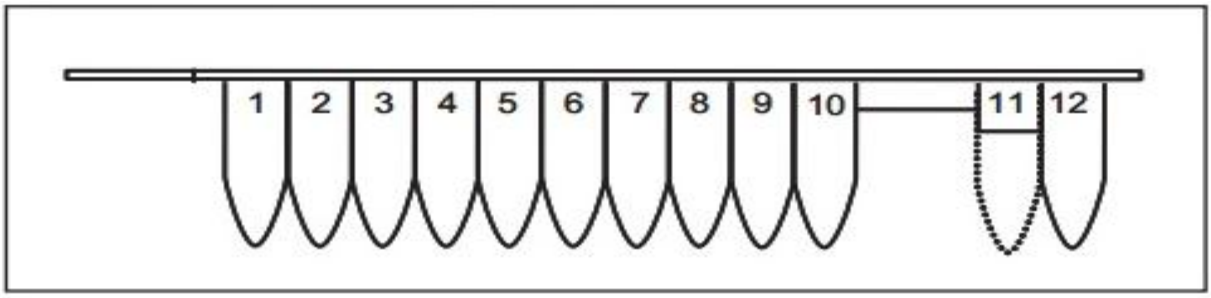


Figure 9. PrepFiler Express™ Cartridge. Tube 1 contains lysis buffer, tube 2 contains suspended magnetic particles, tube 3 contains a binding solution, tubes 4 through 6 contain a wash buffer, and tube 7 contains an elution buffer. Tube 12 is in a heated chamber that functions for elution (photo from Life Technologies).

The cartridge rack was then loaded into the instrument by simply setting it into place. The Prepfiler™ sample tubes containing the lysate, the AutoMate Express™ Tips (Life Technologies), and the PrepFiler™ elution tubes (Life Technologies) were then added into the tip and tube rack (Figure 10). Once the tip and tube rack was loaded, it was set into the instrument in front of the cartridge rack. After completion of the automated extraction, the purified DNA was eluted in a volume of 50 µL and stored at 4°C.

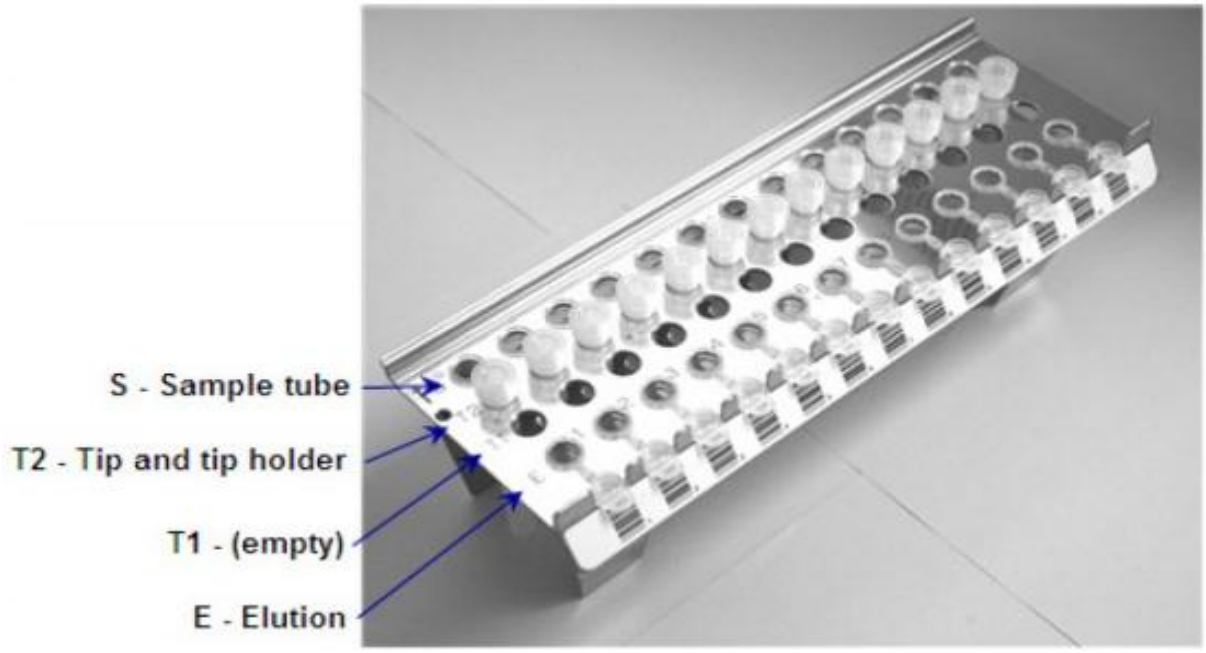


Figure 10. Tip and Tube Rack. The tip and tube rack is filled with necessary components used in the automated extraction process. Row S holds the PrepFiler™ sample tubes containing the lysate. Row T2 contains the PrepFiler™ Tips inserted in tip holders. Row E contains the opened PrepFiler™ elution tubes, into which the extracted DNA is eventually dispensed (photo from Life Technologies).

DNA Quantification

The DNA extracts were quantified using a reduced volume reaction with the Quantifiler® Human DNA Quantification Kit (Life Technologies). The UNTCHI protocol for "Human DNA Quantification using Reduced Reaction Volume Applied Biosystems Quantifiler® Human DNA Quantification Kit" was followed. The quantification assay was run on an Applied Biosystems® 7500 Real-Time PCR System (Life Technologies). The results were compared with a standard dilution series to determine the quantity of DNA to be used for STR and mtDNA amplification.

STR Amplification

Nuclear DNA was amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) following the UNTCHI protocol for "STR Amplification". The thermal cycling conditions were as follows: 1) 95°C for 11 minutes; 2) 94°C for 20 seconds; 3) 59°C for 3 minutes; 4) 28 or 29 cycles of 2) and 3); 4) 60°C for 10 minutes; 4°C indefinitely. When possible, 0.5 to 1.0 ng of DNA was added to a maximum of 10µL of DNA in the 25µL reaction volume.

Nuclear DNA was also amplified using an AmpFLSTR® MiniFiler™ PCR Amplification Kit (Life Technologies) following the standard UNTCHI protocol for "STR Amplification". The thermal cycling conditions were as follows: 1) 95°C for 11 minutes; 2) 94°C for 20 seconds; 3) 59°C for 2 minutes; 4) 72°C for 1 minute; 5) 30 cycles of 2) through 4); 6) 60°C for 45 minutes; 4°C indefinitely. When possible, 0.5 to 1.0 ng of DNA was added to a maximum of 10µL of DNA in the 25µL reaction volume.

Capillary Electrophoresis and Data Analysis

The PCR products obtained from using the Identifiler® Plus and MiniFiler™ PCR Amplification Kits were electrophoresed on a 3130*xl* Genetic Analyzer (Life Technologies) and on a 3500XL Genetic Analyzer (Life Technologies). The UNT Center for Human Identification protocol for "Data Collection Using the Applied Biosystems 3130*xl* Genetic Analyzer" was followed. The data were analyzed using GeneMapper® *ID-X* software (Life Technologies) under the interpretation guidelines set by validation studies performed in the UNTCHI Missing Persons Laboratory. The 3500XL Genetic Analyzer has not been validated for use with casework;

therefore the data were interpreted under the same guidelines as those set for the 3130*xl* Genetic Analyzer.

mtDNA Sequencing

Mitochondrial DNA sequence data was obtained from each of the bone samples tested. The mtDNA regions HV1 and HV2 were amplified separately following the UNTCHI protocol for "mtDNA Amplification". The thermal cycling conditions were as follows: 1) 95°C for 11 minutes; 2) 95°C for 10 seconds; 3) 61°C for 30 seconds; 4) 72°C for 30 seconds; 5) 36 cycles of 2) through 4); 6) 70°C for 10 minutes; 7) 4°C indefinitely. Immediately following the amplification of HV1 and HV2, the PCR products were purified following the UNTCHI protocol for "mtDNA Amplicon Purification with ExoSap-IT®". Five microliters of ExoSap-IT® (Affymetrix®/USB® Products, Santa Clara, CA) were added to each sample tube containing the HV1 and HV2 PCR products and were processed according to the protocol. The PCR products from HV1 and HV2 were quantified using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) DNA 1000 Assay according to the UNT Center for Human Identification protocol for "Post-Amplification Quantitation". Cycle Sequencing was performed using the BigDye® Terminator™ v1.1 Cycle Sequencing Kit (Life Technologies). Thermal cycling conditions were as follows: 1) 96°C for three minutes; 2) 96°C for 15 seconds; 3) 50°C for 10 seconds; 4) 60°C for 3 minutes; 5) 25 cycles of 2) through 4); 4°C indefinitely. Immediately following cycle sequencing, each sample was prepared for electrophoresis using Performa® DTR 96-Well Standard Plates (EdgeBiosystems, Inc., Gaithersburg, MD) according to the UNTCHI protocol for "Preparing Samples for Electrophoresis Using Edge Gel Filtration". The samples were electrophoresed on a 3130*xl* Genetic Analyzer, and the data were analyzed in Sequencher®

version 5.1 sequence analysis software (Gene Codes Corporation, Ann Arbor, MI) using the revised Cambridge Reference Sequence to determine variances in sequence (15).

CHAPTER III

RESULTS

FastPrep-24 Pulverization of Skeletal Remains

The overall goal of Specific Aim 1 was to determine if the FastPrep®-24 instrument could sufficiently pulverize human bone samples into a fine powder in single-use disposable tubes without the need for liquid nitrogen. To then determine if the DNA extracted from the bone powder generated with the FastPrep®-24 instrument would yield at least equivalent amounts of DNA that could produce STR profiles and mtDNA sequence data similar to the conventional method of pulverization using a Freezer/Mill and liquid nitrogen.

The efficiency of the FastPrep®-24 instrument's grinding ability was determined by visual inspection of the bone fragment and by weighing the bone before and after processing to determine the percentage of bone remaining. Grinding was tested on the FastPrep®-24 instrument, both with and without the use of liquid nitrogen.

Initial experiments on the FastPrep®-24 were conducted without the inclusion of liquid nitrogen as recommended by MP Biomedicals. Bone samples 1, 4, and 8, representing each of the three groups were chosen. Each bone sample was placed in an individual tube and subjected to 4 cycles, with each cycle being 40 seconds at 6.0 m/s. After the first 40 second run of the instrument, the tubes were checked for physical damage or weakness. The tubes were warm to

the touch and were scarred at the top and bottom relative to the position that they were seated in the instrument. Upon completion of the fourth cycle, the instrument lid was extremely hot to the touch and had significantly loosened. The lid was removed and it was determined that a washer was loose and had come off of the instrument. There was black debris in the tray of the instrument where the tubes are held during grinding. A small piece of the screw where the lid attaches had broken off during one of the cycles. Thus, the instrument may not be able to withstand the consistent use in a forensic laboratory. However, the aforementioned issues did not affect the efficiency of the instrument.

The samples in this experiment were visually inspected to determine if any mass from the bone fragment was lost and if any powder was generated. All of the bone samples appeared to be the same size and were only smoother around the edges. In each of the 3 tubes with a bone sample and a fourth tube with only the grinding matrices and no bone, a powdery substance was observed after grinding. Based upon a visual inspection of the bone fragments there was no difference in size prior to or after the 4 cycles. The source of the powdery substance was determined to be from the garnet shards because they are reduced to a powder after the run of the instrument.

The next set of experiments was designed to see if the addition of liquid nitrogen to the sample would improve the pulverization using the FastPrep-24® instrument. Bone fragments from samples 3, 5, and 9 were placed in a tube to which liquid nitrogen was directly added. The instrument was run through 4 cycles of 6.0 m/s for 40 seconds, with a five minute rest period in between cycles. Each bone was weighed before grinding to determine a start weight and then after grinding to determine an end weight. The percentage of fragment remaining was determined. There was very little powder generated from bone samples 3 and 9; however, bone

sample 5 was completely reduced by the grinding of the FastPrep®-24 (Table 2). Sample 5 was different from the others in that it was a rib bone as opposed to a femur or other long bone. Rib bones are not typically the primary type of sample used for DNA analysis. It was determined that the FastPrep-24® is not appropriate to use in a forensic laboratory and that the cryogenic grinding method is the most appropriate method to pulverize skeletal remains that are to be used for DNA analysis.

Sample	Start Weight (g)	End Weight (g)	Percentage of the Fragment Remaining (%)
3.A	0.5859	0.5574	95.14
3.B	0.4899	0.4373	89.26
5.A	0.2463	Powdered	0
5.B	0.1483	Powdered	0
9.A	0.4337	0.4179	96.36
9.B	0.5041	0.4841	96.03

Table 2. FastPrep-24® Grinding Efficiency. Start and end weights and percentage of fragment remaining for each sample. Each A sample represents those samples without the addition of liquid nitrogen in the sample tube. Each B sample represents the samples with the addition of liquid nitrogen to each sample tube.

DNA Extraction Using the AutoMate Express™

The main purpose of Specific Aim 2 was to evaluate the utility of the DNA extraction process from bone samples using the AutoMate Express™ Forensic DNA Extraction System in conjunction with the PrepFiler Express BTA™ Lysis Buffer. Furthermore, to then determine if the DNA extracted using the AutoMate Express™ Forensic DNA Extraction System would yield an amount of DNA that was equivalent to or greater than the amount recovered using the current extraction method. In addition the STR profiles and mtDNA sequence data must be comparable to or better than results obtained using the current organic extraction method.

Incubation in PrepFiler Express BTA™ Lysis Solution and DNA Recovery

Incubation in the PrepFiler Express BTA™ Lysis solution is an essential part of the DNA extraction process. It is during this step that cells are lysed, proteins bound to DNA are degraded, and the bone matrix is partially dissolved releasing DNA that was trapped in the bone matrix. Initial studies performed by Life Technologies suggested that increasing incubation times beyond 2 hours in the PrepFiler Express BTA™ Lysis solution did not significantly improve DNA recovery. In order to determine the effect of increased incubation time, 9 samples were evaluated with the standard 2 hour incubation and an overnight, 18 hour incubation. Approximately 1 g of bone was powdered for each of the 9 samples and 6 aliquots, 150 mg each, were distributed. For each bone, 3 aliquots each were incubated at 2 hours and at 18 hours in the PrepFiler Express BTA™ Lysis solution. Following the specified incubation time, samples were then extracted using the standard AutoMate Express™ procedure.

The quantity of DNA recovered from each bone was established by taking the average from the Quantifiler® Human DNA Quantification Kit from the 3 replicates. Table 3 displays the total DNA recovered from each bone sample processed with the two different incubation times. Bone samples 7 and 8 were not included for comparison purposes because the DNA yield was not typical of what is normally seen from bone fragments. Sample 7 was unusual because it had tissue attached when it was ground, and sample 8 was a relatively fresh sample. In 5 of the remaining 7 samples, a greater total recovery was obtained with the 2 hour incubation. Only 2 samples (samples 1 and 6) yielded a higher amount of DNA when the incubation time was longer. Longer incubation time appeared to be deleterious. Figure 11 is a graphical representation of the total DNA recovery when using a 2 hour incubation versus an 18 hour incubation period

Sample	2 hour Average Quantity (ng)	18 hour Average Quantity (ng)
1	1.55	1.70
2	0.21	0.00
3	0.92	0.51
4	0.72	0.47
5	0.86	0.23
6	1.95	2.70
7	116	158
8	24.9	29.4
9	0.73	0.47

Table 3. Total DNA Recovered with 2 Hour Versus 18 Hour Incubation Time in the PrepFiler Express BTA™ Lysis Solution. Total DNA recovered from the average of the quantification values obtained when 150 mg of each sample was processed in triplicate.

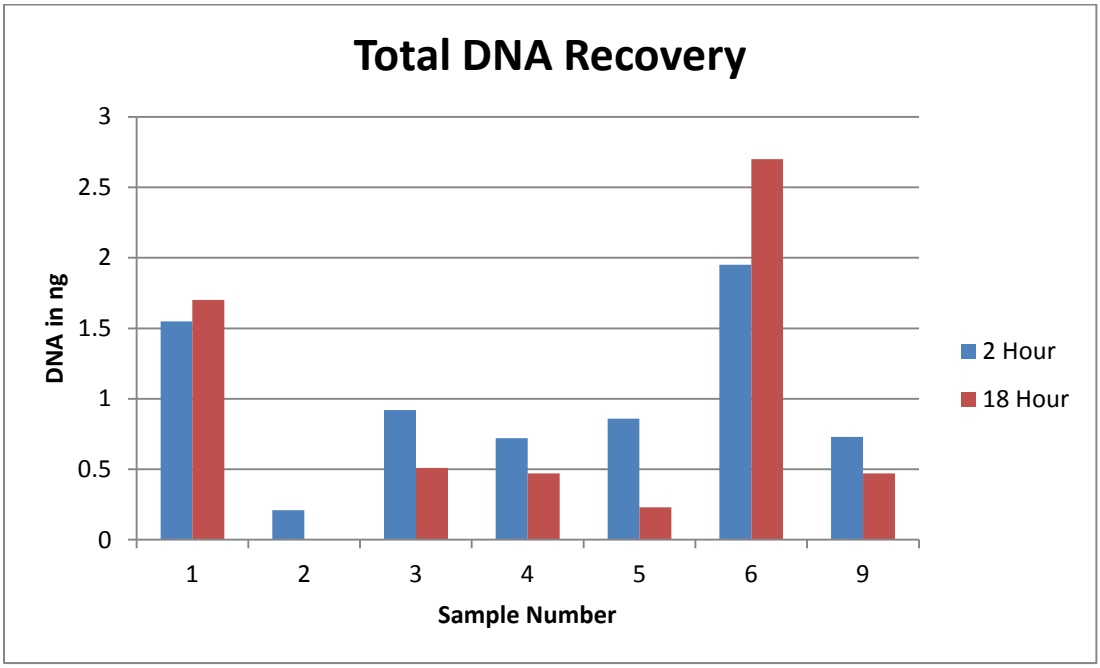


Figure 11. Total DNA Recovered with 2 Hour Versus 18 Hour Incubation Time in PrepFiler Express BTA™ Lysis Solution. A graphical representation of the average total DNA recoveries for 7 of the 9 bone samples tested with differing incubation times.

A Mann-Whitney U Test was performed to determine if there was a statistically significant difference between the DNA concentrations (ng/µL) and the incubation time in the PrepFiler Express BTA™ Lysis solution. This test was not statistically significant ($p=0.178$) at the 0.5 level, which meant that the null hypothesis could not be rejected and DNA concentration

was not statistically different between the two different incubation times in the PrepFiler Express BTA™ Lysis solution. The mean concentration of the samples processed with the 2 hour incubation was 0.0198 ng/µL with a standard deviation of 0.0150. The mean concentration of the samples processed with the 18 hour incubation was 0.0177 ng/µL with a standard deviation of 0.0194.

Incubation in PrepFiler Express BTA™ Lysis Solution and STR Data Quality

All of the samples with positive quantification results were amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles. The replicates from sample 2 with an 18 hour incubation period and one of the replicates from sample 5 with an 18 hour incubation period were the only samples not amplified. The 3500XL Genetic Analyzer was used to separate the amplified products from each of the samples, and the data were analyzed using GeneMapper®*ID-X* software. Currently the 3500XL has not been validated for use in casework and the interpretation thresholds from the validation of the 3130*xl* were used. The minimum interpretation threshold that was set by the UNTCHI Missing Persons Laboratory was 70 relative fluorescence units (rfu) for heterozygous alleles. Alleles in heterozygous loci must meet this threshold, and the peak height ratio cannot be less than 50%. A homozygous peak at a locus was only reported if the allele peak height was greater than or equal to 200 rfu.

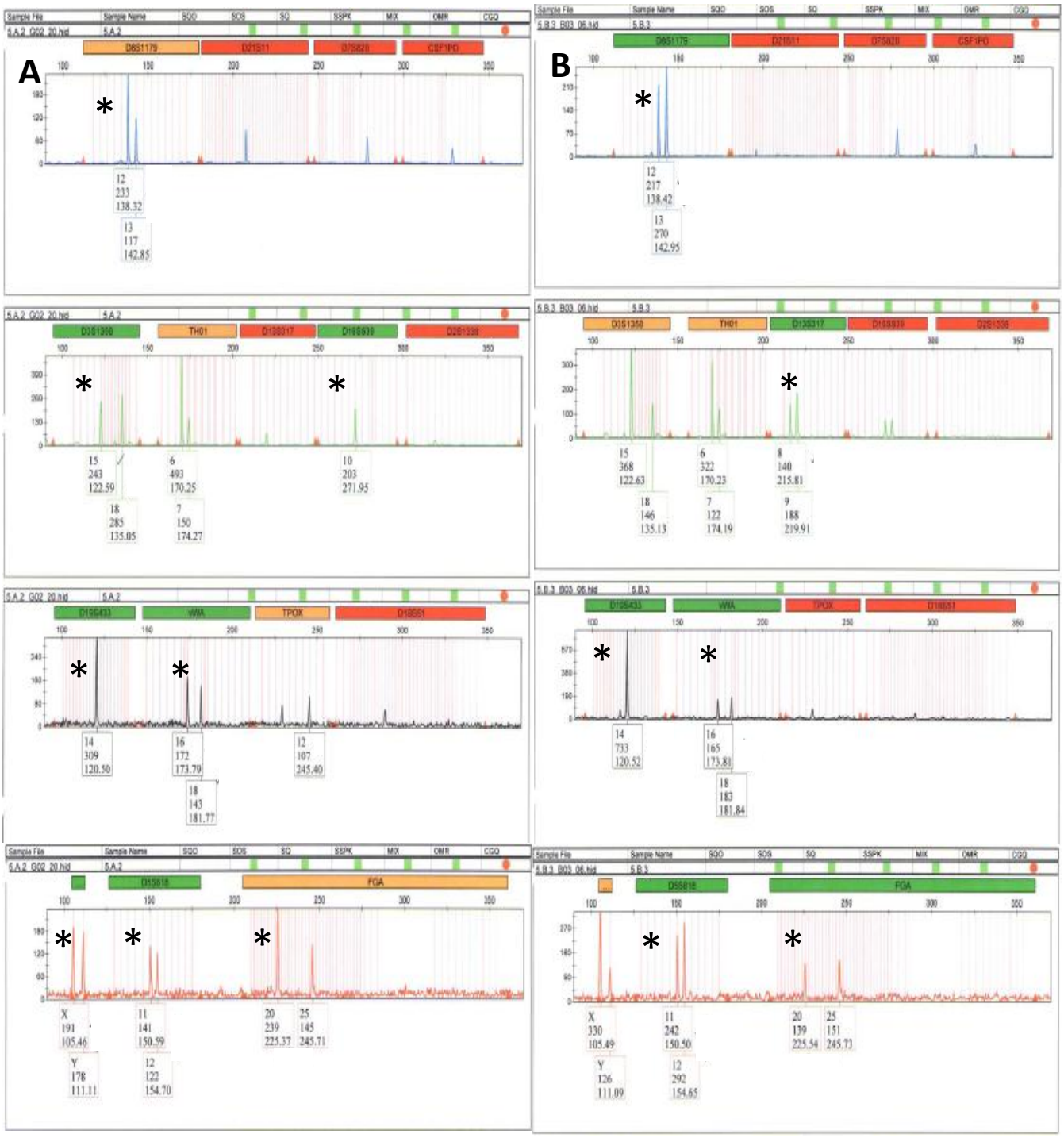


Figure 12. Electropherogram Showing 2 Hour Versus 18 Hour Incubation Time in PrepFiler Express BTA™ Lysis Solution. 12A is an electropherogram for bone sample 5 incubated for 2 hours in the PrepFiler Express BTA™ Lysis Buffer. 12B is an electropherogram for bone sample 5 incubated for 18 hours in the PrepFiler Express BTA™ Lysis Buffer. More reportable alleles were obtained with the 2 hour incubation for sample 5. Reportable loci are marked with an asterisk (*).

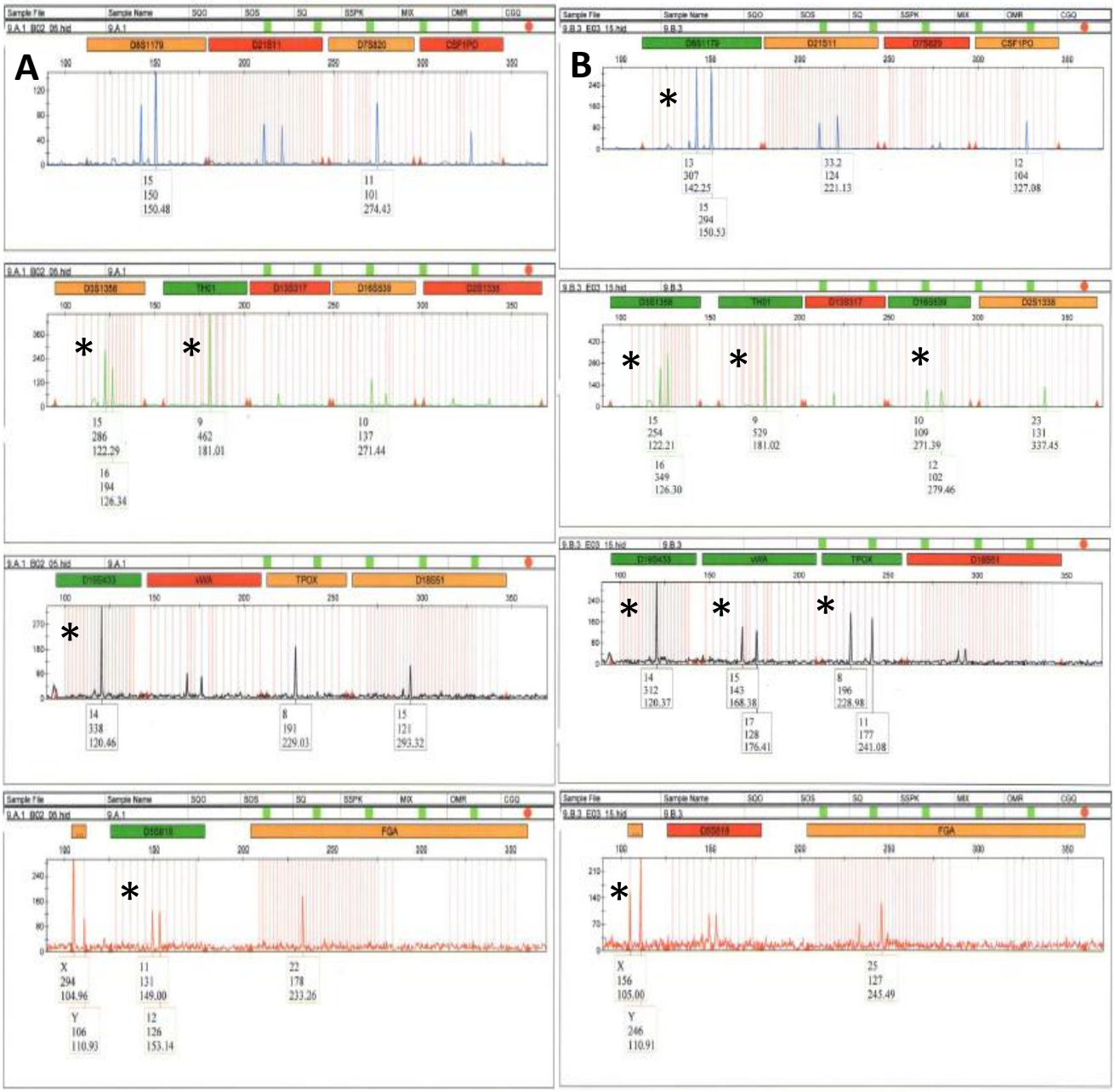


Figure 13. Electropherogram Showing 2 Hour Versus 18 Hour Incubation Time in PrepFiler Express BTA™ Lysis Solution. 13A is an electropherogram for bone sample 9 incubated for 2 hours in the PrepFiler Express BTA™ Lysis Buffer. 13B is an electropherogram for bone sample 9 incubated for 18 hours in the PrepFiler Express BTA™ Lysis Buffer. More reportable alleles were obtained with the 18 hour incubation for sample 9. Reportable loci are marked with an asterisk (*).

Figure 12 shows two electropherograms that were generated from sample 5. Figure 12A is an electropherogram from sample 5 when it was incubated for only 2 hours in the PrepFiler Express BTA™ Lysis solution. When the same sample was incubated in the PrepFiler Express BTA™ Lysis solution for 18 hours there were less reportable alleles (Figure 12B). Reportable loci are marked with an asterisk. Figure 13 shows two electropherograms that were generated from sample 9. Figure 13A is an electropherogram that was recovered from sample 9 when it was incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution. When sample 9 was incubated in the PrepFiler Express BTA™ Lysis solution for 18 hours there were more reportable alleles generated (Figure 13B). Reportable loci are marked with an asterisk.

	Number of Reportable Alleles	
Sample Number	**2 hour**	**18 hour**
1	26	21
3	10	18
4	9	8
5	15	5
6	21	18
7	32	32
8	32	32
9	6	14

Table 4. Number of Reportable Alleles with 2 Hour Versus 18 Hour Incubation Time in the PrepFiler Express BTA™ Lysis Solution. Number of reportable alleles were determined from a composite profile of the 3 replicate amplifications for each sample.

The numbers of reportable alleles that are shown in Table 4 were determined based upon a composite profile using the results of the 3 replicate samples. A composite profile is determined by confirming the presence of reportable alleles in at least 2 out of the 3 replicates. Four samples (bones 1, 4, 5, and 6) contained more reportable alleles using the 2 hour incubation

period; 2 samples (bones 7 and 8) gave the same number of reportable alleles with the 2 hour incubation and 18 hour incubation periods; and 2 samples (bones 3 and 9) contained more reportable alleles using an 18 hour incubation period. A graphical representation of the number of reportable alleles for each bone sample at 2 hour and 18 hour incubation periods are shown in Figure 14.

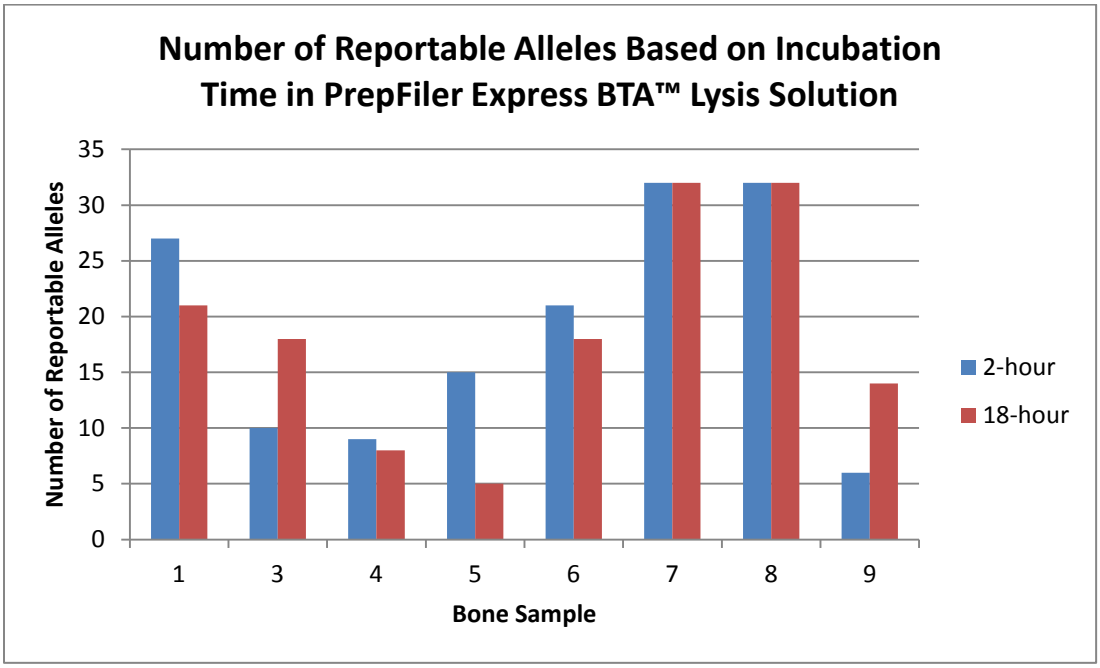


Figure 14. Number of Reportable Alleles with 2 Hour Versus 18 Hour Incubation in the PrepFiler Express BTA™ Lysis Solution. A graphical representation of the number of alleles that were generated with the two different incubation times.

DNA Recovery from Larger Quantities of Bone Powder (150 mg Versus 500 mg)

The structure of bone is not homogeneous, and the osteocytes are sporadically located throughout the bone (Figure 1). Since the recovery of DNA from older bone samples is typically lower, it has been suggested that larger quantities of bone powder should be used for isolating DNA. Seven of the nine original samples had sufficient powder to extract a 500 mg aliquot. In

order to sufficiently lyse 500 mg of bone, the amount of PrepFiler Express BTA™ lysis solution was increased to 1.1 mL. After incubation and centrifugation, approximately 700 µL of solution was recovered. The AutoMate Express™ is pre-programmed to only load a total of 230 µL of the lysate. The programming of the instrument cannot be modified by the user, so the lysate was divided into three equal volumes, approximately 230 µL each, and each was loaded into a separate PrepFiler™ sample tube. Extracted DNA was eluted into 3 tubes containing 50 µL each. Preliminary tests were performed using a defined amount of control DNA (9947A) to determine if it would be beneficial to pool the 3 extracts together and concentrate them using an Amicon® Ultra-4 Centrifugal Filer Unit. However, there was a significant loss seen with the control DNA suggesting that an alternative method to pool and concentrate the 3 aliquots is needed.

Total DNA recovery was determined for each bone sample that was processed starting with 150 mg of bone powder versus 500 mg of bone powder (Table 5). Data were used from previous extractions of 150 mg of bone powder using the standard 2 hour incubation, and the total DNA recovery was determined from the average of the quantification values obtained when 150 mg of each sample was processed in triplicate. The total DNA recovery from a 150 mg aliquot of bone powder was compared to the total DNA recovery from a 500 mg aliquot of bone powder. Since 500 mg represents 3.33 times as much powder, the yield of DNA from 500mg of bone powder should be 3.33 times as much as compared to 150 mg of bone powder. Only one of the 500 mg samples tested yielded more than 3.33 times the amount of DNA as compared to a 150 mg aliquot (sample 1).

Bone Sample	Total DNA Recovered (ng)	
	150 mg	500 mg
1	1.55	5.58
3	0.92	0.00
4	0.72	2.08
6	1.95	4.65
7	116.00	254.00
8	24.90	17.95
9	0.73	0.59

Table 5. Total DNA Recovered with 150 mg of Bone Powder Versus 500 mg of Bone Powder. Total recovered DNA in nanograms for bone samples which started out with two different amounts of bone powder.

Figure 15 is a graph showing the total DNA recovery for bone samples when two different amounts of bone powder were extracted. Only sample 1 demonstrated DNA recovery comparable to the amount of increased bone powder. In samples 4, 6, 7, 8, and 9, the amount of DNA recovered was not proportional to the increased amount of bone powder. The amount of DNA from bone samples 7 and 8 are shown in Table 5, but they are not displayed in the graph since they had a much larger yield in comparison with the other samples.

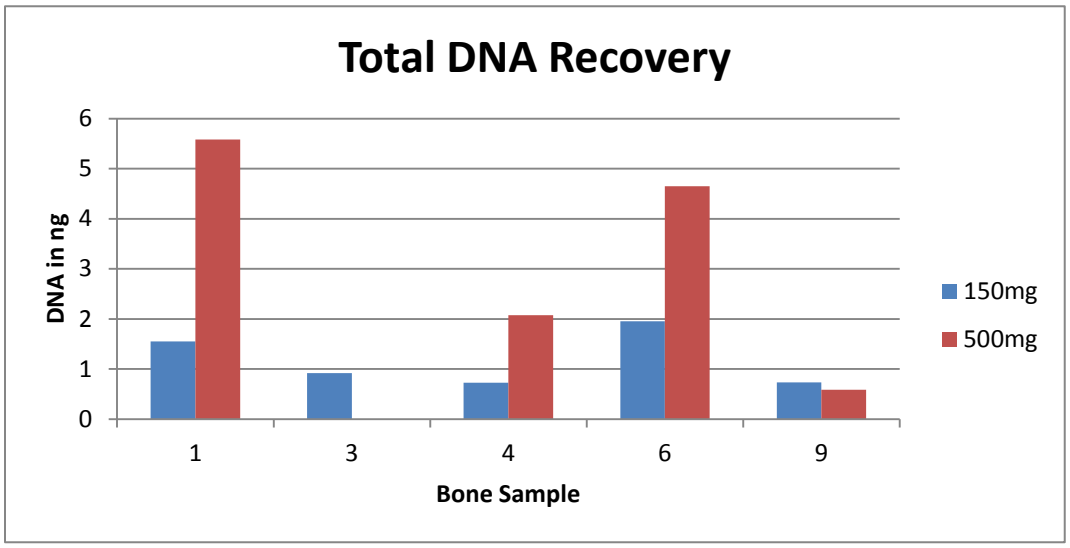


Figure 15. Total DNA Recovered with 150 mg of Bone Powder Versus 500 mg of Bone Powder. A graphical representation of the total DNA recovery in ng for each bone sample tested with differing starting amounts.

Quality of STR Profiles Produced from DNA Extracted on the AutoMate Express™ with Larger Quantities of Bone Powder (150 mg Versus 500 mg)

Each DNA sample extracted from 500 mg aliquots of bone powder was amplified using the AmpFLSTR® Identifler® Plus PCR Amplification Kit at 29 cycles and run on a 3500XL Genetic Analyzer. The data were analyzed in GeneMapper®*ID-X* software. The minimum interpretation threshold and peak height ratio for heterozygous loci remained the same. However, the interpretation guidelines for homozygous loci were increased to 400 rfu when the cycle number was increased to 29. The profiles from 500 mg samples amplified for 29 cycles were compared to the data from the composite profiles of the 150 mg replicates that were amplified for 28 cycles. Table 6 shows the number of alleles that would be considered reportable.

	Number of Reportable Alleles	
Bone Sample	150 mg	500 mg
1	27	28
3	10	0
4	9	14
6	21	20
7	32	30
8	32	32
9	6	18

Table 6. Reportable Alleles with 150 mg of Bone Powder Versus 500 mg of Bone Powder. The number of reportable alleles for each of the tested samples. Samples 2 and 5 are not shown because they were not tested with 500 mg starting material. The interpretation guidelines from the UNTCHI Missing Persons Laboratory were used to determine allele calls. The 150 mg triplicates were amplified for 28 cycles, while 500 mg samples were amplified for 29 cycles.

Three of the samples (samples 1, 4, and 9) generated more reportable alleles when 500 mg of bone powder was used, and four of the samples (samples 3, 6, 7, and 8) generated an either equal or greater number of reportable alleles when 150 mg of bone powder was extracted in triplicate when amplified for one less cycle. The data suggest that there is not an advantage in isolating DNA from larger amounts of bone powder.

Quality of the mtDNA Sequence Data Produced from DNA Extracted on the AutoMate Express™ with Larger Quantities of Bone Powder (150 mg Versus 500 mg)

Mitochondrial DNA sequence data were generated from samples extracted from two different amounts of bone powder. Figure 16 shows mtDNA sequence data from both extractions of bone sample 9. High quality mtDNA sequence data were obtained for both of the starting amounts of bone powder.

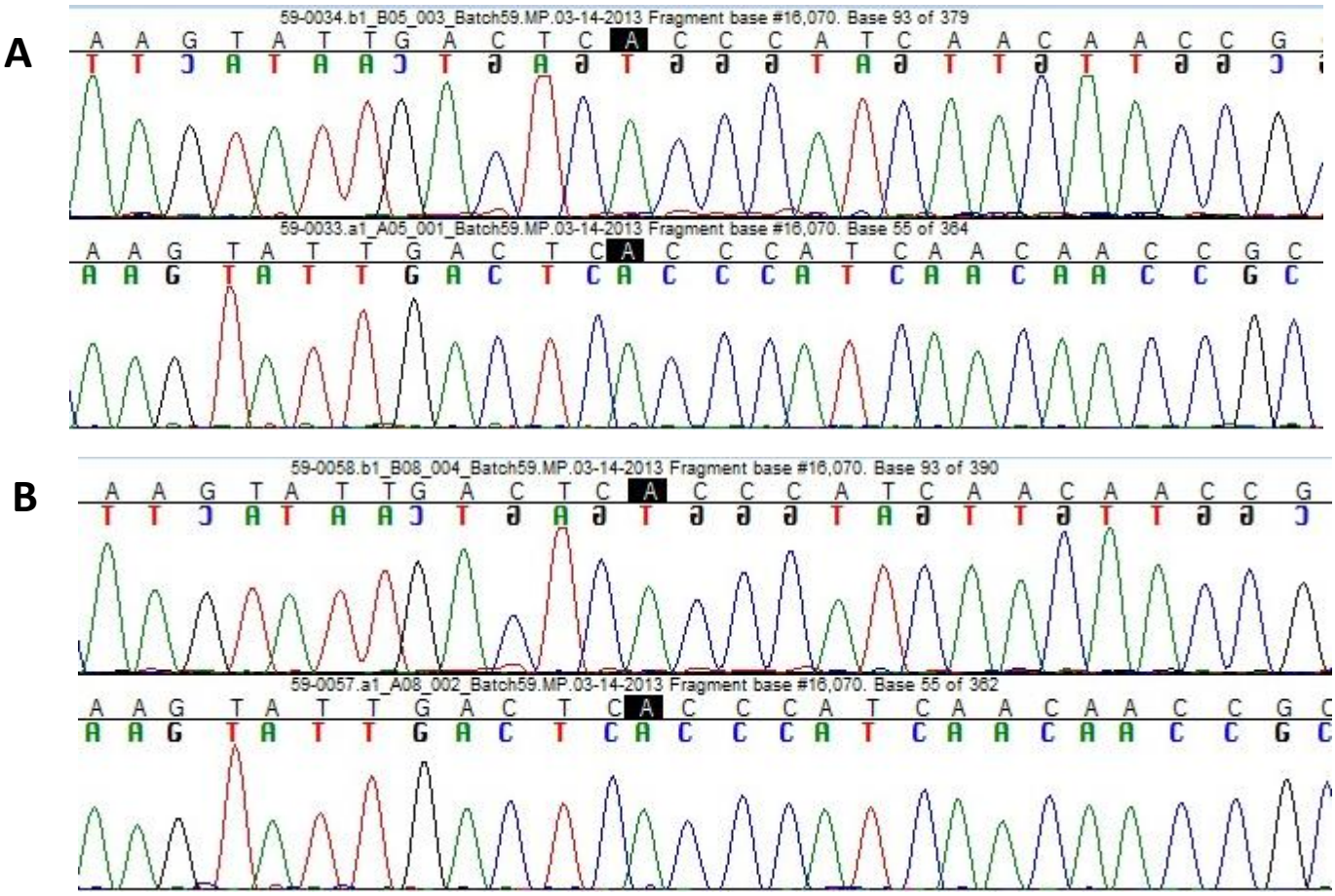


Figure 16. mtDNA Sequence Data Comparing 150 mg of Bone Powder Versus 500 mg of Bone Powder. 16A is sequence data obtained when 150 mg of bone powder was extracted using the AutoMate Express™. 16B is sequence data obtained when 500 mg of bone powder was extracted using the AutoMate Express™.

Comparison to Data Generated from UNTCHI Missing Persons Laboratory

In order to implement a new methodology in the UNTCHI Missing Persons Laboratory, data would have to be compared to determine if the quantity of DNA recovered and the quality of genetic data obtained are better or at least comparable to that obtained using the conventional organic extraction method. Quantification data, STR data using the AmpLSTR® Identifiler® Plus PCR Amplification Kit, STR data using the AmpFLSTR® MiniFiler™ PCR Amplification Kit, and mtDNA sequence data were provided by the UNTCHI Missing Persons Laboratory for comparison with bones processed with the AutoMate Express™ in this study.

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

The quantification data obtained from extractions of two different amounts of bone powder using the AutoMate Express™ were compared to the quantification values provided by the UNTCHI Missing Persons Laboratory in which 1 g of bone powder was used with a conventional organic extraction (Table 7).

	AutoMate Express™		**Organic Extraction**
Bone Sample	450 mg (3x150 mg)	500 mg	1 g
	Total DNA Recovered (ng)		Total DNA Recovered (ng)
1	4.65	5.58	1.93
2	0.62	Not Tested	0.00
3	2.76	0.00	0.00
4	2.21	2.07	3.16
5	2.58	Not Tested	0.58
6	5.81	4.65	30.2
7	326.55	253.50	904.00
8	74.70	18.00	12.44
9	2.21	0.59	4.28

Table 7. Comparison of Total DNA Recovered between the Two Extraction Methods. The total DNA recovery obtained from 450 mg of bone powder (2 hour incubation period) and 500 mg of bone powder extractions using the AutoMate Express™ and from extractions of 1 g of bone powder using the conventional organic method.

In five of the samples (samples 1, 2, 3, 5, and 8), the lysates processed on the AutoMate Express™ yielded more total DNA than those obtained using the organic method. The amount of DNA recovered from sample 7 was smaller using the AutoMate Express™; however, the amount of DNA recovered was more than sufficient to produce a complete STR profile. All of the samples that yielded more DNA using the AutoMate Express™ were from the 150mg aliquots of bone powder extracted in triplicate.

A Mann-Whitney U Test was performed to determine if there was a statistically significant difference between the total DNA recoveries between the two different types of extractions. This test was not statistically significant ($p=0.605$) at the 0.5 level, which meant that the null hypothesis could not be rejected. The total DNA recovery was not statistically different between bone samples processed using the standard organic extraction and those processed using the AutoMate Express™.

Comparison of STR Profiles between DNA Extracted on the AutoMate Express™ Versus the Standard Organic Method

STR profiles from DNA extracted on the AutoMate Express™ were amplified using the AmpFLSTR® Identifler® Plus PCR Amplification Kit at 28 cycles and compared to profiles from the same bone samples extracted by the UNTCHI Missing Persons Laboratory amplified at 29 cycles. Table 8 shows the number of loci that were reportable utilizing UNTCHI Missing Persons Laboratory standard interpretation guidelines. Four of the samples (samples 1, 4, 6, and 9) generated more reportable loci when DNA was isolated with the standard organic extraction. Three of the samples (samples 2, 3, and 7) generated the same number of reportable loci between the two types of extractions. Two of the samples (samples 5 and 8) generated more reportable loci with DNA isolated using the AutoMate Express™ instrument. The data suggest that in 5 out

of 9 samples tested, DNA extracted using the AutoMate Express™ instrument was able to generate a number of reportable loci that was equal to or greater than the number obtained from DNA isolated using the standard organic extraction method.

	AutoMate Express™		**Organic Extraction**	
Bone Sample	150 mg (2 hour Incubation)		1 g	
	Number of Loci	Amount of DNA Added to Reaction (ng)	Number of Loci	Amount of DNA Added to Reaction
1	8	0.565	10	0.097
2	0	0.050	0	Undetermined
3	0	0.351	0	Undetermined
4	0	0.193	4	0.158
5	1	0.227	0	0.029
6	6	0.571	7	0.151
7	16	0.500	16	0.452
8	16	0.500	2	0.622
9	0	0.188	13	0.214

Table 8. Comparison of the Number of Reportable Loci between the Two Extraction Methods Using the AmpFLSTR® Identifler® Plus PCR Amplification Kit. Comparison of the number of reportable loci according to the interpretation guidelines set by the UNTCHI Missing Persons Laboratory obtained when amplifying extracts from the AutoMate Express™ and the conventional organic extraction method with the AmpFLSTR® Identifler® Plus PCR Amplification Kit.

DNA samples that were isolated on the AutoMate Express™ were amplified using the AmpFLSTR® MiniFiler™ PCR Amplification Kit and run on a 3130*xl* Genetic Analyzer. The profiles were interpreted using GeneMapper® *ID-X* software under interpretation guidelines set by the UNTCHI Missing Persons Laboratory for the AmpFLSTR® MiniFiler™ PCR Amplification Kit. The UNTCHI Missing Persons Laboratory provided electropherograms showing samples which were extracted using the conventional organic method. The data suggest that the AmpFLSTR® MiniFiler™ PCR Amplification Kit produced comparable results when used with the two different extraction methods (data not shown).

Comparison of mtDNA Sequence Data Between AutoMate Express™ and Organic Extraction Method

A comparison was performed between mtDNA sequence data obtained from samples that were extracted on the AutoMate Express™ and mtDNA sequence data provided by the UNTCHI Laboratory from samples extracted using the conventional organic method. The mtDNA sequence data obtained from the DNA extracted using the AutoMate Express™ were consistent with the data obtained from DNA isolated using the standard organic extraction method and all base calls were concordant. Figure 17A shows a region of HV1 from Sample 6 that was obtained from the UNTCHI Missing Persons Laboratory. It is compared to the same region of HVI from sample 6 extracted using the AutoMate Express™ (Figure 17B).

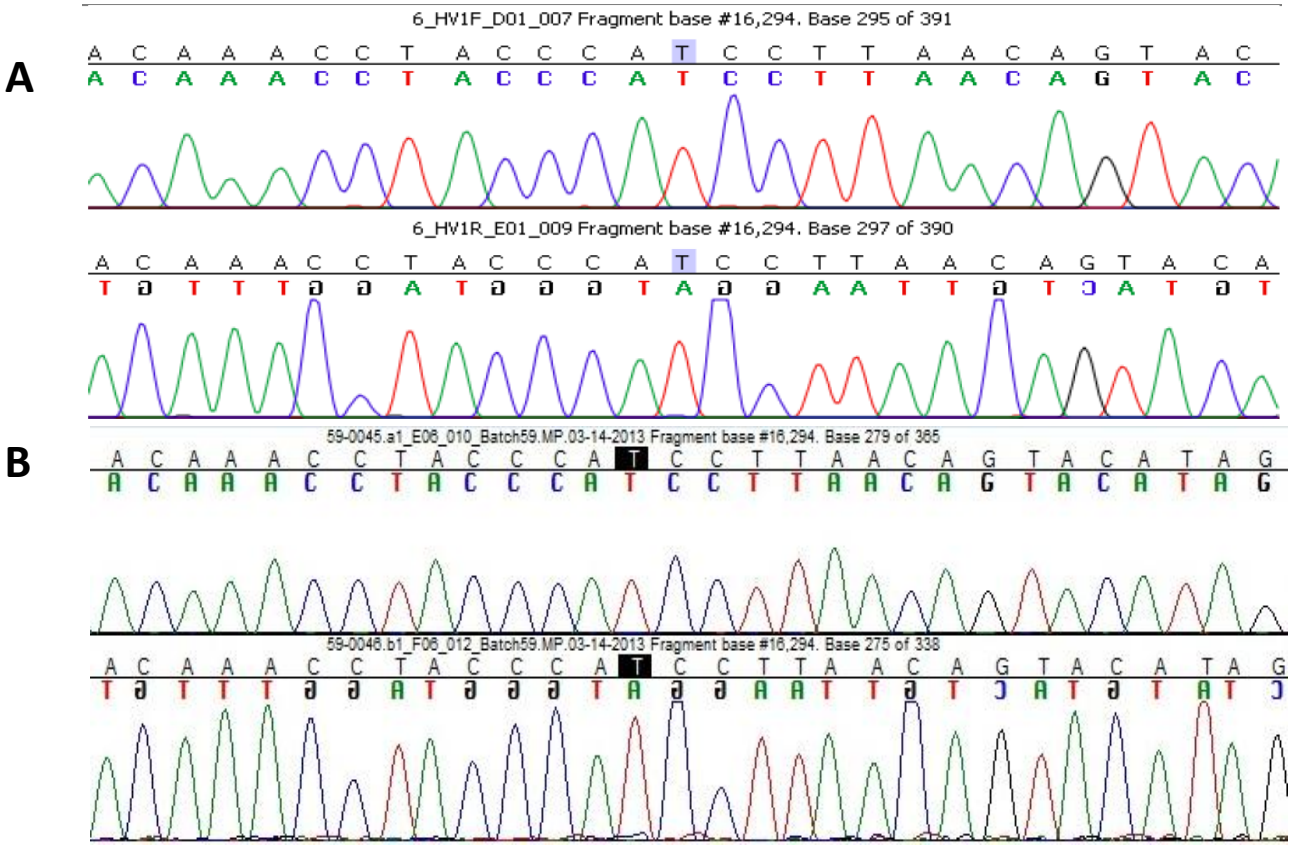


Figure 17. mtDNA Sequence Data Comparing Extraction Methods. 17A shows mtDNA sequence data from the UNTCHI Missing Persons Laboratory that was obtained with an organic extraction. 17B shows mtDNA sequence data that was obtained using the AutoMate Express™.

CHAPTER IV

CONCLUSIONS

The large number of unidentified remains and unsolved missing persons cases is a critical problem within the United States. Each day there are between 85,000 and 100,000 active missing person's cases. In approximately half of these cases, there has been no contact with the missing individual for greater than a year. Thousands of individuals, both children and adults, vanish every year under suspicious circumstances, and their cases remain unsolved. For many years there has been speculation as to the number of unidentified decedents that are retained at medical examiners, coroners and law enforcement agencies. The report issued by the Bureau of Justice Statistics on the census of "Medical Examiner and Coroner's Offices, 2004" indicated that these agencies had records on approximately 13,500 unidentified human decedents. Jeffrey Sedgwick, then Director of the Bureau of Justice Statistics, had suggested that the true number of remains was probably far higher than the 13,500 reported. Prior estimates had suggested that the number of unidentified human decedents could potentially be 40,000 or more. The 2004 report indicated that approximately 4,400 unidentified human decedents are received by medical examiners and coroners each year, and that after one year, approximately 1,000 decedents remained unidentified and became "cold cases" (16). Without the collection and submission of a bone sample for DNA testing, the chance of identifying the decedent is potentially eliminated. If an STR profile and/or a mtDNA haplotype could be obtained from a bone sample, it could be entered into the Unidentified Person (UHR) Database in the Combined DNA Index System

(CODIS). The UHR profiles are compared in CODIS to the DNA profiles from Family Reference Samples (FRS) that are obtained from samples provided by family members with a missing loved one. The family reference samples are typically collected by law enforcement agencies with support from the National Missing and Unidentified Persons System (NamUs).

UNTCHI, with financial support from the National Institute of Justice (NIJ), has been responsible for the identification of over 1,000 human decedents. The identifications have been made with the DNA recovered from skeletal remains. Since 2003, UNTCHI has processed nearly 4,000 bone samples (personal communication with Dr. Arthur Eisenberg, Co-director of UNTCHI, March 30, 2013). There are a large number of samples throughout the United States that still require processing and DNA analysis. However, the current procedures to obtain DNA from skeletal remains are laborious, time-consuming, and not amenable to automation. These procedures require a significant number of physical manipulations which could result in an increased risk of sample loss or the potential for contamination with extraneous DNA.

The purpose of this project was to develop an optimized procedure for the extraction of DNA from skeletal remains by finding alternatives to the methodologies that are currently in place in forensic DNA laboratories. New methods could help improve the DNA analysis of skeletal remains, reduce the amount of time it takes to process samples, and reduce the potential for sample loss and contamination. A streamlined, automated process for DNA extraction from skeletal remains could increase the number of samples a laboratory can process and reduce the large number of unidentified decedents and unsolved missing persons cases that currently plague the United States.

The initial step in the extraction of DNA from skeletal remains requires the powdering of the sample, making the cells trapped within the bone matrix more accessible. The current

methodology used to pulverize the bone into powder, involves the use of a Freezer/Mill and liquid nitrogen. The liquid nitrogen is a necessary component of this process since it freezes the bone making it brittle and easier to powder. The FastPrep-24® has been used effectively to pulverize other types of biological specimens. It was tested in this project to determine if it could pulverize human bone samples for forensic DNA extractions.

FastPrep-24® for the Pulverization of Skeletal Remains

MP Biomedicals had suggested that the FastPrep-24® instrument could be used for the pulverization and powdering of small pieces of bone for DNA extraction. However, at the time of this study, there were no publications found demonstrating that it had been successfully used on human bone. MP Biomedicals had developed a series of grinding matrices that come prepackaged in sterile 2 mL and 15 mL single use tubes. The instrument is capable of processing either 24 individual 2mL tubes or 12 individual 15 mL tubes simultaneously. MP Biomedicals proposed that human bone samples could be pulverized, with the appropriate grinding matrix, without the need for liquid nitrogen. The suggested advantages of the FastPrep-24® instrument were: 1) simultaneous processing of 12 to 24 samples; 2) disposable, single use tubes would eliminate the cleaning and re-use of the metal end caps and impactors; and, 3) liquid nitrogen was not required. Although the conventional procedure for powdering bones using a Freezer/Mill with liquid nitrogen is very effective, it has a number of limitations that led to the evaluation of the FastPrep-24® instrument.

Initially 3 bone samples, one from each age category, were fragmented into 50 to 100 mg pieces to test the FastPrep-24®. MP Biomedicals recommended several different grinding matrices, either individually or in combination, to pulverize the bone fragments. A combination of two of the lysing matrices (garnet shards and zirconium oxide coated ceramic beads) was

considered the most likely to powder human bone. Unfortunately, there was very little evidence of powdering after the instrument was run (4 cycles at 6.0 m/s for 40 seconds each) with this combination of lysing matrices. All of the bone samples, with exception of bone 5, appeared to be the same size. The only noticeable difference was that the edges of the bone fragments appeared to be smoother and more rounded.

Although it was suggested that liquid nitrogen would not be needed, it was included in one of the experiments, in an attempt to make the bone fragments more brittle and more amenable to pulverization. Three additional bones were tested, with the addition of liquid nitrogen directly to each sample tube. The bone samples were each weighed to determine a starting weight before grinding. Liquid nitrogen was added on top of the sample and the lysing matrices, directly to the 15 mL conical tube. The tubes were placed in the instrument, and the run was started. After the 4 cycles were completed, two of the three bone samples appeared to be the same size, with only the exterior surface of the bone showing a polished appearance. Only one of the samples tested with liquid nitrogen was powdered. The sample (bone 5) that powdered was from a rib bone. Empirical studies have shown that long bones, such as a femur or tibia, most often yield DNA, and are the first choice for forensic testing. The 9 bone samples used in this study were: 7 femur samples, 1 humerus sample, and 1 rib sample. Rib bones have a thin outer cortex of compact bone; however, they are primarily made up of trabecular bone. Trabecular bone is less dense, containing more hollow space, and typically yields less DNA. The lysing matrices were able to powder trabecular bone much more effectively than they could compact bone fragments.

The same 3 bone samples were tested without liquid nitrogen in the sample tubes, and the results were identical to those with liquid nitrogen. The two compact bone fragments were not

pulverized, whereas bone sample 5 had been powdered. The addition of liquid nitrogen had no influence on the effectiveness of the FastPrep-24® in pulverizing compact bone. The only bone sample powdered by the FastPrep-24® instrument was a fragment of rib bone. Since the FastPrep-24® was ineffective at powdering compact bone using currently available lysing matrices, it was determined that using a Freezer/Mill with liquid nitrogen is still the most practical method for powdering bone. This process has been optimized for pulverizing bones and is currently used by the UNTCHI Missing Persons Laboratory. Although this process has limitations, it is manageable and produces bone powder that could be effectively used with the PrepFiler Express BTA™ Lysis solution and the AutoMate Express™.

Incubation in PrepFiler Express BTA™ Lysis Solution and DNA Recovery

Bone powder (150 mg) from each of the nine different samples was extracted in triplicate on the AutoMate Express™ following both a 2 hour and 18 hour incubation period in the PrepFiler Express BTA™ Lysis solution (Table 3). A longer incubation time was tested to determine it could increase the quantity of DNA recovered and/or the quality of the resulting DNA profiles. The results from bone sample 7 and 8 were not used in this study since the amount of DNA recovered from these two samples was significantly higher than the other 7 samples. Thus, they were considered outliers. The amount of DNA recovered in 5 of the remaining 7 samples (bones 2, 3, 4, 5, and 9) was higher when the 2 hour incubation was used. Only 2 of the samples (bones 1 and 6) had a higher yield DNA after the 18 hour incubation. The longer incubation time in the PrepFiler Express BTA™ Lysis solution was not advantageous. In fact, for 5 of the 7 samples, there was a reduction in the amount of DNA recovered. Therefore,

incubating the bone powder in the PrepFiler Express BTA™ Lysis solution overnight (18 hours) was not used in further studies.

Incubation in PrepFiler Express BTA™ Lysis Solution and STR Data Quality

A composite STR profile was developed for eight of the nine bone samples using the profiles generated with the DNA from the triplicate extractions. An allele was included in the composite profile, if it was reportable (based upon UNTCHI interpretation guidelines) in at least two of the three independent, amplification reactions. Bone sample 2 did not yield any reportable alleles with the DNA extracted following either the 2 hour or 18 hour incubation in the PrepFiler Express BTA™ Lysis solution (Table 4). DNA extracted from four of the eight bone samples (bones 1, 4, 5, and 6) produced a composite profile with more reportable alleles with the 2 hour incubation in the PrepFiler Express BTA™ Lysis solution. DNA from two of the eight samples (bones 7 and 8) produced a complete composite profile with the same number of reportable alleles with both the 2 hour and 18 hour incubation. DNA from two samples (bones 3 and 9) had composite profiles with more reportable alleles when 18 hour incubation was used. The overall quality of the STR profiles and the amount of genetic data obtained after an incubation of 18 hours did not justify incubating the bone powder in the PrepFiler Express BTA™ Lysis solution for longer periods of time.

DNA Recovery with Increased Quantities of Bone Powder (150mg Versus 500mg) Using the AutoMate Express™

In an attempt to recover larger quantities of DNA, two different amounts of bone powder (150 mg and 500 mg) were extracted using the AutoMate Express™. The amount of DNA recovered from 500 mg of bone powder was compared to the amount recovered from 150 mg.

The 500 mg sample has 3.33 times more powder than the 150 mg aliquot, and if the extraction process was scalable, the amount of DNA recovered from 500 mg of powder should be approximately 3.33 times greater than the 150mg aliquot of the same powder. The data indicated (Table 5) that only 1 of the 7 bone samples (bone 1) yielded at least 3.33 times more DNA extracted from the 500mg aliquot compared to the 150 mg aliquot of bone powder. Based on this observation, it does not appear that using 500 mg of bone powder was as effective as extracting DNA from 150mg of bone powder. In order to extract 500 mg of bone powder, the volume of the PrepFiler Express BTA™ Lysis Buffer was increased from 230 µL to 1,100 µL per sample. The reduced efficiency seen in extracting DNA from a 500 mg sample cannot be justified. To increase the total recovery of DNA from a bone sample, it is suggested that three replicates of 150 mg of powder be processed.

Quality of the STR Profiles Produced from DNA Extracted on the AutoMate Express™ with Larger Quantities of Bone Powder (150 mg Versus 500 mg)

DNA samples extracted from both 150 mg and 500 mg aliquots of bone powder were amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit and the numbers of reportable alleles were determined (Table 6). In three of the seven samples (bones 1, 4 and 9), DNA extracted from a single 500 mg aliquot of bone powder produced more reportable alleles than the DNA extracted from the 150 mg aliquot of bone powder. DNA extracted from bone 8 produced an equivalent number of reportable alleles from the two different amounts of bone powder. In the remaining three samples (bones 3, 6 and 7) the DNA extracted from a 150 mg aliquot of bone powder gave more reportable alleles than the DNA from the 500 mg sample which was amplified at one additional cycle. Again, there was no advantage in extracting DNA from a larger quantity of sample in a single aliquot.

Quality of the mtDNA Sequence Data Produced from DNA Extracted on the AutoMate Express™ with Larger Quantities of Bone Powder (150 mg Versus 500 mg)

Mitochondrial DNA sequence data were obtained from DNA recovered from differing amounts of bone powder (Figure 16). The base calls made were concordant amongst all samples. There was no advantage in the quality of the mtDNA sequence data when extracting mtDNA from more bone powder.

Comparison with Data Generated from UNTCHI Missing Persons Laboratory

The main purpose of this study was to optimize the extraction of DNA from human skeletal remains using the PrepFiler Express BTA™ Lysis Buffer and the AutoMate Express™ instrument. Once optimized, the amount of DNA obtained, the quality of the STR data, and the quality of mtDNA sequence data were compared to assess the feasibility of replacing the standard bone demineralization and DNA organic extraction procedure with a faster, simpler automated method. The data generated in this study will be provided to the UNTCHI Missing Persons Laboratory to make further decisions on the implementation of the AutoMate Express™ in casework.

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

Based on the quantification data, it appears that the samples extracted on the AutoMate Express™ produced comparable amounts of DNA as UNTCHI's standard demineralization and organic extraction method. Five out of the 9 samples extracted on the AutoMate Express™ yielded more DNA than the organic extraction method.

Comparison of STR Profiles between DNA Extracted on AutoMate Express™ Versus the Standard Orgranic Method

The STR profiles obtained using the AmpFLSTR® Identifler® Plus PCR Amplification Kit with the AutoMate Express™ extractions were similar to the STR profiles obtained using the DNA recovered with the organic extraction method. Four of the samples (samples 1, 4, 6, and 9) generated more reportable loci when DNA was isolated with the standard organic extraction. Three of the samples (samples 2, 3, and 7) generated the same number of reportable loci between the two types of extractions. Two of the samples (samples 5 and 8) generated more reportable loci when DNA was isolated with the AutoMate Express™ Instrument. The data suggest that in 5 out of 9 samples tested, DNA extractions using the AutoMate Express™ instrument were able to generate a number of reportable loci that were equal to or greater than the number obtained from DNA isolated using the standard organic extraction method amplified at one additional cycle. The STR profiles generated using the AmpFLSTR® MiniFiler™ PCR Amplification Kit were similar to the results with the AmpFLSTR® Identifler® Plus PCR Amplification Kit (data not shown). The quality of the DNA profiles produced with the AutoMate Express™ is at least comparable to the DNA recovered with the organic extraction procedure currently used by the UNTCHI Missing Persons Laboratory.

Comparison of the mtDNA Sequence Data Generated with the DNA Extracted on the AutoMate Express™ and the Standard Organic Method

The quality of the mtDNA sequence data was concordant between the two extraction methods. The HV1 and HV2 regions of the mtDNA genome were amplified from DNA that was recovered with both extraction methods. The sequence data was 100% concordant between the DNA extracted on the AutoMate Express™ and the DNA extracted by the UNTCHI Missing Persons Laboratory.

Overall Conclusions

The FastPrep-24®, in theory, could be beneficial to forensic laboratories, unfortunately it was not capable of routinely powdering long bones (humeri and tibias). It did successfully powder a rib bone; however, the amount of DNA recovered from rib bones is less than what is typically recovered from an equivalent amount of a long bone. Since the amount of DNA recovered from a bone sample is often the limiting factor in obtaining a full STR profile, it is important that the DNA recovery be maximized from the most productive bone sample (compact bones). Until different lysing matrices can be developed or until the speed and grinding ability of the instrument is improved, the FastPrep-24® is not suitable for the extraction of DNA from bone samples for the identification of human skeletal remains. The Freezer/Mill with liquid nitrogen has the ability to powder all types of bone samples, including compact bone. As a result, it is not recommended to use the FastPrep-24® for the pulverization of bone samples at this time.

The data from this project has demonstrated that the AutoMate Express™ in conjunction with the PrepFiler Express BTA™ Lysis Buffer provided an effective method for the extraction of DNA from human skeletal remains. The DNA recovered from a 150mg of bone powder was sufficient to generate reproducible STR profiles and high quality mtDNA sequence data. The quality of the DNA extracted using the AutoMate Express™ instrument produced STR profiles and mtDNA sequence data comparable to the data produced with the UNTCHI conventional organic extraction procedure. In some of the bone samples, the DNA from the AutoMate Express™ produced STR data that was better than the STR results generated from the DNA extracted by the UNTCHI Missing Person Laboratory.

The quantity of DNA recovered from 150 mg of bone powder varied from sample to sample. This is similar to samples extracted with the conventional demineralization and organic

extraction procedure. Increased amounts of bone powder did not result in a proportional increase in the DNA yield. When extracting the DNA from 500 mg of bone powder, a larger volume of the PrepFiler Express BTA™ Lysis solution was required. The appropriate volume of the PrepFiler Express BTA™ Lysis solution for 500 mg of bone powder was determined to be approximately 1,100 µL. After the 2 hour incubation period the lysate was centrifuged to pellet any residual powder, and approximately 700 µL of lysate was recovered. The AutoMate Express™ is pre-programmed to load only 230 µL of lysate; therefore, the lysate from the 500 mg of bone powder was processed in 3 separate columns. The AutoMate Express™ extraction, consisting of 150mg of bone powder in 230µL of the PrepFiler Express BTA™ Lysis solution along with an incubation time of 2 hours prior to loading, was the most effective and cost-efficient procedure. This method takes less than 3 hours from the time the bones are powdered and will save at least one full day per bone to extract DNA. It will also reduce the potential risk of contamination from extraneous DNA because of the minimal number of transfers from one tube to another. The results from this project have shown that extracting DNA from skeletal remains with the AutoMate Express™ extraction method will expedite the results and potentially increase the amount of genetic data and ultimately UNTCHI's capacity for processing bone samples.

Future Studies

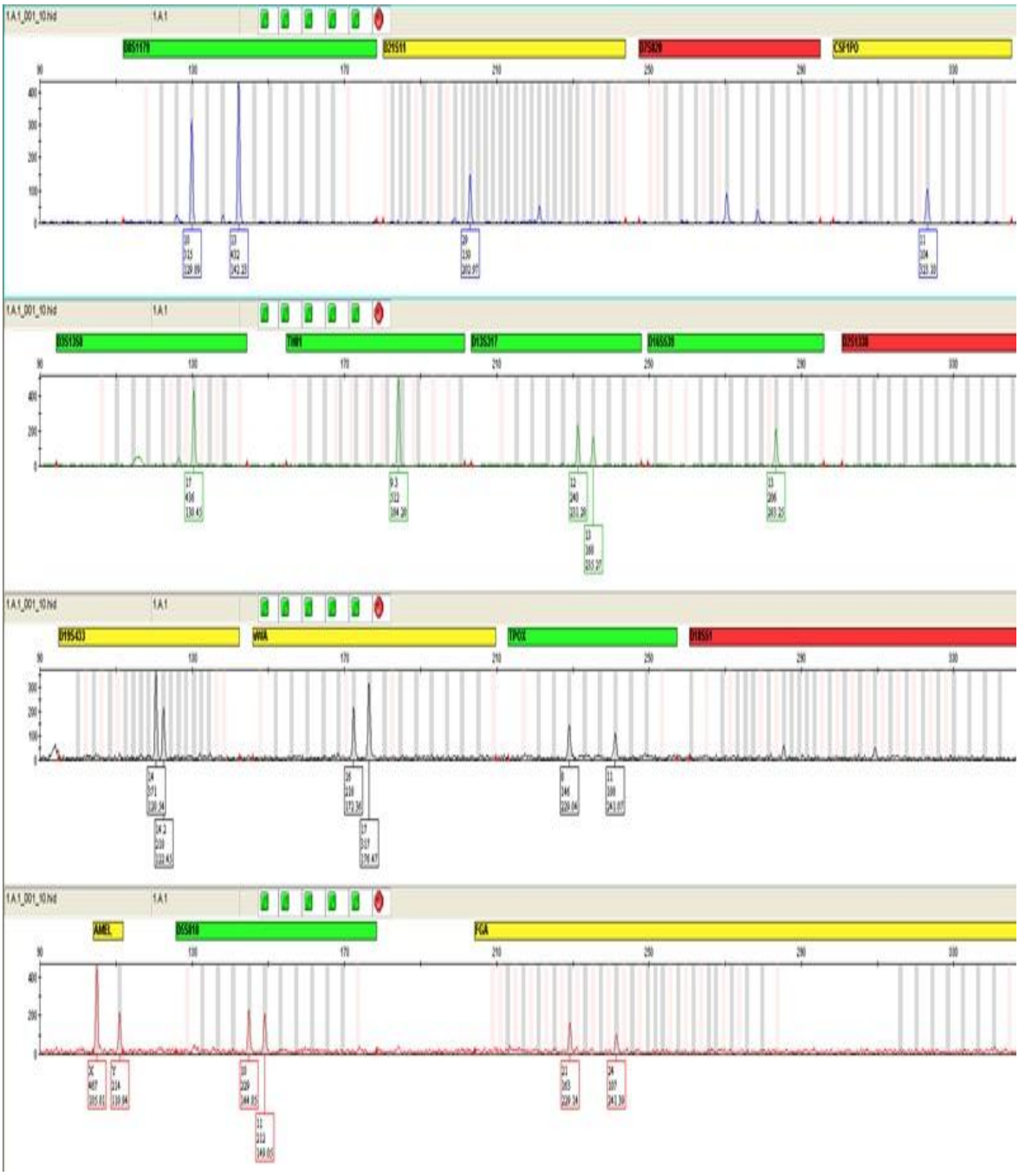
Human bone samples have been sent to MP Biomedicals to test the FastPrep-24™ with a new stainless steel lysing matrix that they are developing. They will continue to optimize the FastPrep-24™ to pulverize skeletal remains and other resistant biological samples. UNTCHI will contact Life Technologies to determine if the AutoMate Express can be re-programmed to

initially load a larger volume of the lysate solution onto the PrepFiler columns in order to allow more bone powder to be processed in a single column. An increased amount of bone powder may require additional washes or larger wash volumes. It would be advantageous to process increased amounts of bone powder and then elute the DNA in a single tube. UNTCHI's Missing Persons Laboratory will develop the appropriate studies to further validate the implementation of the AutoMate Express™ with the PrepFiler Express BTA™ Lysis Buffer in casework.

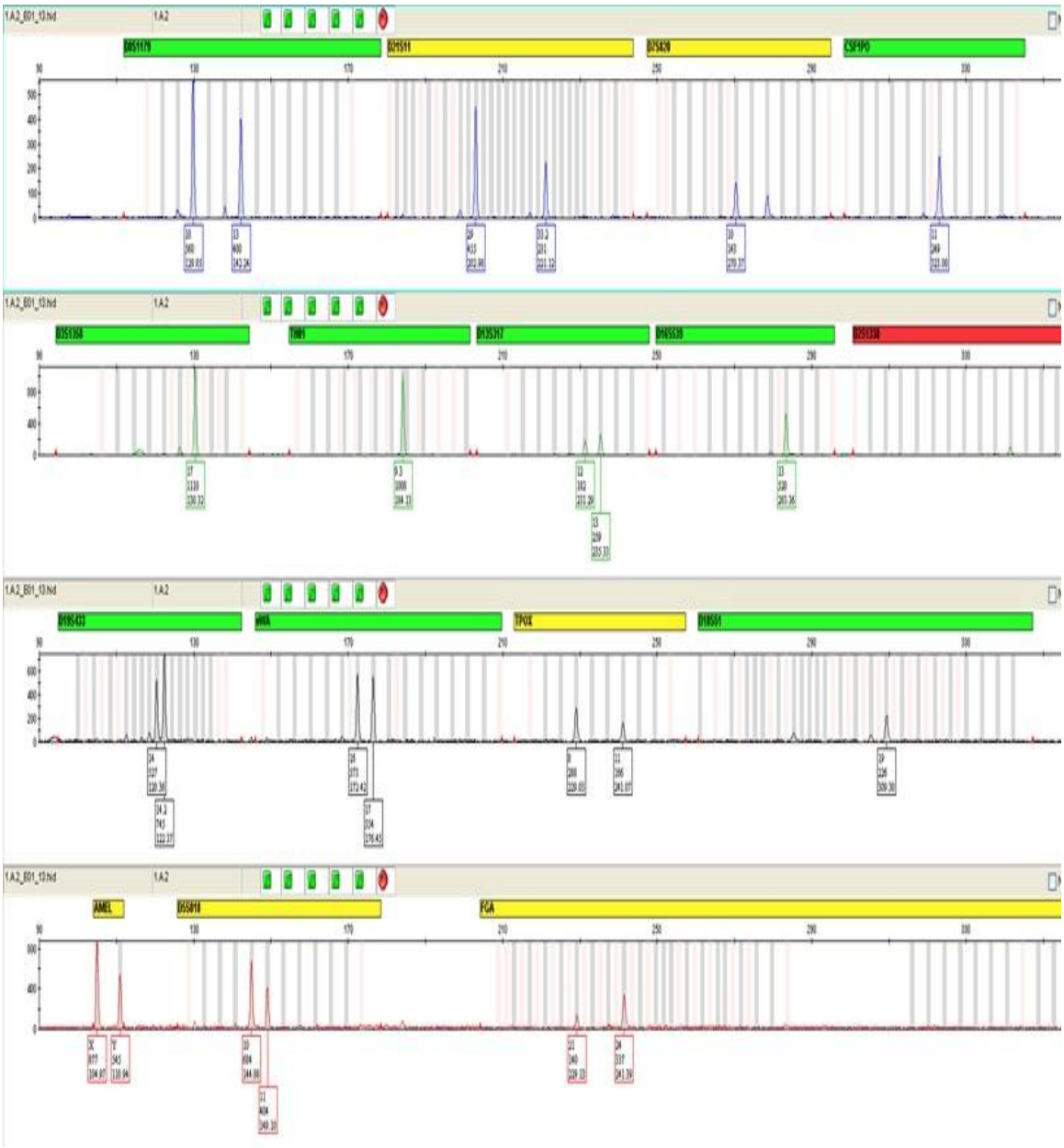
APPENDIX A

ELECTROPHEROGRAMS

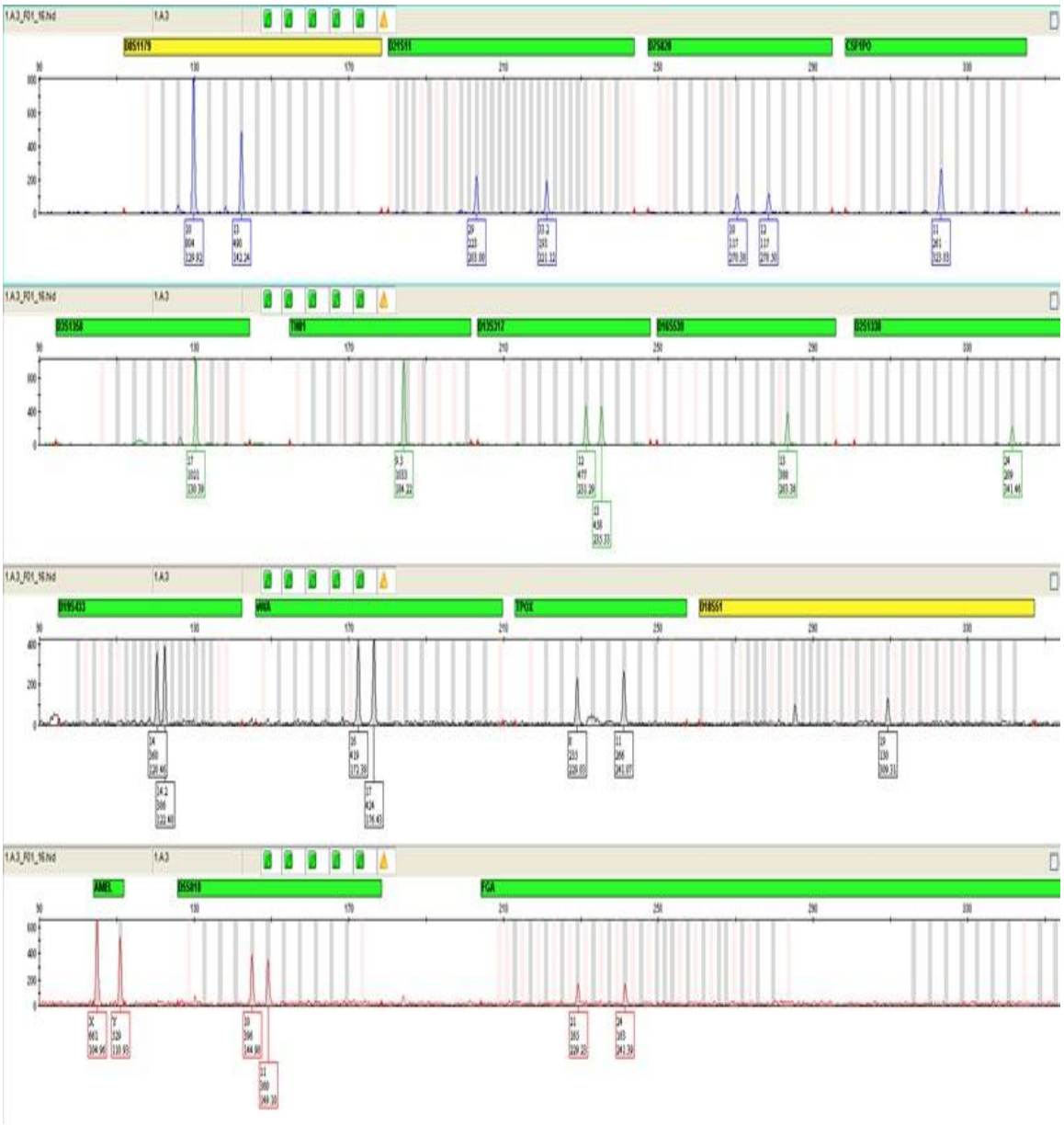
Sample 1 – First triplicate of sample 1 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 1



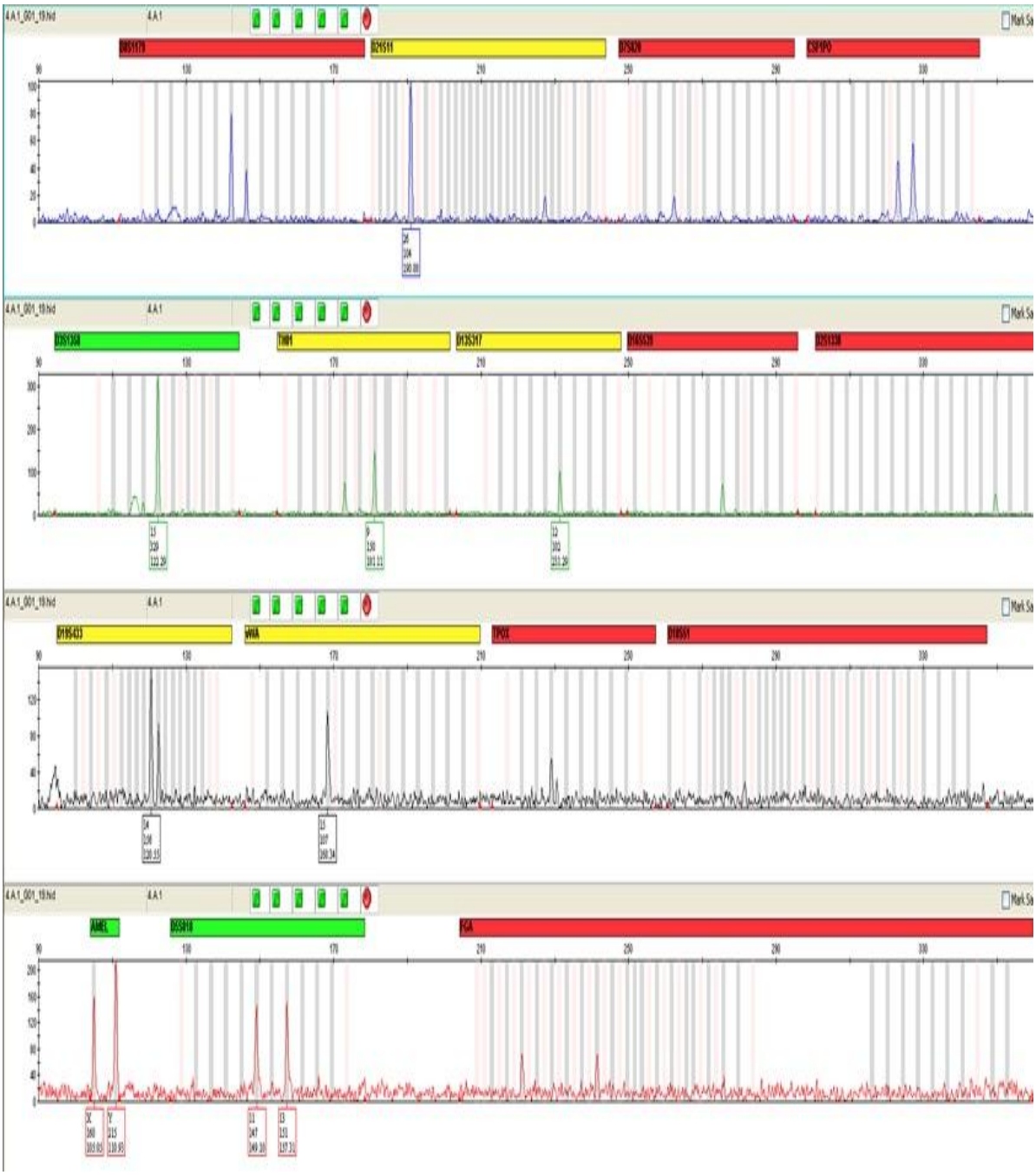
Sample 1 – Second triplicate of sample 1 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 1



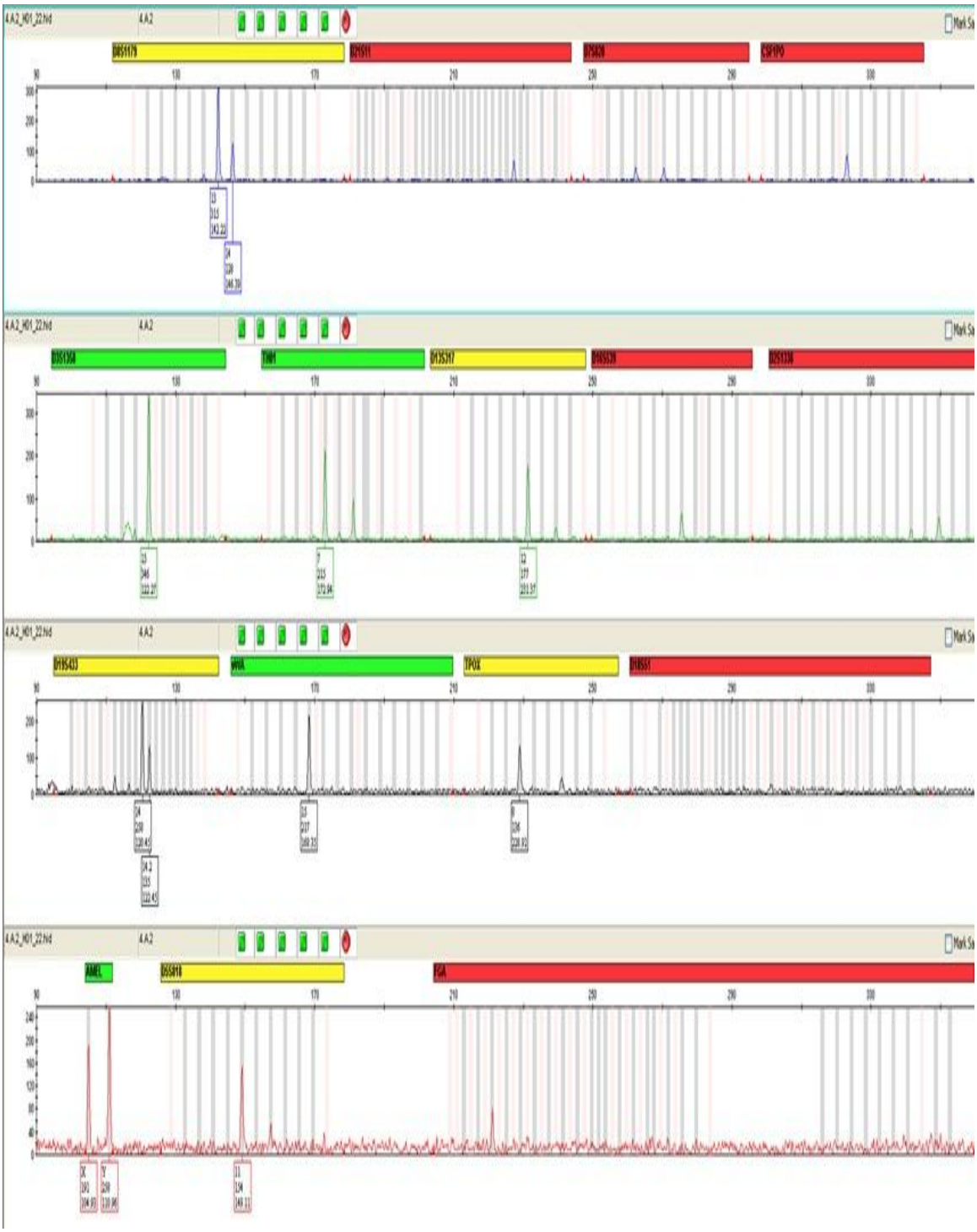
Sample 1 – Third triplicate of sample 1 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 1



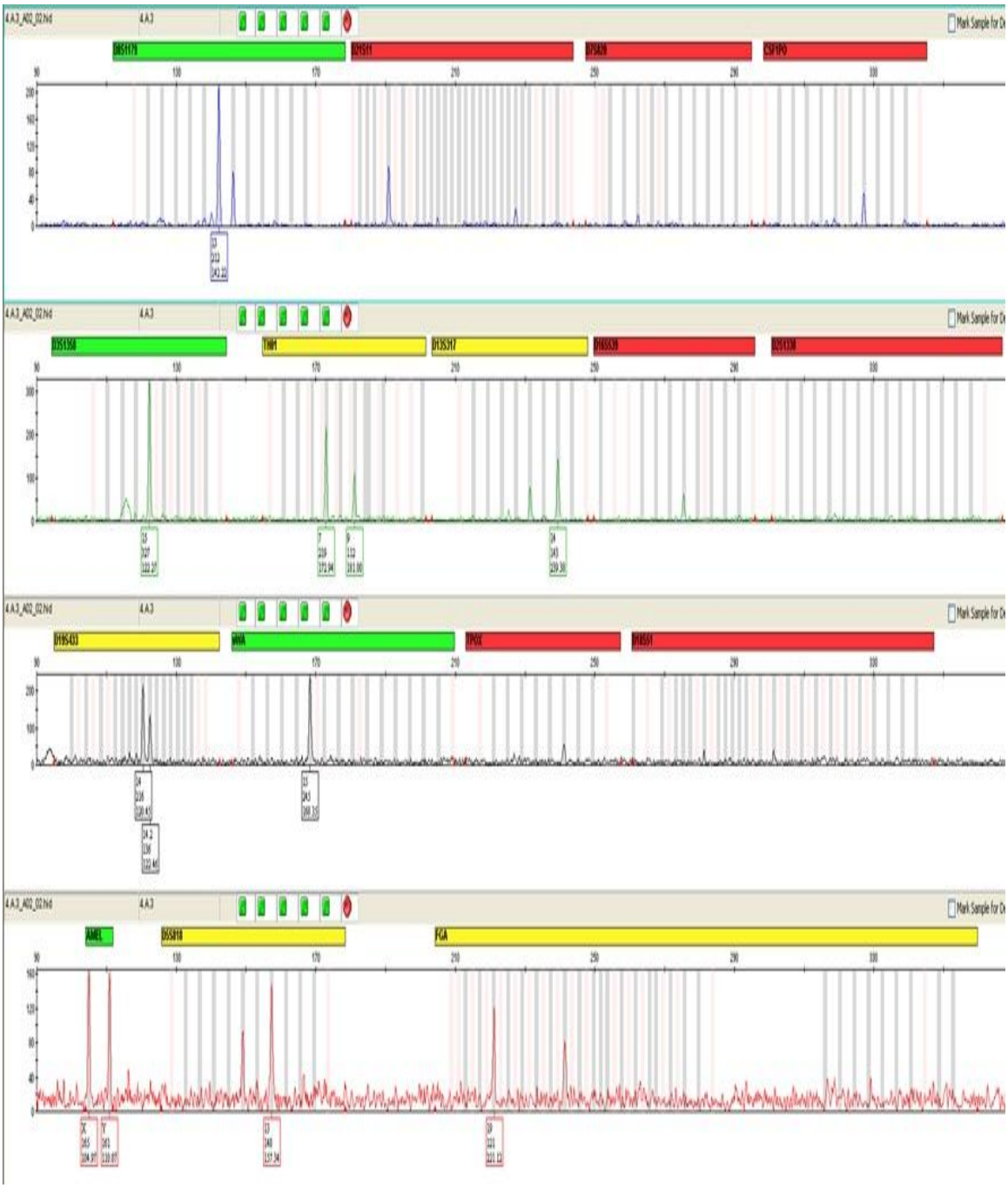
Sample 4 – First triplicate of sample 4 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 4



Sample 4 – Second triplicate of sample 4 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 4



Sample 4 – Third triplicate of sample 4 incubated for 2 hours in the PrepFiler Express BTA™ Lysis solution used to determine the composite profile for sample 4



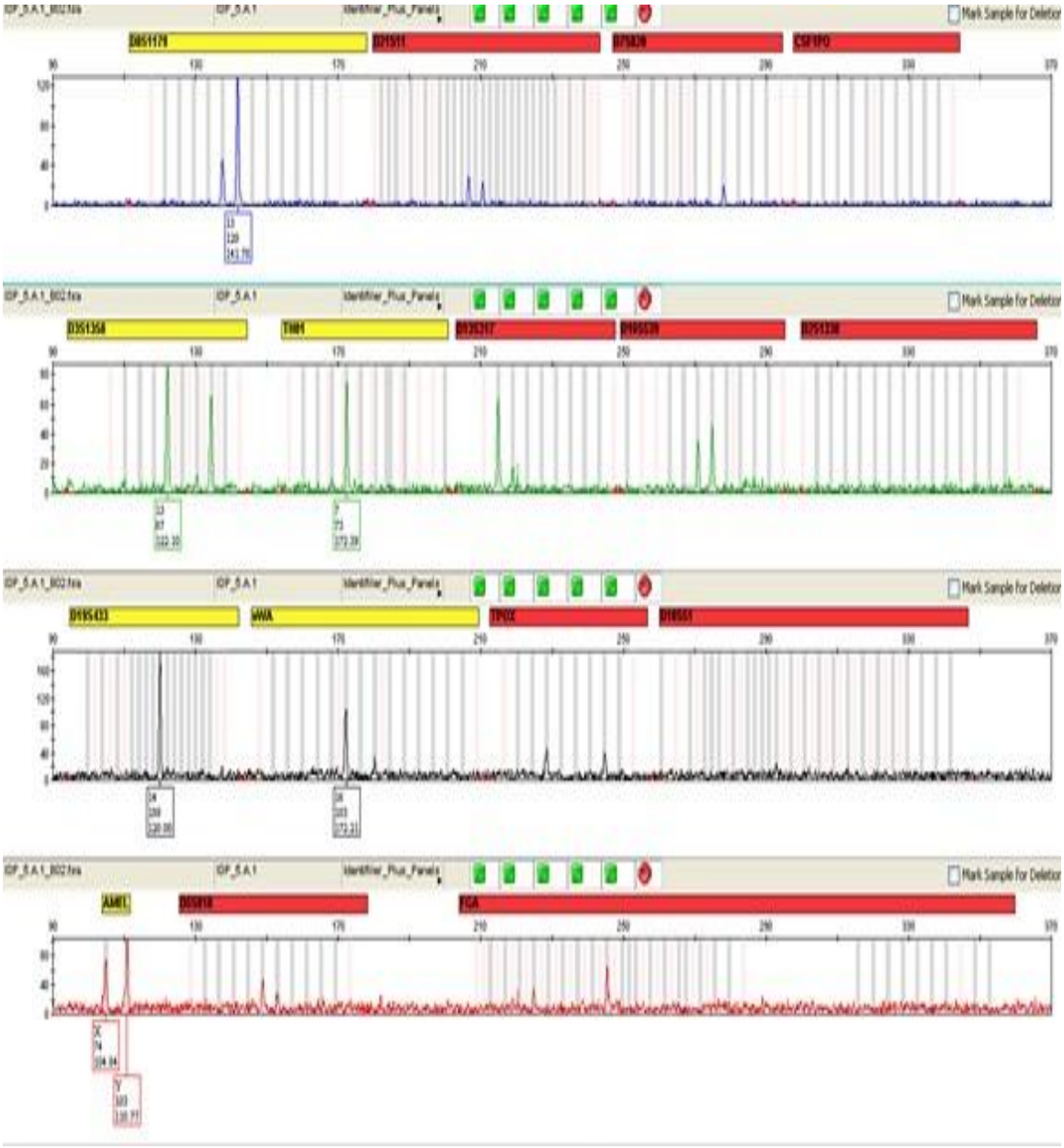
A table showing the reportable alleles for each replicate of sample 1. If an allele is reportable in 2 out of 3 samples it is considered a true allele and is part of the composite profile according to the UNTCHI Missing Persons Laboratory.

Locus	Replicate 1	Replicate 2	Replicate 3	Composite
D8S1179	10,13	10,13	10,13	10,13
D21S11		29,33.2	29,33.2	29,33.2
D7S820			10,12	
CSF1PO		11,11	11,11	11,11
D3S1358	17,17	17,17	17,17	17,17
TH01	9.3,9.3	9.3,9.3	9.3,9.3	9.3,9.3
D13S317	12,13	12,13	12,13	12,13
D16S359	13,13	13,13	13,13	13,13
D2S1338			24	
D19S433	14,14.2	14,14.2	14,14.2	14,14.2
vWA	16,17	16,17	16,17	16,17
TPOX	8,11	8,11	8,11	8,11
D18S51		19		
Amelogenin	X,Y	X,Y	X,Y	X,Y
D5S818	10,11	10,11	10,11	10,11
FGA	21,24		21,24	21,24

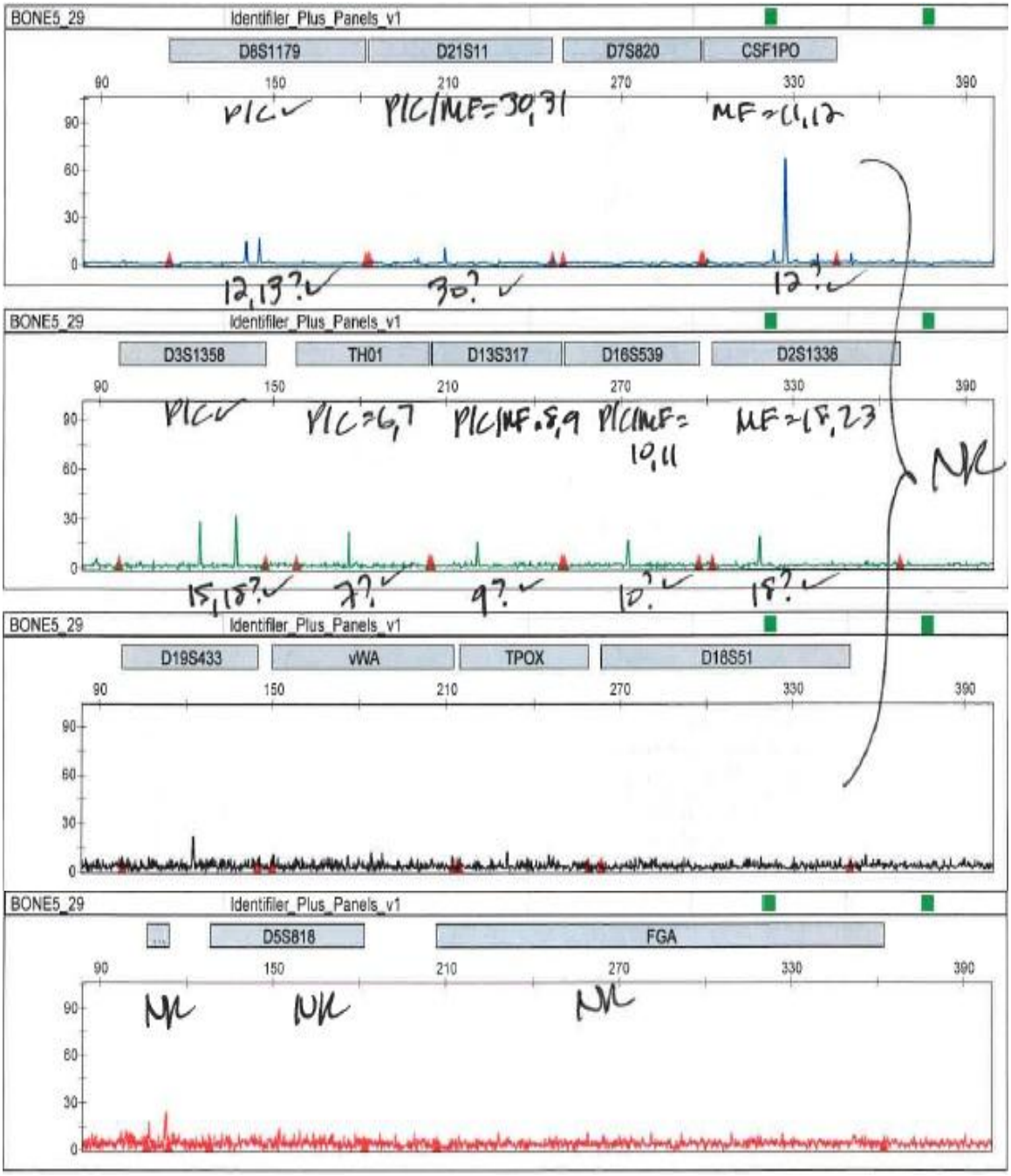
A table showing the reportable alleles for each replicate of sample 1. If an allele is reportable in 2 out of 3 samples it is considered a true allele and is part of the composite profile according to the UNTCHI Missing Persons Laboratory

Locus	Replicate 1	Replicate 2	Replicate 3	Composite
D8S1179			13	
D21S11				
D7S820				
CSF1PO				
D3S1358	15,15	15,15	15,15	15,15
TH01		7	7,9	7
D13S317				
D16S359				
D2S1338				
D19S433		14,14.2	14,14.2	14,14.2
vWA		15,15	15,15	15,15
TPOX				
D18S51				
Amelogenin	X,Y	X,Y	X,Y	X,Y
D5S818	11,13			
FGA				

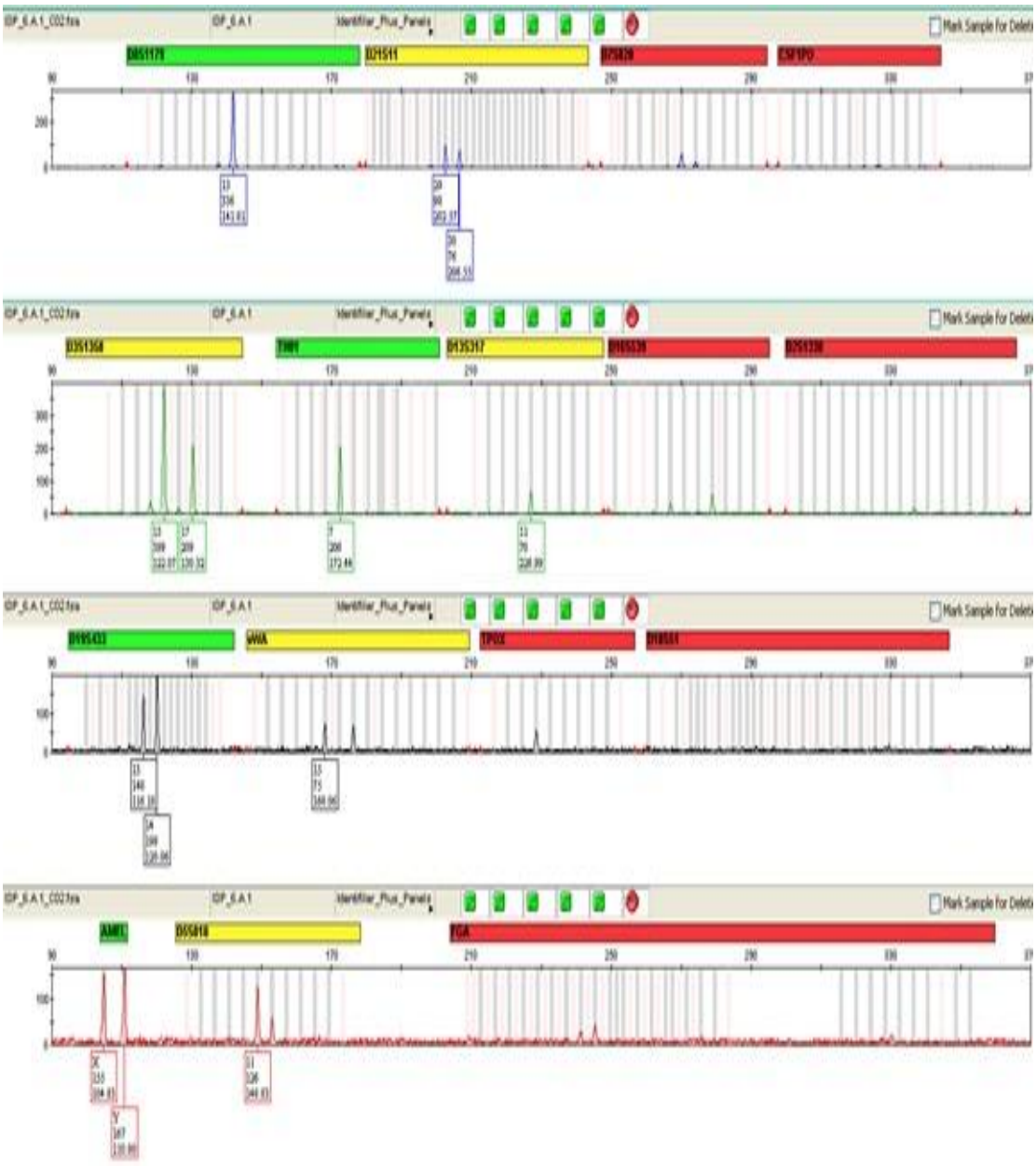
Sample 5 –Sample 5 extracted using the AutoMate Express and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles.



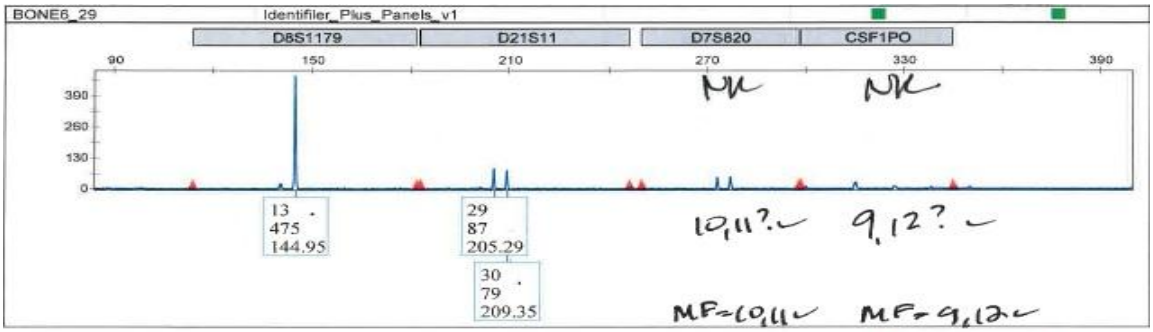
Sample 5 – Sample 5 extracted using the standard organic extraction procedure and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 29 cycles.

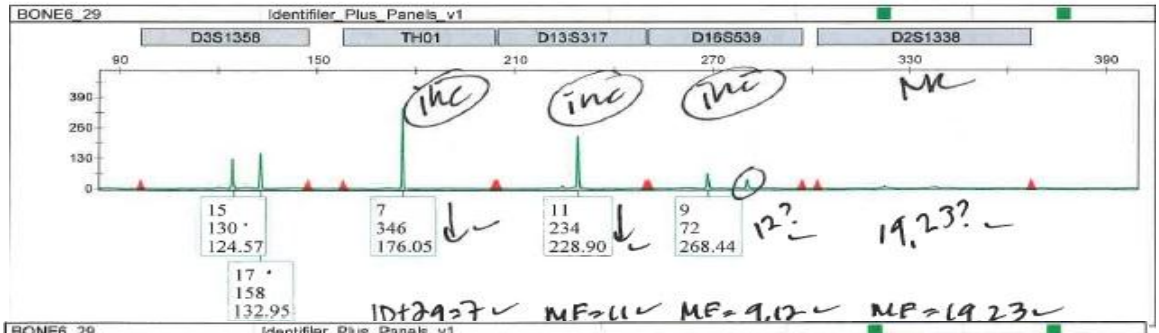


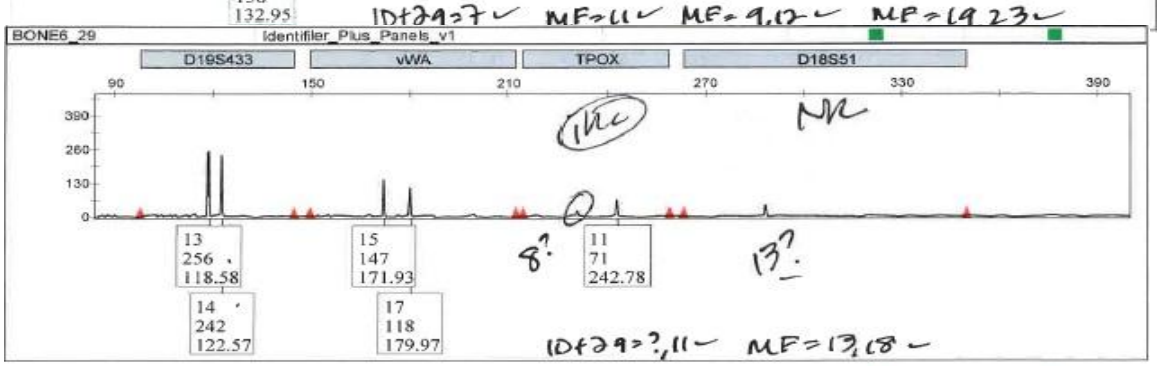
Sample 6 – Sample 6 extracted using the AutoMate Express and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles.

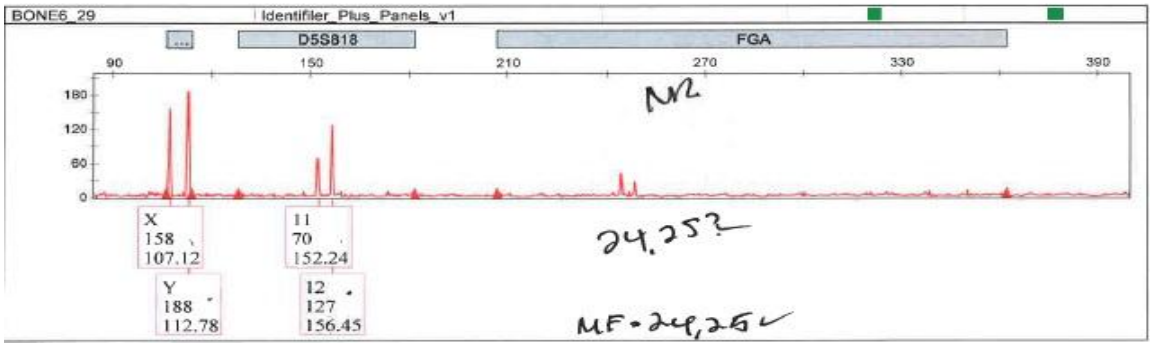


Sample 6 – Sample 6 extracted using the standard organic extraction procedure and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 29 cycles.

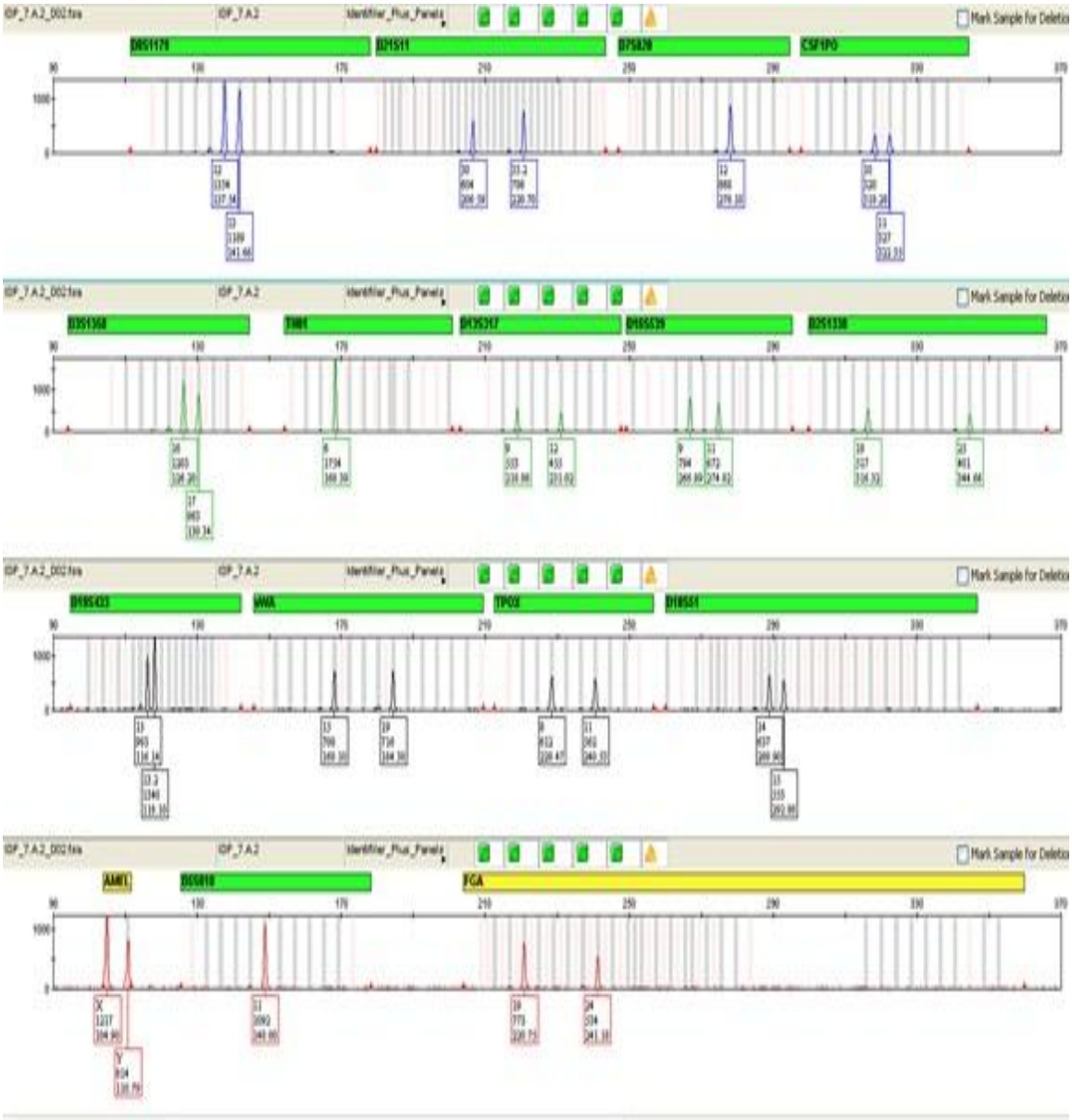




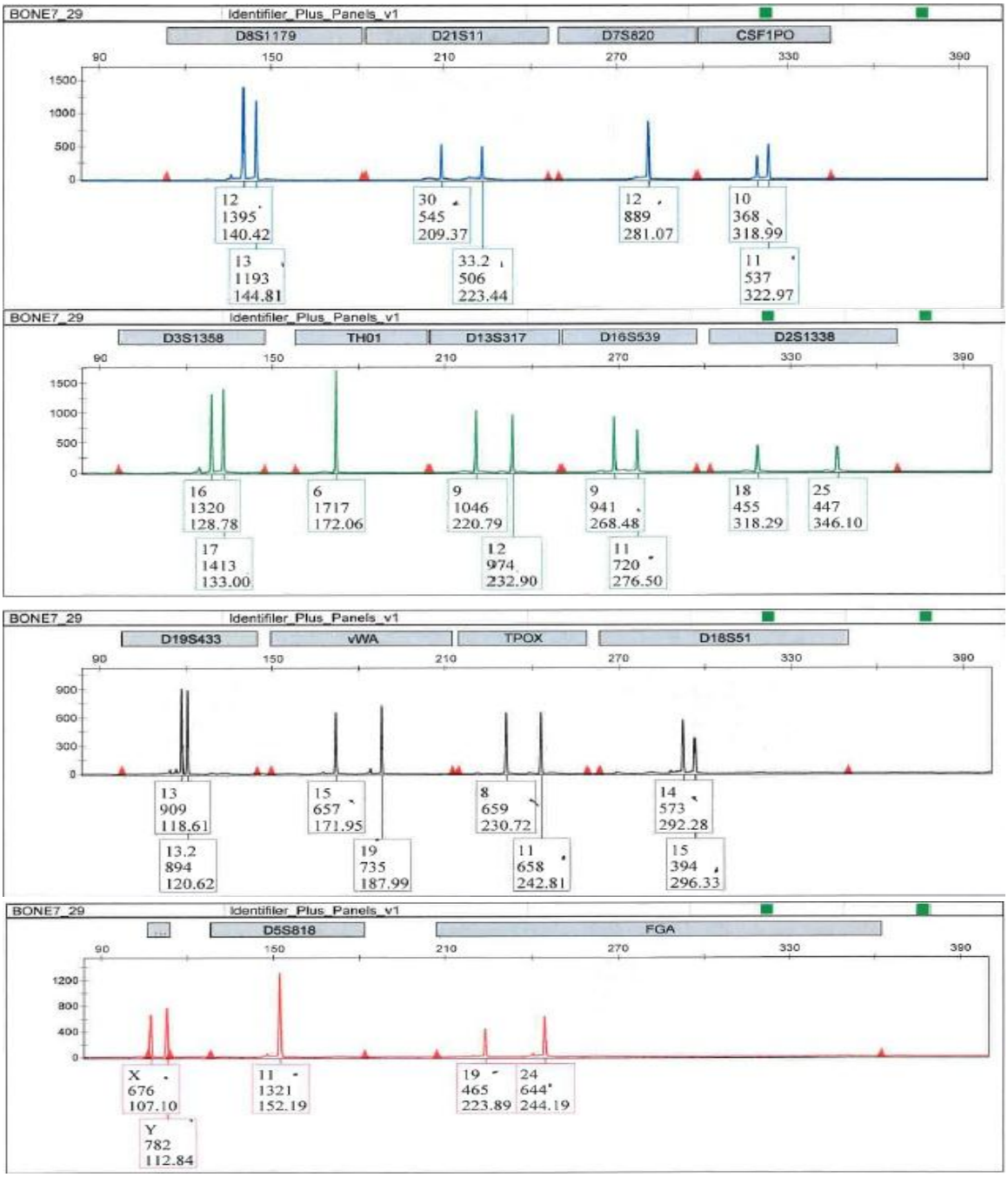




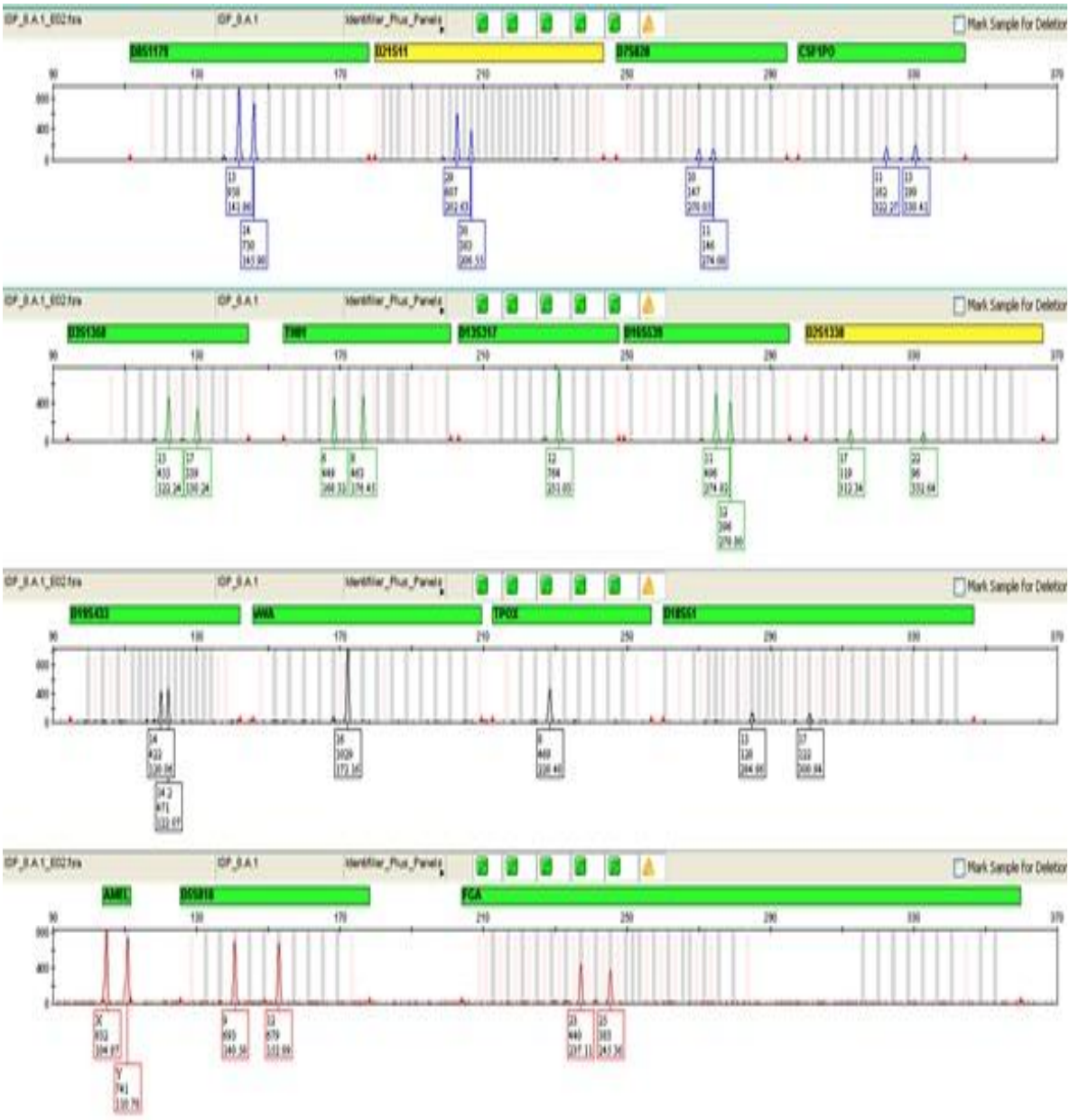
Sample 7 – Sample 7 extracted using the AutoMate Express and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles.



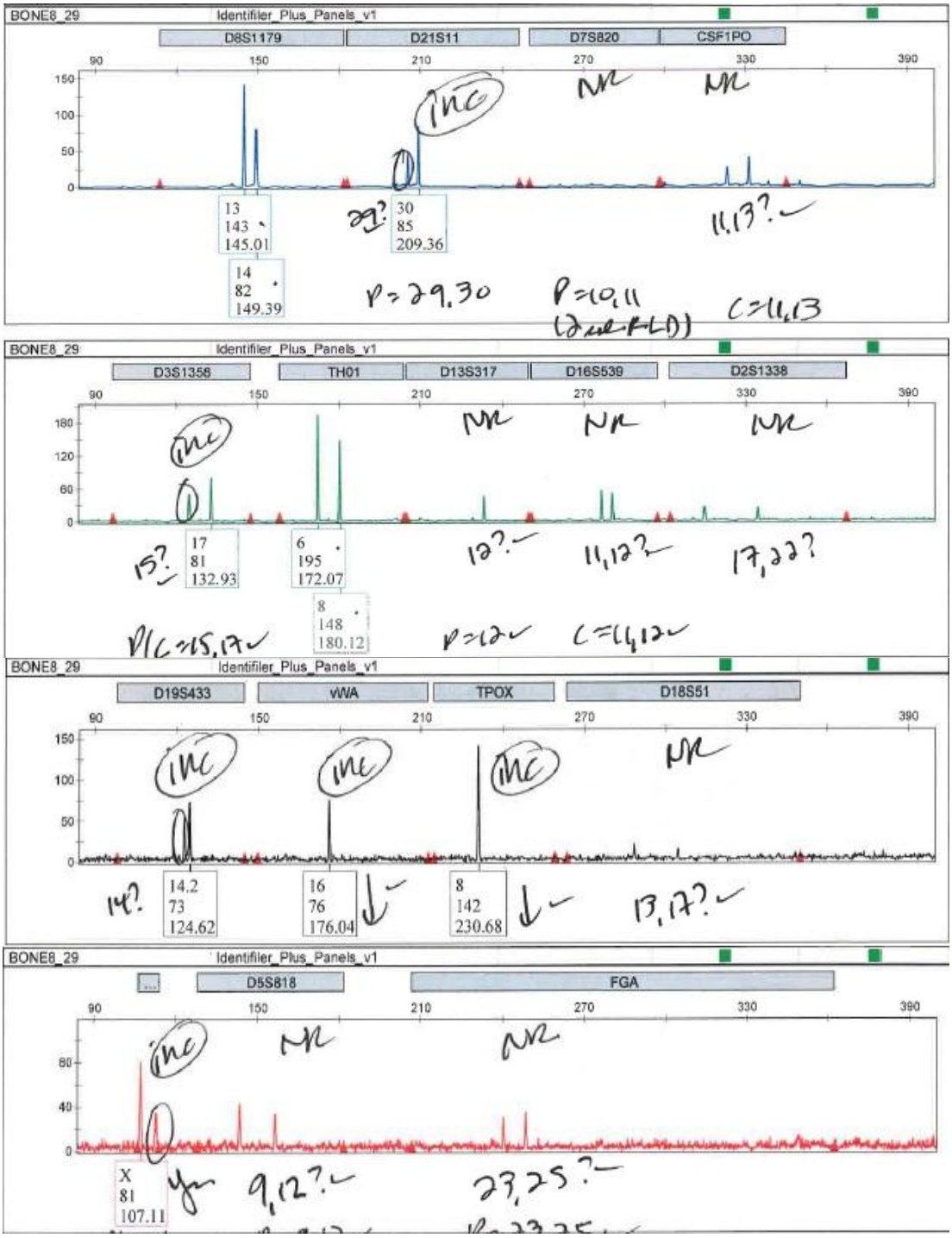
Sample 7 – Sample 7 extracted using the standard organic extraction procedure and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 29 cycles



Sample 8 – Sample 8 extracted using the AutoMate Express™ and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 28 cycles.



Sample 8 – Sample 8 extracted using the standard organic extraction procedure and amplified with the AmpFLSTR® Identifiler® Plus PCR Amplification Kit at 29 cycles.



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