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Effects of Amicon Ultra-4 Centrifugal Devices on DNA Yield From Bone Samples and Effects of Amplicon Rx Post-PCR Treatment onRelative Florescence Units Obtained During Capillary Electrophoresis

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The ability to recover DNA and perform DNA analysis from skeletal remains is a valuable tool for identification of missing persons and unidentified remains. DNA extraction from bone often yields low levels of DNA, which can inhibit analysis and impact genetic profiles. Efficient recovery of DNA from bones is therefore vital.

In this study, three molecular weight cut-off columns were evaluated. The Amicon® 10,000 NMWL filter device proved to be most efficient at retaining amplifiable DNA and obtaining optimal genetic analysis results. Additionally, the ability of Amplicon Rx™, a post-PCR treatment, to improve genetic profiles by providing a boost in RFUs obtained during capillary electrophoresis was evaluated.

EFFECTS OF AMICON® ULTRA-4 CENTRIFUGAL DEVICES ON DNA YIELD FROM BONE SAMPLES AND EFFECTS OF AMPLICON Rx™ POST-PCR TREATMENT ON RELATIVE FLORESCENCE UNITS OBTAINED DURING CAPILLARY ELECTROPHORESIS

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CHAPTER 1

INTRODUCTION

The use of DNA analysis is a cornerstone in the field of forensics. It has been utilized extensively to help solve traditional criminal cases such as sexual assaults and homicides. In addition, it is often the sole means of identification in missing person cases where skeletal remains may be all that are recovered. In the United States, there are approximately 85,000 – 100,000 active missing persons cases on any given day (1). In a typical year, medical examiners' and coroners' offices report handling approximately 4,400 unidentified decedents, 1,000 of which remain unidentified at the end of the year (2). The ability to recover DNA and perform DNA analysis from skeletal remains is a valuable tool for identification of missing persons and unidentified remains (3). DNA analysis provides the strongest evidence of identification in cases such as these, which rely on human remains.

Although approximately 99.7% of human DNA is identical between individuals, there exist regions that are highly variable (4). These regions contain short, repeated DNA sequences called short tandem repeats (STRs). The highly variable nature of STRs allows forensic scientists to target them for analysis and use them for genetic identification of individuals. The repeating units that make up STRs are 2-7 base pairs in length and the size of STRs containing these repeats range from approximately 100 to 400 base pairs. They work well in the forensic setting because STRs are easily amplified using the polymerase chain reaction (PCR); however, the

larger size STRs can be more difficult to amplify from degraded samples. Longer DNA fragments are more susceptible to damage than shorter fragments, which interfere with PCR amplification and subsequent analysis.

DNA typing methods have improved since their first use, but DNA extraction remains a challenge for some samples, including bone. DNA typing generally requires DNA extraction, quantification, amplification, electrophoresis, and data analysis. The extraction step in any DNA typing protocol is critical and must be capable of providing a sample that can be successfully profiled through amplification. The goal of extraction is to (i) lyse the cells, allowing the release of DNA, (ii) separate the DNA from other cellular components, and (iii) purify the DNA for further analysis (4). Successful extraction procedures maximize DNA recovery and eliminate compounds that can inhibit downstream analysis. The physical and chemical composition of bone that serves to protect DNA from degradation also serves as a physical barrier to extraction reagents, making extraction from bone more challenging that other forensic samples (5).

Current protocols for DNA extraction from bone involve pulverization of the bone into a fine powder and then a demineralization step prior to extraction. The incubation of bone powder in an ethylenediaminetetraacetic acid (EDTA) buffer serves two purposes: (i) the EDTA helps demineralize the bone, allowing extraction reagents access to the DNA, and (ii) it sequesters magnesium and calcium cations, inactivating enzymes that could degrade DNA. Releasing DNA from the cells via lysis is the first step in the process and is accomplished with the addition of sodium dodecylsulfate (SDS) and Proteinase K. After the DNA is released from the cells, it must be removed from other cellular components like proteins and lipids. The addition of organic reagents, such as phenol chloroform isoamyl alcohol (PCIA), is used to separate the DNA. When centrifuged, the degraded and denatured proteins are separated into an organic layer away from

the aqueous layer containing the DNA. The aqueous layer, which contains DNA, is then removed and purified further.

Another commonly used method for extracting and purifying DNA from bone involves the use of silica. This method utilizes the negative charge of DNA molecules, which, in the presence of a high concentration of chaotrophic salts, will bind to silica particles. The high concentration of chaotrophic salts disrupts the structure of the DNA molecules, which allows positively charged ions to form a salt bridge between the negatively charged DNA molecules and silica. This results in binding of the DNA molecules to the silica particles. The salts, impurities, and unwanted cellular components are then washed from the bound DNA in several steps. In the final step, the DNA is eluted in TE buffer or water (4). In this project, a QIAquick® (QIAGEN Inc., Valencia, CA) silica-based spin column is used for further purification.

The current methods employed for obtaining DNA from degraded or inhibited samples such as bone do not always provide full STR profiles regardless of the extraction method. Degradation of DNA is a primary concern, but the presence of endogenous and environmental inhibitors that co-extract with DNA can also pose a significant challenge and must be addressed in the extraction process (6). The use of a filtration device can help address this problem by retaining high quality, amplifiable DNA. The smaller, highly degraded and sheared DNA fragments, along with other small molecular weight inhibitors, will pass through the filter.

Amicon® Ultra-4 Centrifugal Filter Devices (EMD Millipore Corporation, Billerica, MA) are used in the extraction process to remove potential PCR inhibitors and concentrate DNA. The filtration devices are characterized by their nominal molecular weight limit (NMWL), which reflects their ability to retain molecules above the specified molecular weight limit; however, retention also depends on the size and shape of the molecule and those with weights close to the

NMWL may only be partially retained. For this reason, the manufacturer recommends choosing a device with a NMWL at least two times smaller than the molecular weight of the solute you are concentrating. The average weight of a DNA base pair is 650 Daltons; the molecular weight of a double-stranded DNA molecule can be calculated by multiplying the number of base pairs by 650. The recommendation for nucleic acids is 30,000 (30K) NMWL. The 30K NMWL device protocol is optimized to recover double-stranded DNA fragments ranging in size from 137 to 1,159 base pairs. These DNA molecules would have molecular weights ranging from 89,050 to 753,350, but per the recommendation of using a filter twice as small, these molecular weights would suggest using the 30K filter rather than the 50K. The first aim of this study was to compare DNA recovery using three different Amicon® Ultra-4 Centrifugal Filter Devices in two ways: (i) by evaluating the amount of amplifiable DNA recovered, and (ii) by evaluating total recovered DNA.

In addition to concentrating and filtering the DNA, the filtration devices can aid in the removal of inhibitors. Removal of inhibitors during the extraction process is critical because these compounds can interfere with PCR amplification, which in turn, can inhibit profiling results. Another aim of this study was to examine possible inhibition associated with the three different Amicon® filter devices.

Research being conducted within the University of North Texas Center for Human Identification on DNA extraction from bone samples using the Amicon® 50K NMWL filter device shows a loss of DNA template fragments smaller than 1,000 base pairs. The microfluidic separation of DNA in the extract using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) shows the presence of small DNA template fragments prior to filtration, but an absence of these fragments after the use of the Amicon® 50K filtration device. For forensic

purposes and STR analysis, the loss of these template fragments may be significant. Targeted STR regions for forensic DNA analysis range from 100 to 400 base pairs. The small DNA template fragments being lost during filtration could potentially contain these targeted STR regions, thus the loss of these small DNA template fragments may be negatively affecting DNA typing results. Another specific aim was to evaluate the loss of small DNA fragments using the three different Amicon® filter devices by evaluating DNA fragment sizes using the Agilent 2100 Bioanalyzer.

The smallest filtration device, the 10K NMWL device, may be most efficient at retaining small DNA fragments, but will also retain other small molecular weight materials. These other materials may serve to inhibit the polymerase chain reaction (PCR) and lower the quality of results obtained. Manufacturer recommendations for purification and concentration of nucleic acids indicate that the 30K NMWL device is optimal for recovering nucleic acids 137 to 1,159 base pairs in length. The fourth aim of this study was to determine if there was a correlation between the Amicon® filter device used and the quality of the DNA profile obtained. This correlation was examined by evaluating capillary electrophoresis peak height relative florescent units (RFUs) along with the number of reportable alleles in the resulting DNA profiles. It was expected that optimal genetic analysis results would be obtained using the Amicon® Ultra-4 30,000 NMWL centrifugal device due to decreased presence of small molecular weight contaminants and inhibitors in comparison to the 10,000 NMWL device. The 30,000 NMWL device was also expected to outperform the 50,000 NMWL device by retaining an increased quantity of amplifiable DNA fragments.

After DNA extraction and amplification, forensic scientists use capillary electrophoresis to size, separate, and detect STR products. Capillary electrophoresis separates ions based on

charge as voltage is applied and then separates based on size as the ions move through the polymer-filled capillary. Samples move into the capillary via an electrokinetic injection that lasts only a few seconds, as negatively charged particles are drawn to and injected into the capillary. The amount of sample drawn into the capillary is limited by injection time, and only 4-8% of the reaction mixture is sampled; the remaining 92-96% of amplicons are not injected or analyzed. STR amplicons compete for injection with unincorporated dNTPs and primers, salts, and other negatively charged components in the reaction mixture (7). Post-PCR clean-up methods aim to remove these negatively charged products from the reaction mixture, which allows the injection of more amplicons, thus increasing the RFU signal intensity and improving the STR profile obtained (8).

Amplicon Rx™ Post-PCR treatment (IFI Independent Forensics, Lombard, IL) is an easy three-step process that promises to improve weak results and profiles. The PCR reaction is mixed with a low salt binding buffer and added to the column. The buffer facilitates binding of PCR amplicons while allowing primers, dNTPs, and salts to wash away. The purified amplicons are eluted with formamide and analyzed with an appropriate size standard.

Amplicon Rx™ Post-PCR treatment is marketed as a product that increases peak heights and the number of alleles obtained for a profile. Developmental validation of Amplicon Rx™ showed an average peak height RFU boost of 4.7 fold with low template samples. Although the company did not include DNA extracted from bone in the validation study, bone often contains relatively low amounts of DNA. The final specific aim of this study was to evaluate the ability of Amplicon Rx™ post-PCR treatment to boost RFU signal and improve poor STR results; this was evaluated by (i) examining the number of reportable alleles in untreated versus treated samples, and (ii) comparing peak height RFUs obtained during capillary electrophoresis. It was expected

that Amplicon Rx™ post-PCR treatment would increase peak height RFUs obtained from DNA extracted from bone samples, which could result in an increase in the number of alleles that exceed the detection threshold and thus improve the STR profiles obtained.

CHAPTER II

MATERIALS AND METHODS

Sample Selection

Three cadaver bones were obtained from the UNTHSC Willed Body Program and used to obtain samples: a left humerus, a right humerus, and a left tibia. Three replicates were collected from each bone. Samples from the left humerus were labeled "LH," samples from the right humerus were labeled "RH," and samples from the left tibia were labeled "LT." Samples were run in triplicate; each replicate was numbered 1, 2, or 3. Each replicate was used with three different Amicon® filters and were labeled as 10K (10,000 NMWL), 30K (30,000 NMWL), or 50K (50,000 NMWL). For example, the third replicate collected from the left tibia used to test the 10K Amicon® filter was labeled LT.3.10K.

In addition, three casework bone samples were obtained and used to evaluate the Amicon® filters as well as the Amplicon Rx™ post-PCR treatment: sample 0073-14, sample 0079-14, and sample 0080-14. These casework bone samples were previously processed by the UNTCHI missing persons laboratory, and were selected for this study based on the quality of the genetic profiles obtained. Sample 73 generated a full profile with 15 reportable loci. Samples 79 and 80 both generated partial profiles, with nine and seven reportable loci respectively. Each casework bone sample was used to evaluate the three different Amicon® filters and labeled as

10K, 30K, or 50K. For example, the casework sample collected from sample 0079 and used to evaluate the 30K Amicon® filter was labeled 79.30K.

Sample Preparation

Bones were prepared following UNTHSC CHI's "Preparation of Skeletal Remains and Teeth for DNA Extraction" protocol. This procedure ensures that the exterior surfaces of the bones are decontaminated before pulverization. All Dremel® (Dremel, Mount Prospect, IL) and Stryker® (Stryker Corporation, Kalamazoo, MI) saw tools were cleaned and UV crosslinked for a minimum of 30 minutes to prevent contamination from exogenous DNA. To further prevent contamination, sanding and cutting of bone samples was carried out in a negative airflow sanding station. An area of bone measuring approximately 3" x 2" was sanded with a Dremel® tool and thin sections of bone were cut from this portion using a Stryker® saw. The samples were added to individual 50 mL tubes, soaked in 50% bleach for five minutes, rinsed with distilled water three to six times to ensure all bleach was removed, rinsed with 100% ethanol, and set aside to dry.

Bone samples were pulverized to maximize the surface area in contact with demineralization and extraction buffers. Prior to pulverization, all freezer mill equipment was cleaned and UV crosslinked for a minimum of 30 minutes to prevent contamination. Each sample was added to an individual cylinder with an impactor and sealed with end caps. The freezer mill reservoir was filled with liquid nitrogen and allowed to chill for seven minutes before the cylinder containing the sample was inserted. Samples were pulverized for seven minutes, after which they were visually inspected to ensure sufficient pulverization. After complete pulverization, samples were removed and left to thaw to room temperature for

approximately two hours. Cadaver bone samples were weighed in a dead air hood into 0.25 g aliquots and casework bone samples were weighed into 0.50 g aliquots. All samples were then stored at -20°C for DNA extraction.

Demineralization

Demineralization and extraction of the bone powder samples was performed following UNTHSC CHI's "Demineralization Extraction of Skeletal Remains" protocol. A demineralization buffer was prepared with 0.5 g of Sodium N-Lauroylsarcosinate and 50 mL 0.5M EDTA. The EDTA in this buffer both demineralizes the bone and inactivates DNAses that can degrade the extracted DNA by chelating divalent cations such as Mg++ and Ca++ (7). 0.25 g of bone powder from cadaver samples and 0.50g of bone powder from casework samples was added to tubes with 3.0 mL of demineralization buffer and 200 μL of Proteinase K. Samples were incubated on an orbital shaker at 56°C for 16-24 hours and then centrifuged.

DNA Extraction

An equal volume of PCIA (3 mL) was added to the demineralized samples. This process serves to denature and remove proteins as they partition with the organic phase, leaving the DNA in the aqueous phase. After the addition of PCIA, the samples were vortexed and centrifuged to separate the organic and aqueous layers. The aqueous phase was removed and analyzed using the Agilent High Sensitivity DNA Kit (Agilent Technologies) on the 2100 Bioanalyzer to evaluate DNA fragment size.

Amicon® Filtration

All samples were used to evaluate the Amicon® filter devices, 10K, 30K, and 50K NMWL. The aqueous phase from the organic extraction was added to the top of the appropriate Amicon® Ultra-4 Centrifugal Filter Device. The filter devices were centrifuged at 4,000 x g until approximately 50 μL of sample remained in the filter. The largest filters, 50K NMWL, were centrifuged for 12-13 minutes; 30K NMWL filters were centrifuged for 19-20 minutes; and the smallest filters, 10K NMWL, were centrifuged for 35-36 minutes. 2 mL of molecular biology grade water was added to each filter and samples were again centrifuged at 4,000 x g until approximately 50 μL of sample remained in the filter. An additional amount of water was added to each filter to bring the total volume to 100 μL. The filters were rinsed with the residual water and the DNA extracts were transferred to new tubes. Following Amicon® filtration, the samples were reanalyzed using the Agilent system to evaluate DNA fragment sizes. After the final purification step in the extraction protocol using a QIAquick® spin column, the samples were evaluated once more using the Agilent system. All samples were also quantified in duplicate using Quantifiler® Duo to determine the total amount of amplifiable DNA present.

QIAGEN® QIAquick® Purification

QIAquick® purification was performed following UNTHSC CHI's "Demineralization Extraction of Skeletal Remains" protocol. 1 mL of Buffer PB was added to each sample and vortexed. Sample was added to a QIAquick® spin column in increments of 650 μL and centrifuged at 16,000 x g for 30 seconds until all sample had been filtered through the spin column. Flow through was discarded and each column was washed with 750 μL of Buffer PE. Samples were again centrifuged at 16,000 x g for 60 seconds and flow through was discarded.

An additional centrifugation of 60 seconds at 16,000 x g was performed before transferring the spin column to a fresh tube and eluting the bound DNA with 100 μL of Buffer EB. Samples were then centrifuged for 60 seconds, spin columns were discarded, and samples were stored at 4°C.

DNA Quantification, Agilent

The total amount of DNA present was quantified using the Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer following the manufacturer's Agilent High Sensitivity DNA Assay Protocol. The Agilent High Sensitivity DNA Kit is designed for sizing and quantification of amplification products ranging in size from 50 to 7,000 base pairs. The Agilent was used to evaluate DNA fragment size at three points during the extraction process: (i) after the separation of the PCIA aqueous phase and before Amicon® filtration, (ii) after Amicon® filtration and before the QIAquick® purification step, and (iii) after QIAquick® purification of the extracts when DNA extraction is complete.

DNA Quantification, Quantifiler® Duo

Amplifiable DNA was quantified using the Quantifiler® Duo DNA Quantification Kit (Life Technologies, Carlsbad, CA) on a 7500 Real-Time PCR System (Life Technologies) following the UNTHSC CHI "Human and Male DNA Quantification using Applied Biosystems® Quantifiler® Duo Kit" protocol. The Quantifiler® Duo kit is designed to estimate the quantity of amplifiable human DNA. Quantification was performed in duplicate according to manufacturer's instructions.

STR Amplification

Prior to amplification, DNA samples were normalized to a concentration of 1 ng/μL based on quantification values obtained from Quantifiler® Duo. Samples with values less than 1 ng/μL were not normalized. DNA was amplified using the AmpFLSTR® Identifiler® Plus PCR Amplification Kit (Life Technologies) on a GeneAmp® 9700 PCR System (Life Technologies) following UNTHSC CHI "STR Amplification" protocol for 29 cycles. The Identifiler® Plus kit is a short tandem repeat (STR) assay that amplifies 15 tetranucleotide repeat loci plus amelogenin, the gender determining marker.

Capillary Electrophoresis and Data Analysis

The PCR products obtained during amplification were electrophoresed on a 3500 Genetic Analyzer (Life Technologies) using UNTHSC CHI's protocol. The data was analyzed using GeneMapper® *ID-X* (Life Technologies) software. The 3500 genetic analyzer has not been validated for casework, but has been validated within the department. Interpretation guidelines set by those validation studies were used to analyze electropherograms. The minimum interpretation threshold for heterozygous alleles was 100 relative florescence units (RFUs); any allele that did not meet this threshold was not included in analysis. For homozygous alleles, the minimum interpretation threshold was 200 RFUs.

Post Amplification Purification, Amplicon Rx™

Casework samples were used to evaluate the Amplicon Rx™ kit. After samples were amplified, the 25 μL reaction obtained from the amplification reaction for each sample was divided in two; 12.5 μL was used for capillary electrophoresis and 12.5 μL was used to evaluate

the Amplicon Rx™ Post-PCR treatment. 11 μL of each sample was removed from the amplification plate and added to a tube with 55 μL of binding buffer. Samples were vortexed briefly and loaded onto the Amplicon spin column. The columns were centrifuged at 12,000 x g for three minutes. Collection tubes and waste were discarded, and the spin columns were added to fresh collection tubes. A master mix was prepared with 240 μL of formamide and 1.2 μL of GeneScan™ 500 LIZ® Size Standard (Life Technologies). 15 μL of master mix was added to each sample in the spin column, and then incubated at room temperature for five minutes. After spin columns were centrifuged at 12,000 x g for two minutes they were ready for analysis. All flow through collected, approximately 11 μL for each sample, was plated. Samples were then analyzed via capillary electrophoresis on the 3500 Genetic Analyzer using UNTHSC CHI's protocol. All sample data were analyzed using GeneMapper® *ID-X* software following the interpretation guidelines set forth by validation studies performed within the department. The number of reportable alleles and peak height relative florescence units (RFUs) were analyzed to evaluate Amplicon Rx™ treated versus untreated samples.

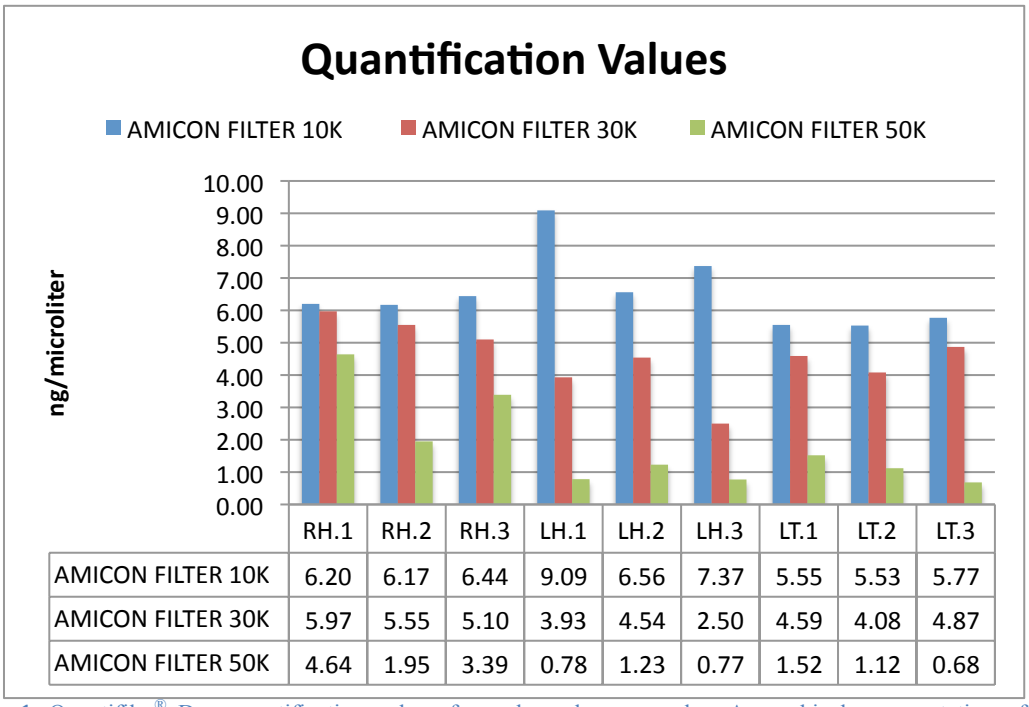
CHAPTER III

RESULTS

The overall goal of **Specific Aim 1** was to compare DNA recovery using three different Amicon® Ultra-4 Centrifugal Filter Devices in two ways: (i) by evaluating the amount of amplifiable DNA recovered using Quantifiler® Duo, and (ii) by evaluating total recovered DNA using the Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer.

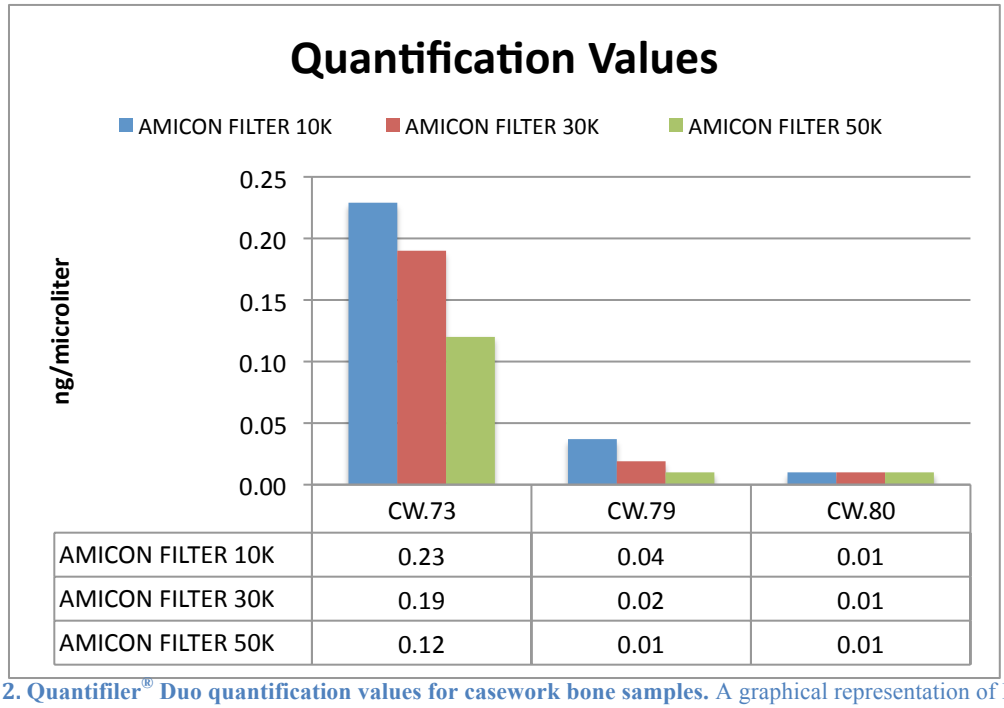
The results of DNA extraction using the three different sizes of Amicon® filter devices were assessed by comparing the amount of amplifiable DNA in each sample using Quantifiler® Duo. The quantification results for the cadaver samples are presented in Figure 1. In every sample, more amplifiable DNA was retained using the 10K filter, with quantification values ranging from 5.5 ng/μL to 9.1 ng/μL. The samples from the 30K filter quantified in the range of 2.5 ng/μL to 6.0 ng/μL. The quantification results for the 50K samples were consistently the lowest, ranging from 0.68 ng/μL to 4.6 ng/μL.

The quantification results for the casework bone samples are presented in Figure 2. Again the smallest filter, the 10K NMWL, retained the greatest quantity of DNA, with quantification values ranging from 0.23 ng/μL to 0.01 ng/μL. Samples from the 30K and 50K filters had quantification values similar to each other, ranging from 0.19 ng/μL to 0.01 ng/μL.



	RH.1	RH.2	RH.3	LH.1	LH.2	LH.3	LT.1	LT.2	LT.3
AMICON FILTER 10K	6.20	6.17	6.44	9.09	6.56	7.37	5.55	5.53	5.77
AMICON FILTER 30K	5.97	5.55	5.10	3.93	4.54	2.50	4.59	4.08	4.87
AMICON FILTER 50K	4.64	1.95	3.39	0.78	1.23	0.77	1.52	1.12	0.68

Figure 1. Quantifiler® Duo quantification values for cadaver bone samples. A graphical representation of DNA quantification values of the samples collected from the three cadaver bone samples and used with three different Amicon® filter devices: 10K, 30K, and 50K.



	CW.73	CW.79	CW.80
AMICON FILTER 10K	0.23	0.04	0.01
AMICON FILTER 30K	0.19	0.02	0.01
AMICON FILTER 50K	0.12	0.01	0.01

Figure 2. Quantifiler® Duo quantification values for casework bone samples. A graphical representation of DNA quantification values of the samples collected from the three casework bone samples and used with three different Amicon® filter devices: 10K, 30K, and 50K.

In addition to the Quantifiler® Duo data, the Agilent data were used to compare DNA recovery from the three Amicon® filter devices. Agilent electropherograms for all cadaver bone samples show that the samples from the 10K filters contain more DNA than the 50K filters. The results are presented in Figure 3, Figure 4, and Figure 5. Generally, the scale on the vertical axis that measures fluorescence decreases as the filter size increases, starting at 400-500 fluorescence units (FU) for the 10K samples, decreasing to 200-300 FU for the 30K samples, and decreasing again to 100 FU for the 50K samples. The highest peak in the 10K samples averaged approximately 500 RFUs, the highest peak in the 30K samples averaged approximately 300 RFUs, and the average was approximately 50-100 RFUs for the 10K samples. A fixed area under the curve (peak area) was measured and in all samples, the peak areas were highest for the 10K samples and lowest for the 50K samples. Peak areas from each run, (i) after Amicon® filtration and (ii) after QIAquick® purification, for all cadaver bone samples are presented in Figure 6. These data also illustrate the difference in DNA concentration of the extracts, which increased after the final purification step with the QIAquick® column.

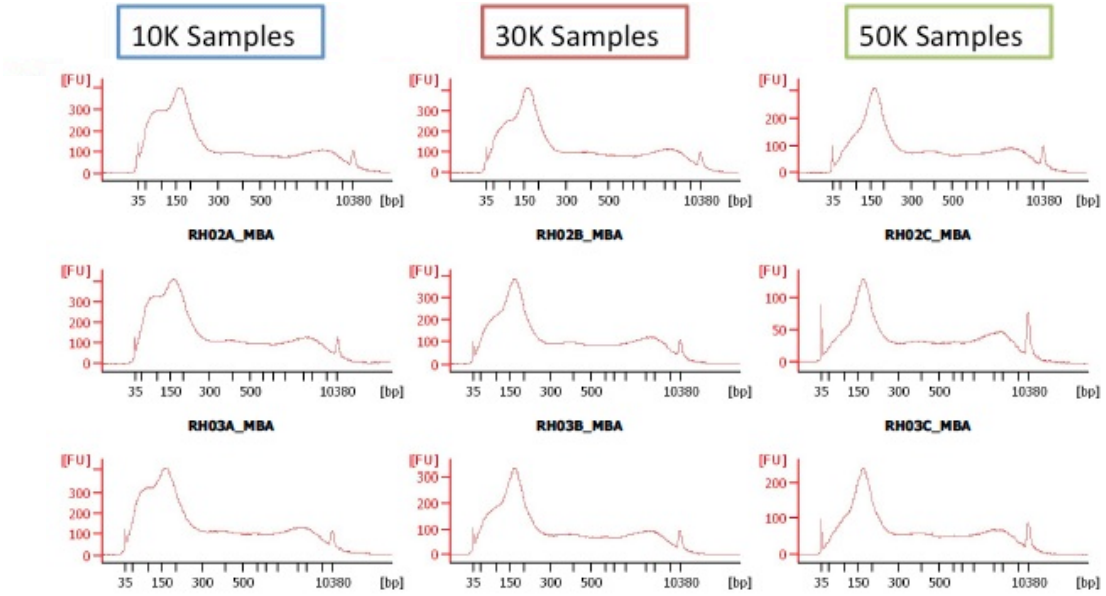


Figure 3. Agilent electropherograms for right humerus samples. Electropherograms for the right humerus replicates and each Amicon® filter device, showing DNA fragment base pair size on the x-axis versus florescence units on the y-axis.

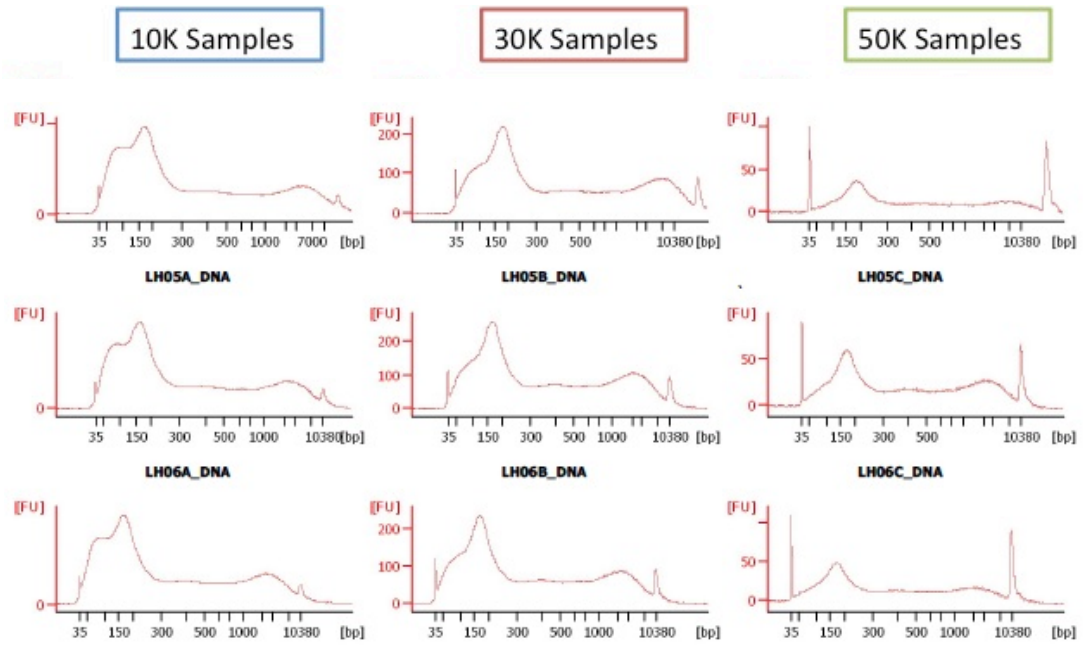


Figure 4. Agilent electropherograms for left humerus samples. Electropherograms for the left humerus replicates and each Amicon® filter device, showing DNA fragment base pair size on the x-axis versus florescence units on the y-axis

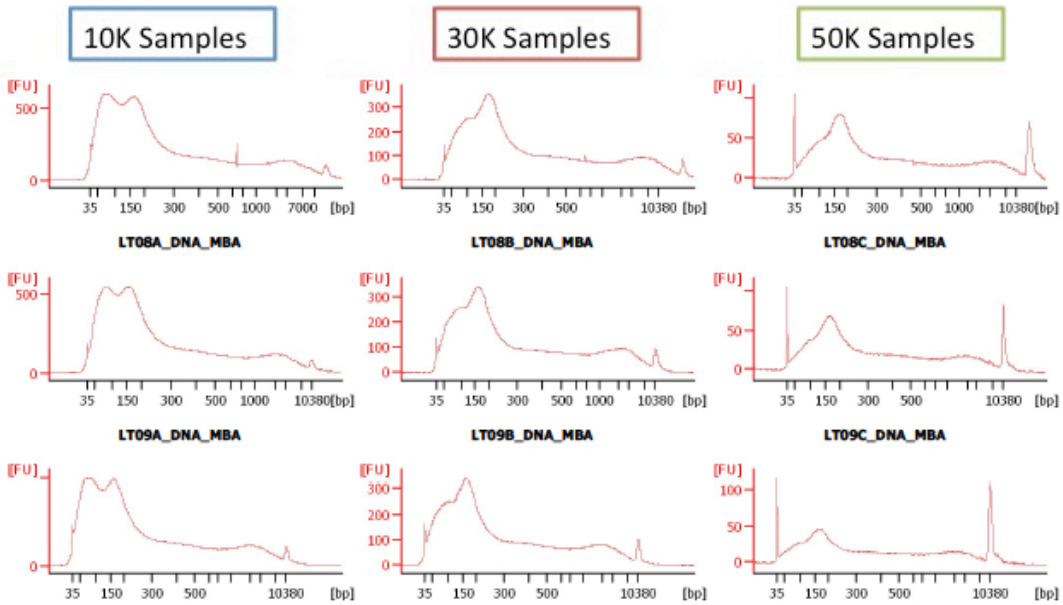


Figure 5. Agilent electropherograms for left tibia samples. Electropherograms for the left tibia replicates and each Amicon® filter device, showing DNA fragment base pair size on the x-axis versus florescence units on the y-axis.

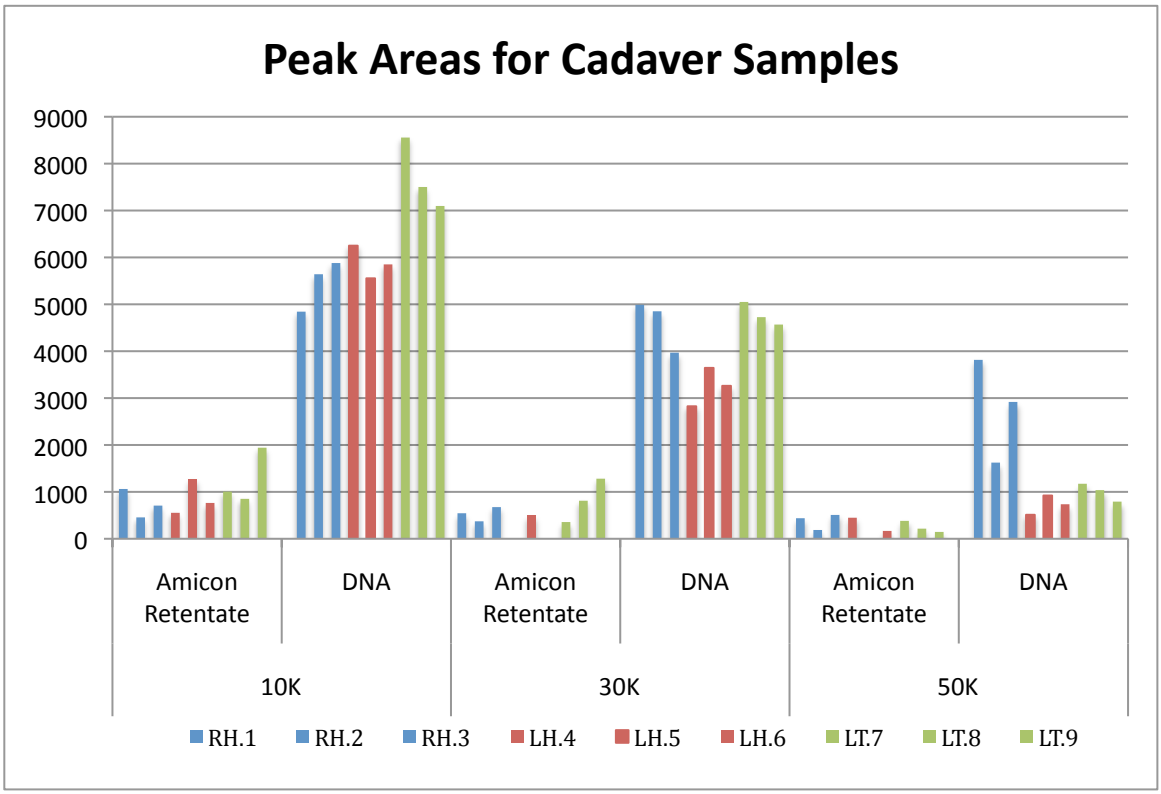


Figure 6. Agilent peak areas for all cadaver bone samples. A graphical representation of Agilent peak areas under the curve for all cadaver samples and replicates at two stages of extraction: after Amicon® filtration, labeled "Amicon Retentate," and after QIAquick® purification and concentration, labeled "DNA." All right humerus replicates are represented by blue peaks, left humerus by red peaks, and left tibia by green peaks.

Replicates from each bone were run together on an Agilent chip and the resulting gels are presented in Figure 7, Figure 8, and Figure 9. The Agilent gels from each run show the majority of DNA in the extracts concentrated at approximately 150-200 base pairs. The optical density of the bands on the gels corresponds to the concentration of the sample; a darker band indicates a higher concentration of DNA at that fragment size. The 10K samples produced a wider darker band, indicating the presence of more DNA in a wider range of small fragments. In contrast, the 50K samples produced a lighter band with fewer DNA fragments less than 150 base pairs in length.

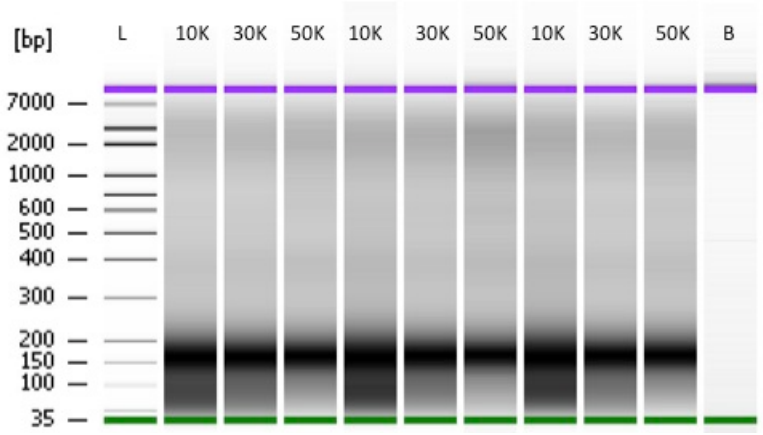


Figure 7. Agilent gel for right humerus samples after complete extraction. The ladder in the first well is used to size DNA fragments in the remaining wells. The three replicates from the right humerus, each tested with the three Amicon® filter devices, were run in the remaining wells. The optical density of the bands corresponds to the concentration of DNA in the sample. The darkest bands on the gel are approximately 150-200 base pairs in size, indicating these fragments are present in the highest concentration.

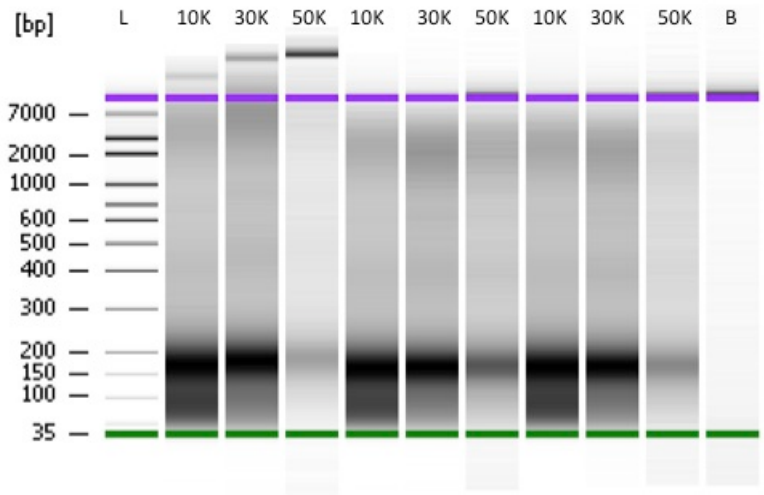


Figure 8. Agilent gel for left humerus samples after complete extraction. The ladder in the first well is used to size DNA fragments in the remaining wells. The three replicates from the left humerus, each tested with the three Amicon® filter devices, were run in the remaining wells. The optical density of the bands corresponds to the concentration of DNA in the sample. The darkest bands on the gel are approximately 150-200 base pairs in size, indicating that these fragments are present in the highest concentration.

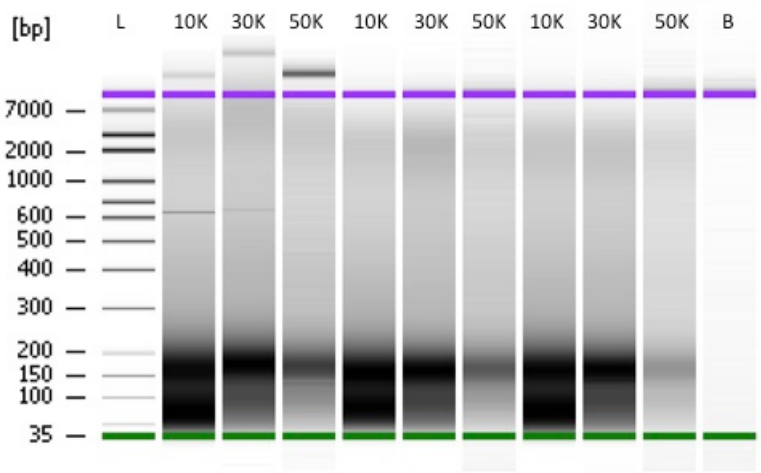


Figure 9. Agilent gel for left tibia samples after complete extraction. The ladder in the first well is used to size DNA fragments in the remaining wells. The three replicates from the left tibia, each tested with the three Amicon® filter devices, were run in the remaining wells. The optical density of the bands corresponds to the concentration of DNA in the sample. The darkest bands on the gel are approximately 150-200 base pairs in size, indicating these fragments are present in the highest concentration.

The goal of **Specific Aim 2** was to evaluate the loss of small DNA fragments using the three different Amicon® filter devices by evaluating DNA fragment sizes using the Agilent High Sensitivity DNA Kit on the 2100 Bioanalyzer.

It was proposed that the DNA would be evaluated on the 2100 Bioanalyzer at three different stages during the extraction process to determine at which stage DNA fragments, if any, were being lost: (i) before Amicon® filtration, (ii) after Amicon® filtration, and (iii) after the final step in the extraction protocol, QIAquick® purification. After processing the first group of samples from the right humerus, samples RH.1, RH.2, and RH.3, it was evident that the samples were too dirty before Amicon® filtration for the electrophoresis to run properly, preventing any fragments from being properly sized. This Agilent run was therefore eliminated from all further samples.

The second Agilent run, after the Amicon® filtration, was successful in two of the three bone sample groups. The samples from the left humerus were still inhibited and electrophoresis in many of the wells failed.

Overall, the Agilent results show DNA fragments present in all samples ranging in size from 50 to more than 7,000 base pairs. This range of DNA fragments is represented by the large continuous peak seen in the Agilent electropherograms for each sample (see Figure 3, Figure 4, and Figure 5). Each broad peak crests at approximately 150-200 base pairs, which is consistent with the darkest bands on the gels (Figure 7, Figure 8, and Figure 9). DNA fragments 150-200 base pairs in size are present in the greatest concentration as indicated by the bands on the gels and the peaks on the electropherograms. This was true for both the Amicon® retentate runs and the DNA extract runs for all samples.

The goal of **Specific Aim 3** was to examine possible inhibition associated with the three different Amicon® filter devices by evaluating cycle thresholds of the internal positive control during quantification with Quantifiler® Duo.

Quantifiler® Duo internal positive control (IPC) cycle threshold (Ct) was evaluated for indications as to the presence of inhibitors. These results for the cadaver bone samples are presented in Figure 10, Figure 11, and Figure 12. There was no significant difference in IPC cycle threshold among the samples from all cadaver bones when compared to the average IPC cycle thresholds of the standards. Therefore, the presence of inhibitors was not detected by Quantifiler® Duo.

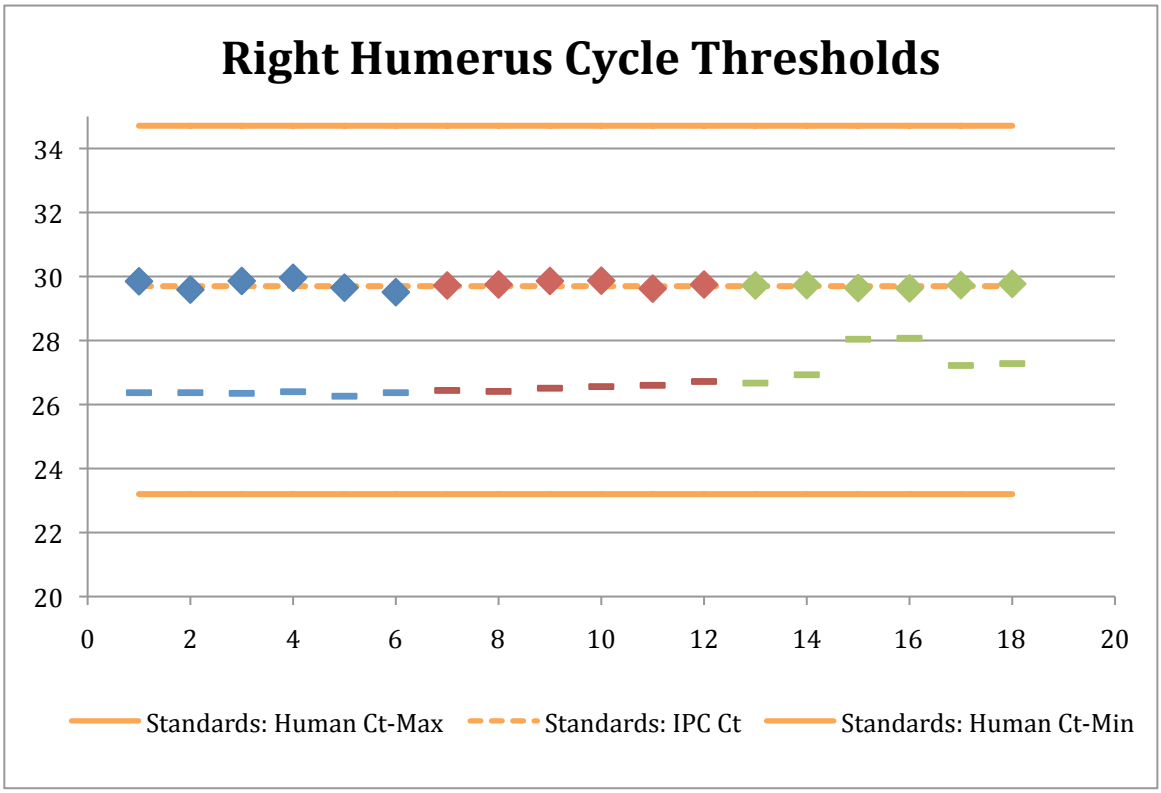


Figure 10. Quantifiler® Duo IPC cycle thresholds for all replicates of the right humerus sample using three Amicon® filter devices. A graphical representation of the Quantifiler® Duo cycle thresholds analyzed to evaluate possible inhibition. Samples were quantified in duplicate; therefore there are two values for each of the three replicates (six markers per filter size). The samples processed with the 10K filter are represented by the blue diamonds and dashes, the 30K by the red, and the 50K by the green. The colored dash marks that correspond to the different filters represent the human Ct values of the right humerus samples, while the dashed orange line represents the IPC Ct of the standards. The solid lines represent the minimum and maximum Ct values of the standards, which correspond to the minimum and maximum quantification values of the standards, 0.023 ng/µL and 50.0 ng/µL respectively.

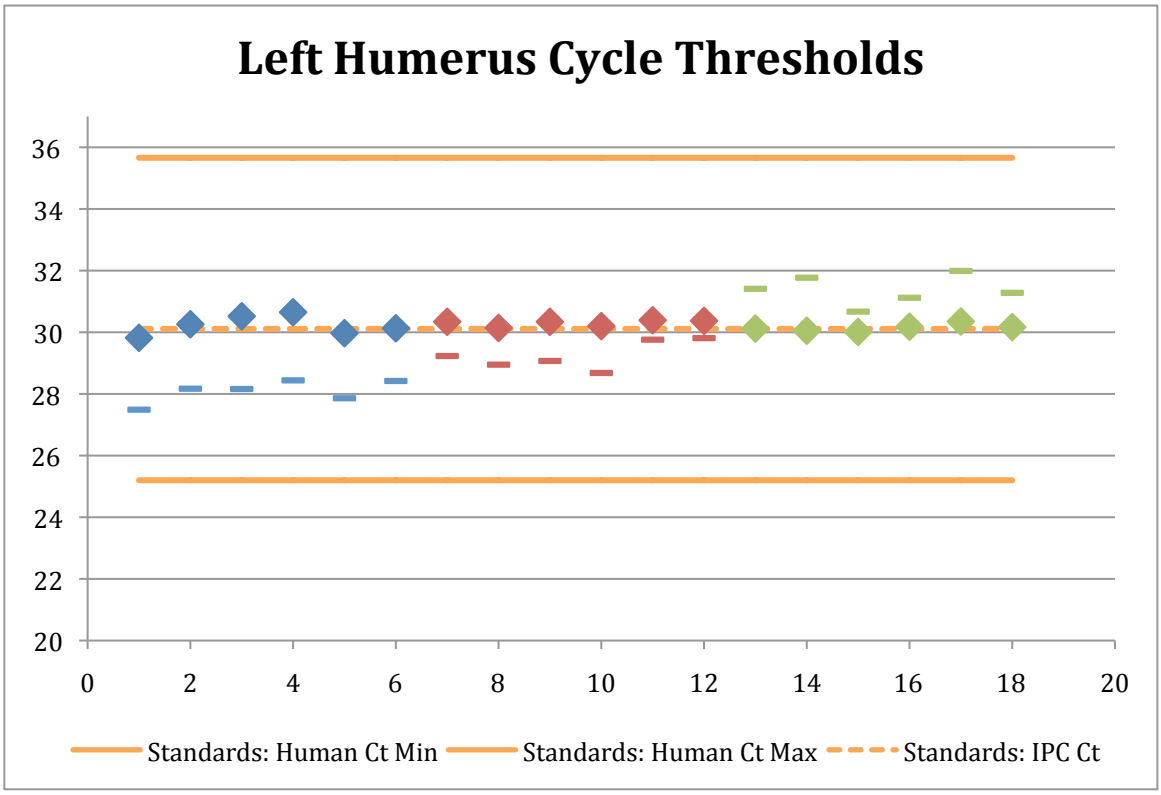


Figure 11. **Quantifiler® Duo IPC cycle thresholds for all replicates of the left humerus sample using three Amicon® filter devices.** A graphical representation of the Quantifiler® Duo cycle thresholds analyzed to evaluate possible inhibition. Samples were quantified in duplicate; therefore there are two values for each of the three replicates (six markers per filter size). The samples processed with the 10K filter are represented by the blue diamonds and dashes, the 30K by the red, and the 50K by the green. The colored dash marks that correspond to the different filters represent the human Ct values of the left humerus samples, while the dashed orange line represents the IPC Ct of the standards. The solid lines represent the minimum and maximum Ct values of the standards, which correspond to the minimum and maximum quantification values of the standards, 0.023 ng/µL and 50.0 ng/µL respectively

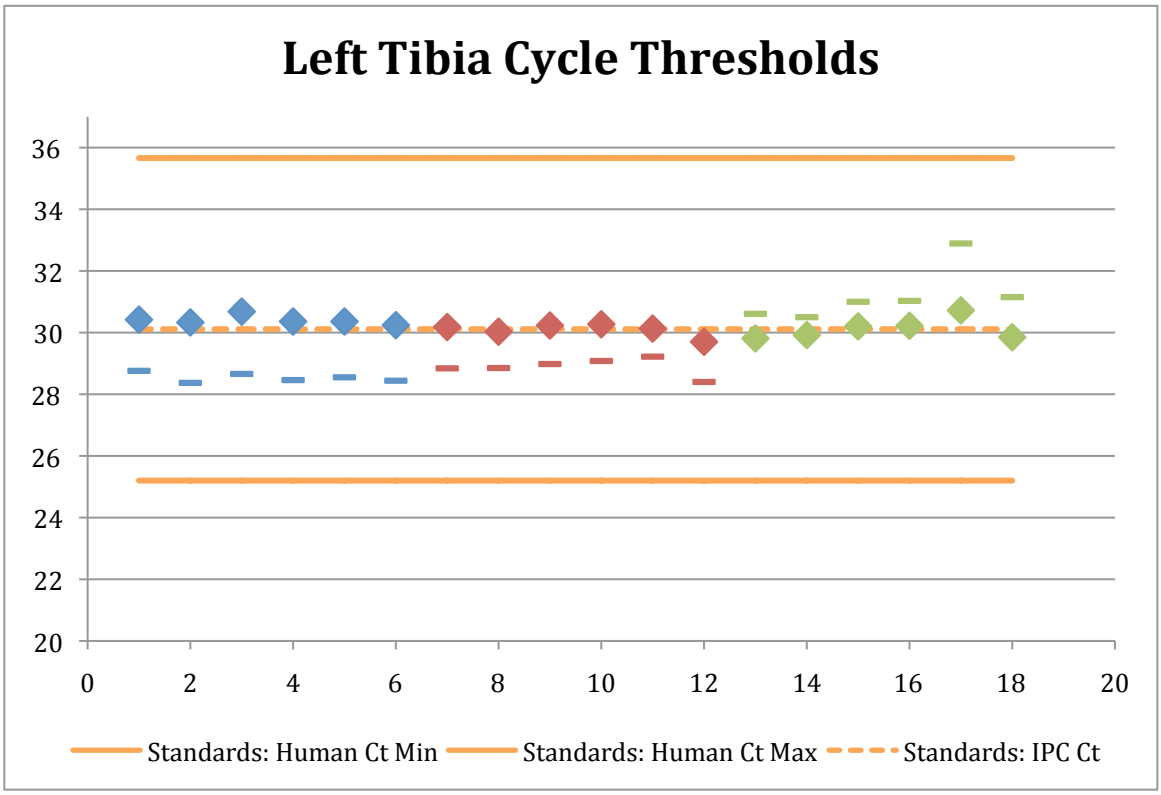


Figure 12. Quantifiler® Duo IPC cycle thresholds for all replicates of the left tibia sample using three Amicon® filter devices. A graphical representation of the Quantifiler® Duo cycle thresholds analyzed to evaluate possible inhibition. Samples were quantified in duplicate; therefore there are two values for each of the three replicates (six markers per filter size). The samples processed with the 10K filter are represented by the blue diamonds and dashes, the 30K by the red, and the 50K by the green. The colored dash marks that correspond to the different filters represent the human Ct values of the left tibia samples, while the dashed orange line represents the IPC Ct of the standards. The solid lines represent the minimum and maximum Ct values of the standards, which correspond to the minimum and maximum quantification values of the standards, 0.023 ng/µL and 50.0 ng/µL respectively.

The goal of **Specific Aim 4** was to determine if there was a correlation between the Amicon® filter device used and the quality of the DNA profile obtained. This correlation was examined by evaluating capillary electrophoresis peak height RFUs along with the number of reportable alleles in the resulting DNA profiles.

For almost all cadaver samples, peak heights were highest for the 10K samples, ranging from 3,493 RFUs to 1,958 RFUs. Overall, peak height averages for the 30K filters fell in the middle, and peak height averages for the 50K samples were the lowest, ranging from 2,545

RFUs to 1,074 RFUs. Peak height averages for all replicates of the cadaver bone samples for each Amicon® filter device are presented in Figure 13.

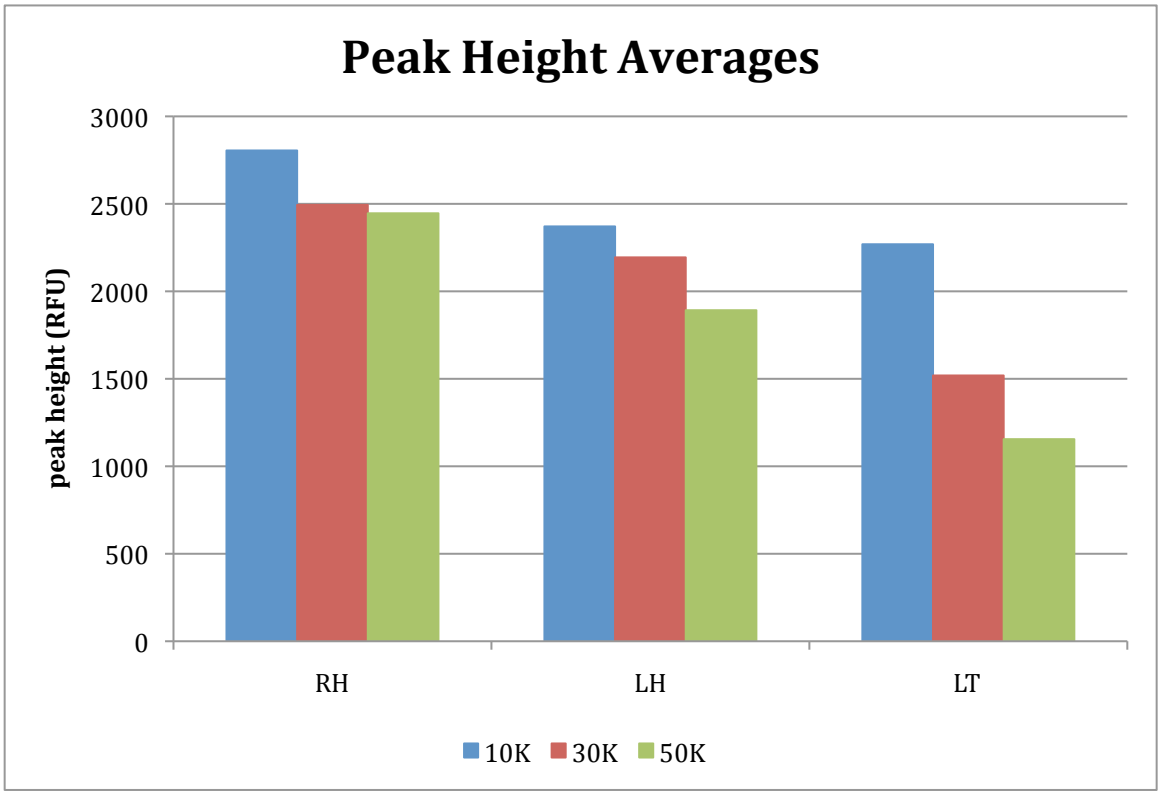


Figure 13. Peak height averages for cadaver samples. Graphical representation of combined peak height averages from capillary electrophoresis for all replicates per Amicon® filter device for each cadaver bone sample.

Peak height averages for casework samples are presented in Figure 14; however, these samples had such low quantification values that it was not possible to normalize the amount of input DNA to 1 ng/µL. The quantification values for the replicates of sample 79, 79.10K, 79.30K, and 79.50K, were 0.04 ng/µL, 0.02 ng/µL and 0.01 ng/µL respectively. The quantification values for the replicates of sample 80 were all 0.01 ng/µL. In each case, 10 µL of sample was added to the polymerase chain reaction for a total DNA input ranging from 0.4 ng/µL to 0.1 ng/µL rather than the standard 1 ng/µL. The resulting difference in peak heights

could be attributed to the difference in quantity of input DNA rather than Amicon® filter. Sample 73 had higher quantification values than the other two casework bones and therefore had higher peak height averages.

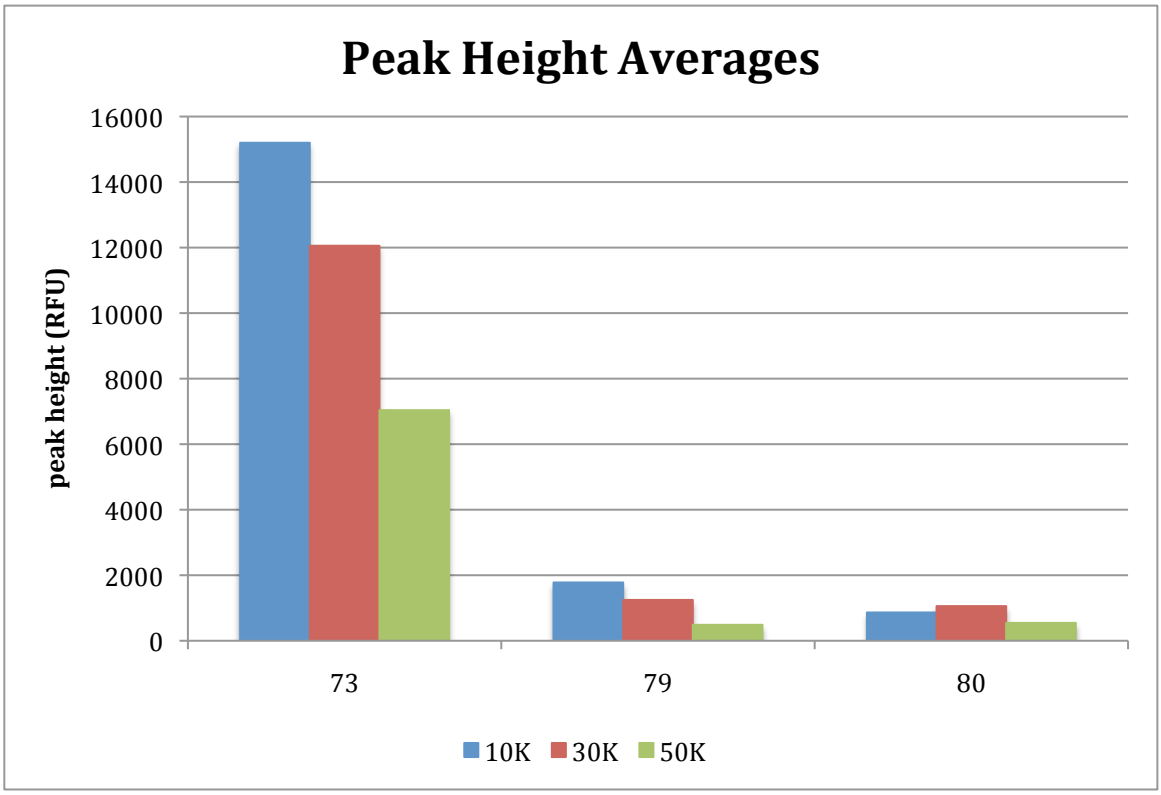


Figure 14. Peak height averages for casework samples. Graphical representation of combined peak height averages from capillary electrophoresis for all replicates per Amicon® filter device for each casework bone sample.

For all samples, both cadaver and casework, allele peak heights were also compared by plotting each allele peak height for the samples from each of the three Amicon® filter devices against the peak heights for the 30K samples and adding a trend line for each set of points. Peak heights for right humerus samples are presented in Figure 15. Samples from the 10K filter generated higher peak heights than the 30K samples, while 50K samples generated lower peak heights on average.

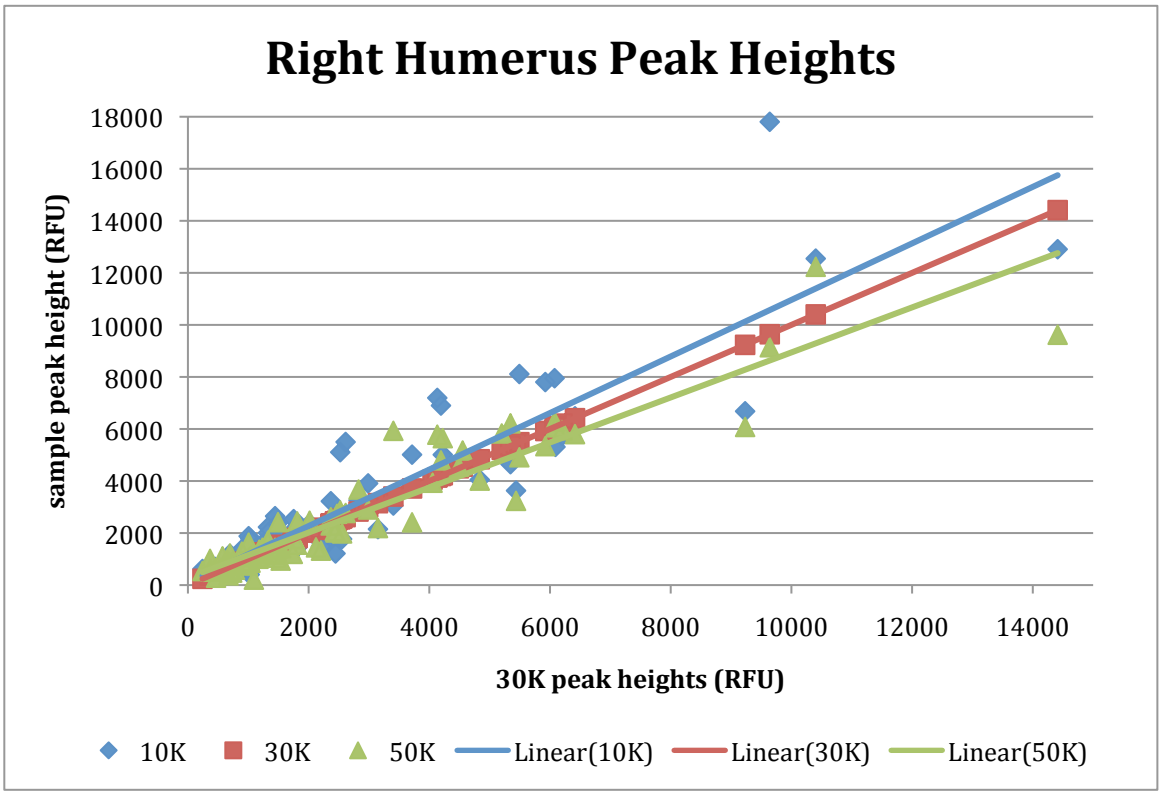


Figure 15. Allele peak heights for all replicates of right humerus samples. Graphical representation of allele peak heights obtained during capillary electrophoresis for right humerus samples. Peak heights from each filter were plotted against peak heights for the 30K filter in order to compare overall peak height trends. When looking at the average trend line of peak heights, the 10K samples generated higher peak heights than the 30K samples, while the 50K samples generated lower peak heights than the 30K samples

Peak heights for left humerus samples are presented in Figure 16 and peak heights for left tibia samples are presented in Figure 17. Trend lines for both cadaver bone samples show the highest peak heights for the 10K samples. The samples from the 30K filter fall in the middle, and the samples from the 50K consistently generate peak heights lower than both the 10K and 30K filters.

Peak heights for casework samples are presented in Figure 18, Figure 19, and Figure 20. Trend lines for samples 73 and 79 follow the same pattern as the other samples; the samples from the 10K filters have the highest peak heights and the samples from the 50K filters have the lowest.

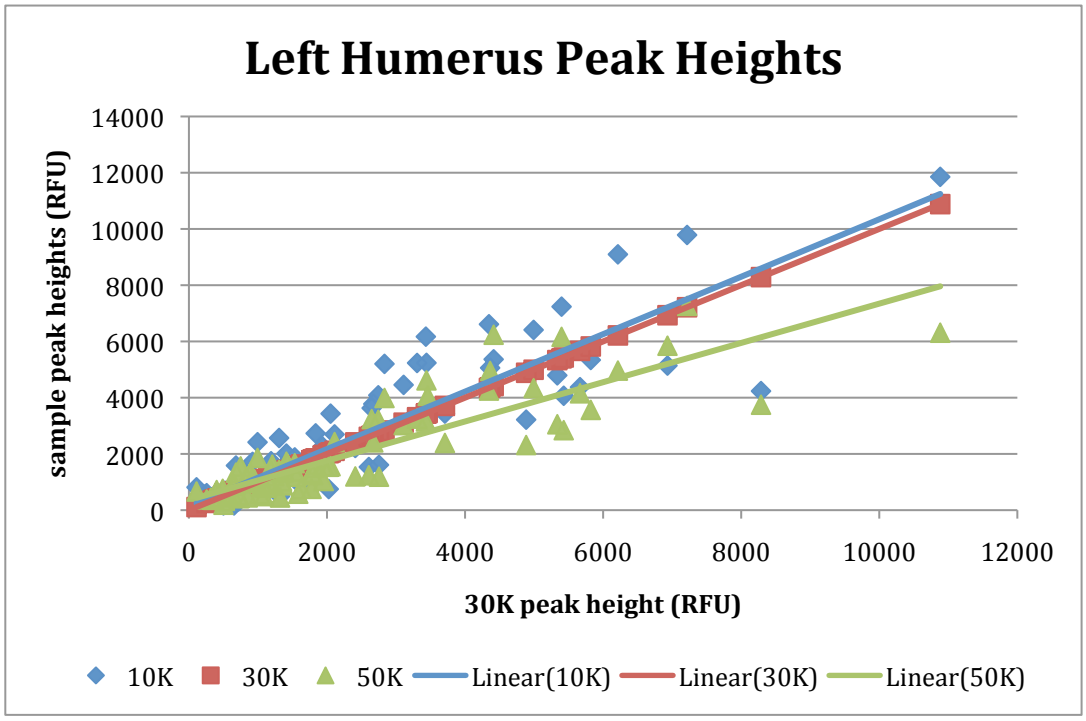


Figure 16 Allele peak heights for left humerus samples. Graphical representation of allele peak heights obtained during capillary electrophoresis for left humerus samples. When looking at the average trend line of peak heights, the 10K samples generated slightly higher peak heights than the 30K samples, while the 50K samples generated much lower peak heights that the 30K samples.

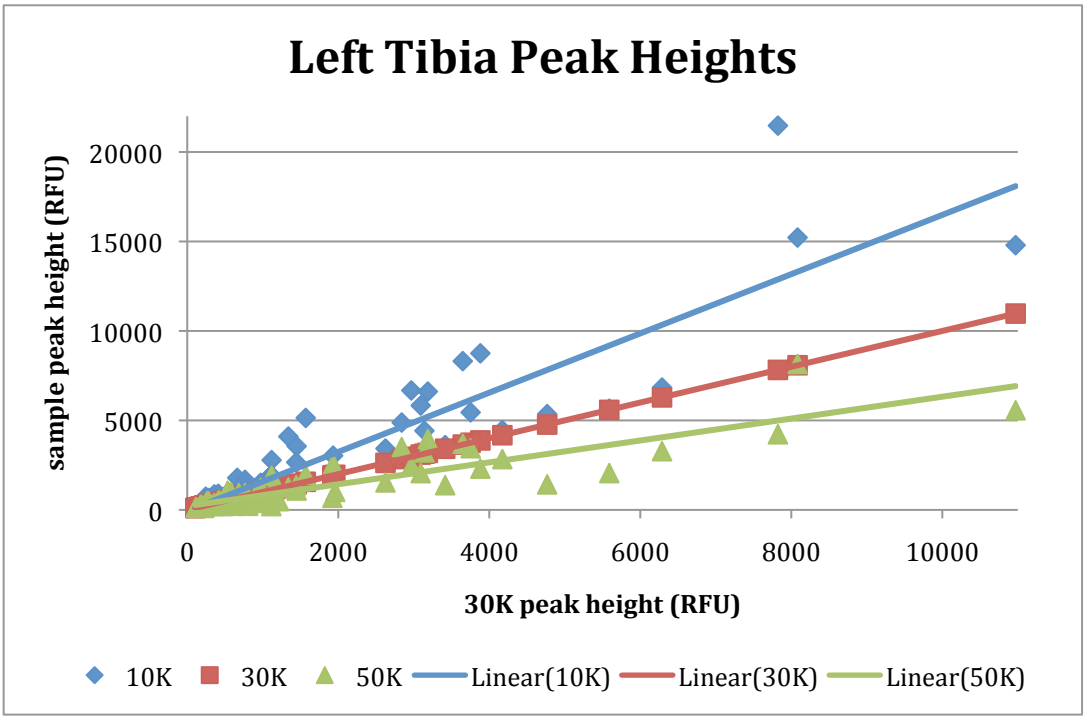


Figure 17. Allele peak heights for left tibia samples. Graphical representation of allele peak heights obtained during capillary electrophoresis for left tibia samples. When looking at the average trend line of peak heights, the 10K samples generated higher peak heights than the 30K samples, while the 50K samples generated lower peak heights than the 30K samples.

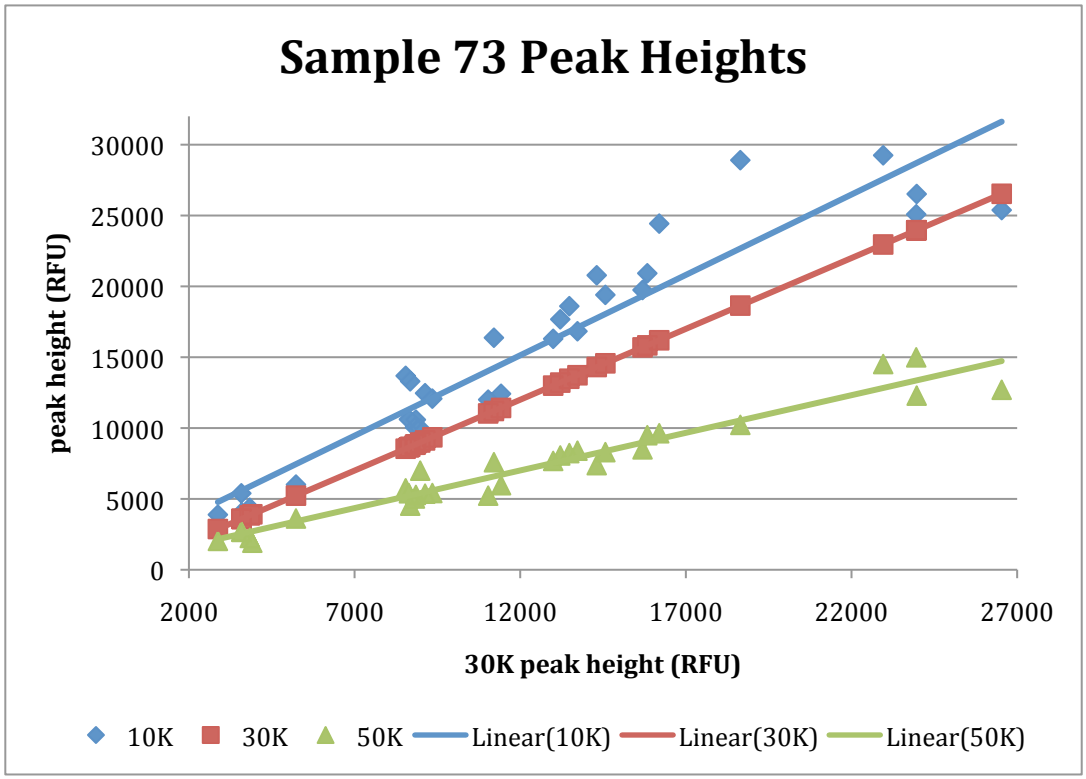


Figure 18. Allele peak heights for casework sample 73. Graphical representation of allele peak heights obtained during capillary electrophoresis for sample 73. When looking at the average trend line of peak heights, the 10K sample generated higher peak heights than the 30K sample, while the 50K sample generated the lowest peak heights of the three replicates.

The trend line of peak heights for casework sample 80 (Figure 20) deviate from the trend seen in the other samples. Sample 80 peak heights from the 30K filter generated the highest peak heights of the three replicates. There were a few alleles in the 10K sample that had higher peaks than the corresponding 30K allele, but overall the 30K alleles had higher peaks than those of the 10K and 50K filter devices. The 50K sample consistently had peak heights much lower than the samples from the other two Amicon® filter devices. Again, this small difference in peak heights could be the result of varying quantities of input DNA.

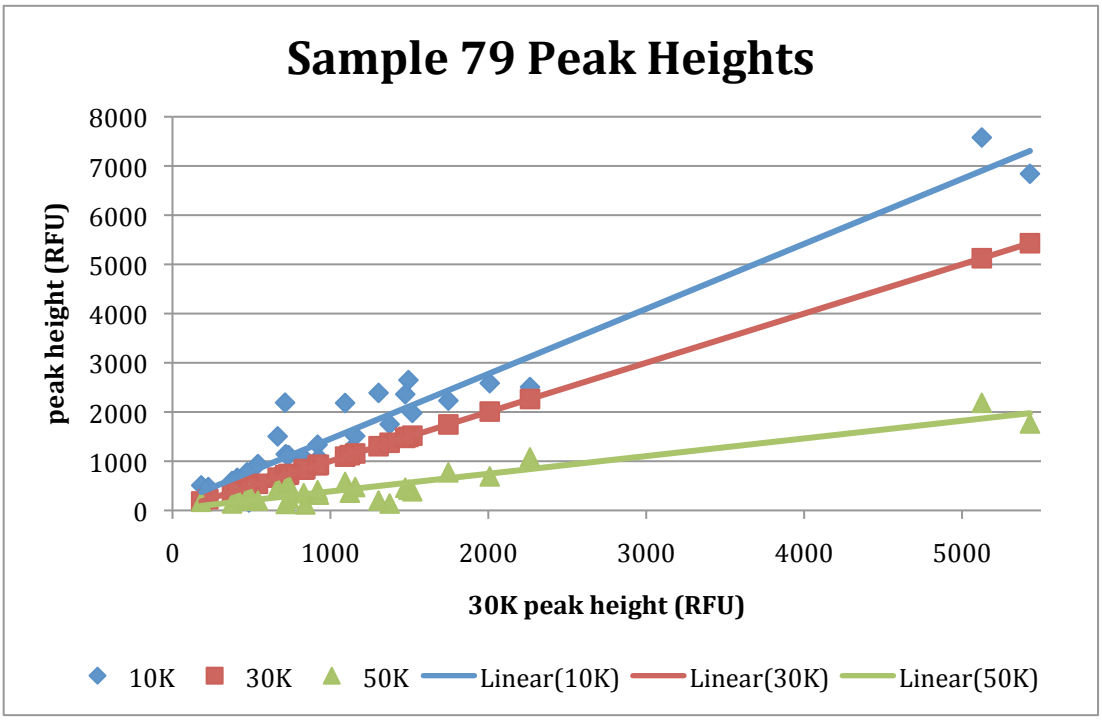


Figure 19. Allele peak heights for casework sample 79. Graphical representation of allele peak heights for sample 79. When looking at the average trend line, the 10K sample generated the highest peak heights while the 50K sample generated the lowest peak heights of the three replicates.

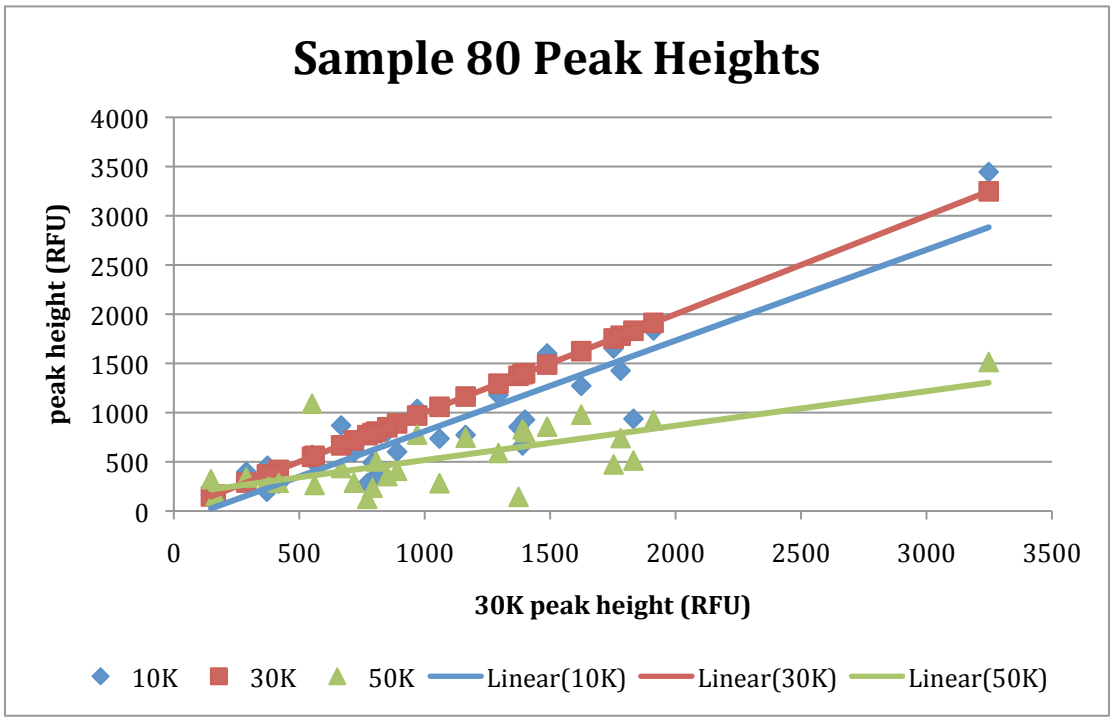


Figure 20. Allele peak heights for casework sample 80. Graphical representation of allele peak heights for sample 80. When looking at the average trend line, the 30K sample generated higher peak heights on average, with the 10K samples averaging just below, and the 50K sample again averaging the lowest peak heights of the three replicates.

The quality of profiles obtained was also assessed by evaluating the number of reportable alleles; reportable alleles are those that met the minimum detection threshold and were included in the DNA profile. The results for the cadaver samples are presented in Figure 21. The three cadaver bones share the same profile so dropout can be assessed across all replicates for all three bones. All instances of dropout were present in the samples taken from the left tibia. Of all nine samples from this bone, only two generated full profiles with no dropout. One replicate from the left tibia, sample LT.1.50K, had five instances of allelic drop out.

The results for the casework samples are presented in Figure 22. Reference profiles of the casework samples were not provided; therefore, dropout can only be evaluated by comparing the number of alleles obtained among the three filters for each sample. For example, the number of alleles obtained for sample 73 is not comparable to the number of alleles obtained from sample 80. There were two instances of dropout in the casework profiles. One allele dropped out of the profile for sample 79 and one allele dropped out of the profile for sample 80; both instances of allelic dropout were in the samples from the Amicon® 50K filter devices.

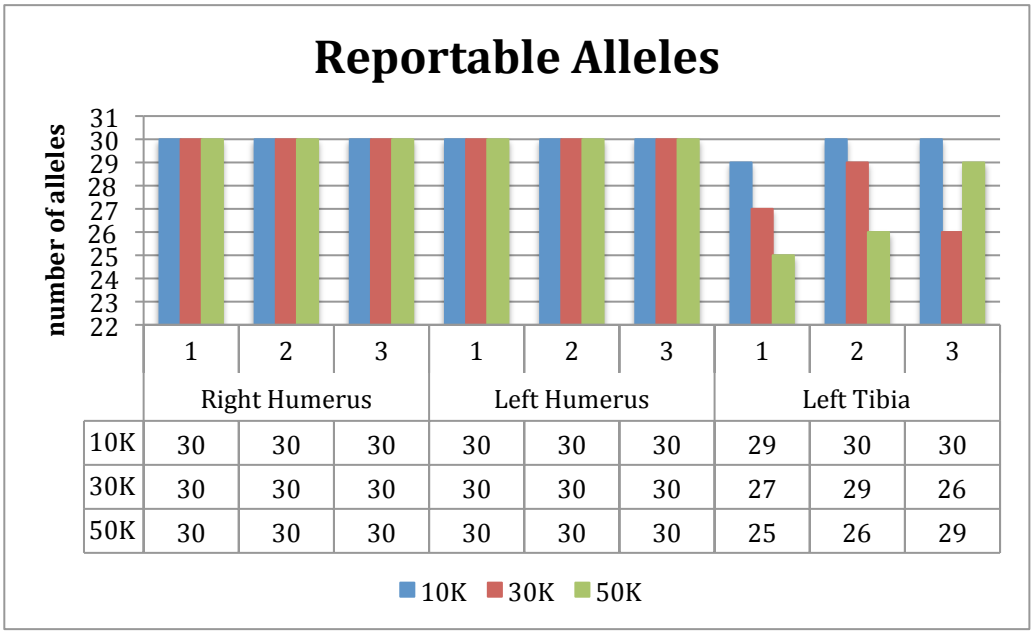


Figure 21. Reportable alleles for cadaver samples. Graphical representation of the number of reportable alleles for each replicate of all cadaver samples.

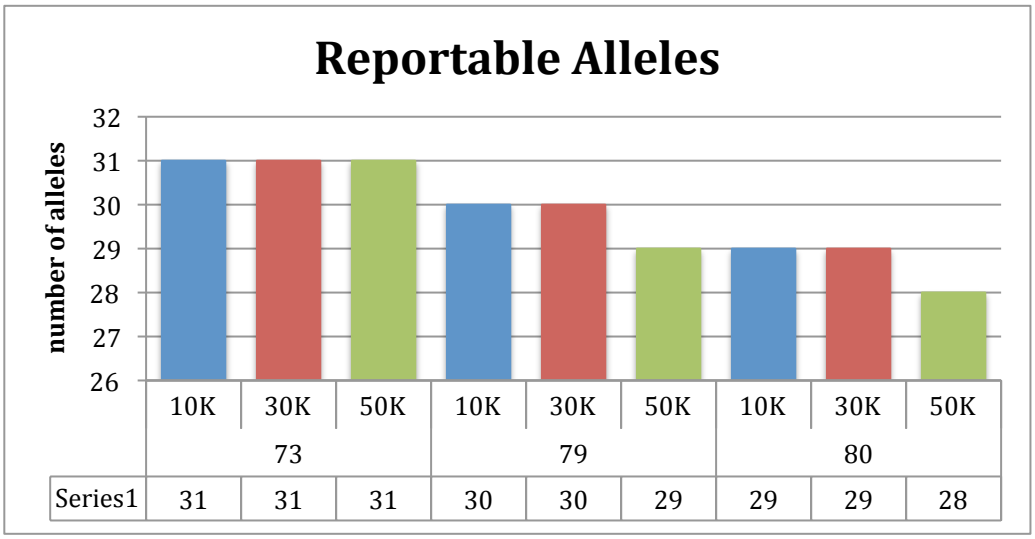
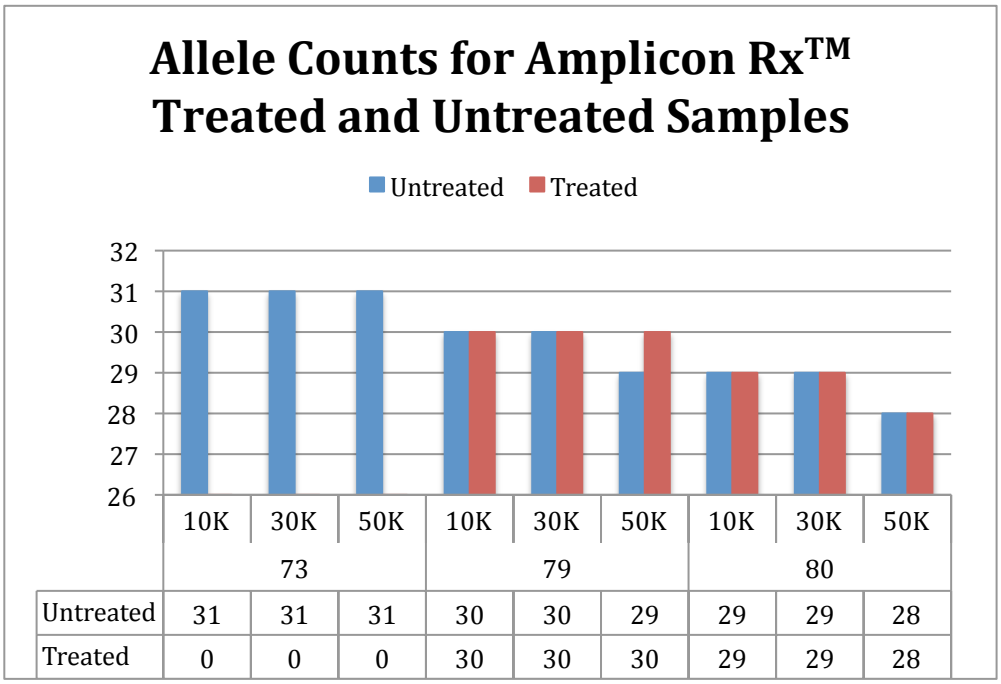


Figure 22. Reportable alleles for casework samples. Graphical representation of the number of reportable alleles for each replicate of all cadaver samples.

The goal of **Specific Aim 5** was to evaluate the ability of Amplicon Rx™ post-PCR treatment to boost RFU signal and improve poor STR results; this product was evaluated by (i) examining the number of reportable alleles in untreated versus treated samples, and (ii) comparing peak height relative florescence units (RFU) obtained during capillary electrophoresis.

The numbers of reportable alleles for all Amplicon Rx™ treated and untreated samples are presented in Figure 23. There was one instance of Amplicon Rx™ treated samples recovering an allele that dropped out in the corresponding untreated sample. This occurred for sample 79.50K; the number of reportable alleles increased by one with the post-PCR treatment. For sample 73, the RFU boost provided by Amplicon Rx™ was so great that the software, GeneMapper® *ID-X*, could not accurately size the peaks. Therefore, no data is included in Figure 20 for Amplicon Rx™ treated samples for casework sample 73.



	10K	30K	50K	10K	30K	50K	10K	30K	50K
	73			79			80		
Untreated	31	31	31	30	30	29	29	29	28
Treated	0	0	0	30	30	30	29	29	28

Figure 23. Allele counts for casework samples before and after Amplicon Rx™ treatment. Graphical representation of allele count comparison between treated and untreated casework samples with three different Amicon® filter devices.

The instance of allele dropout can be seen in the electropherograms for sample 79.50K. The 10 allele at locus CSF1P0 is missing in the electropherogram of the untreated sample. This electropherogram is presented in Figure 24. The CSF1P0 10 allele was recovered by treatment with Amplicon Rx™ and can be seen on the electropherogram for the Amplicon Rx™ treated sample, which is presented in Figure 25.

Allele peak height RFUs of untreated and Amplicon Rx™ treated samples were compared. Peak height RFU comparisons of untreated and treated samples from each Amicon® filter for casework sample 79 are presented in Table 1, Table 2, and Table 3. The average signal boost provided by Amplicon Rx for sample 79.10K was 2.7 times the original peak height RFU.

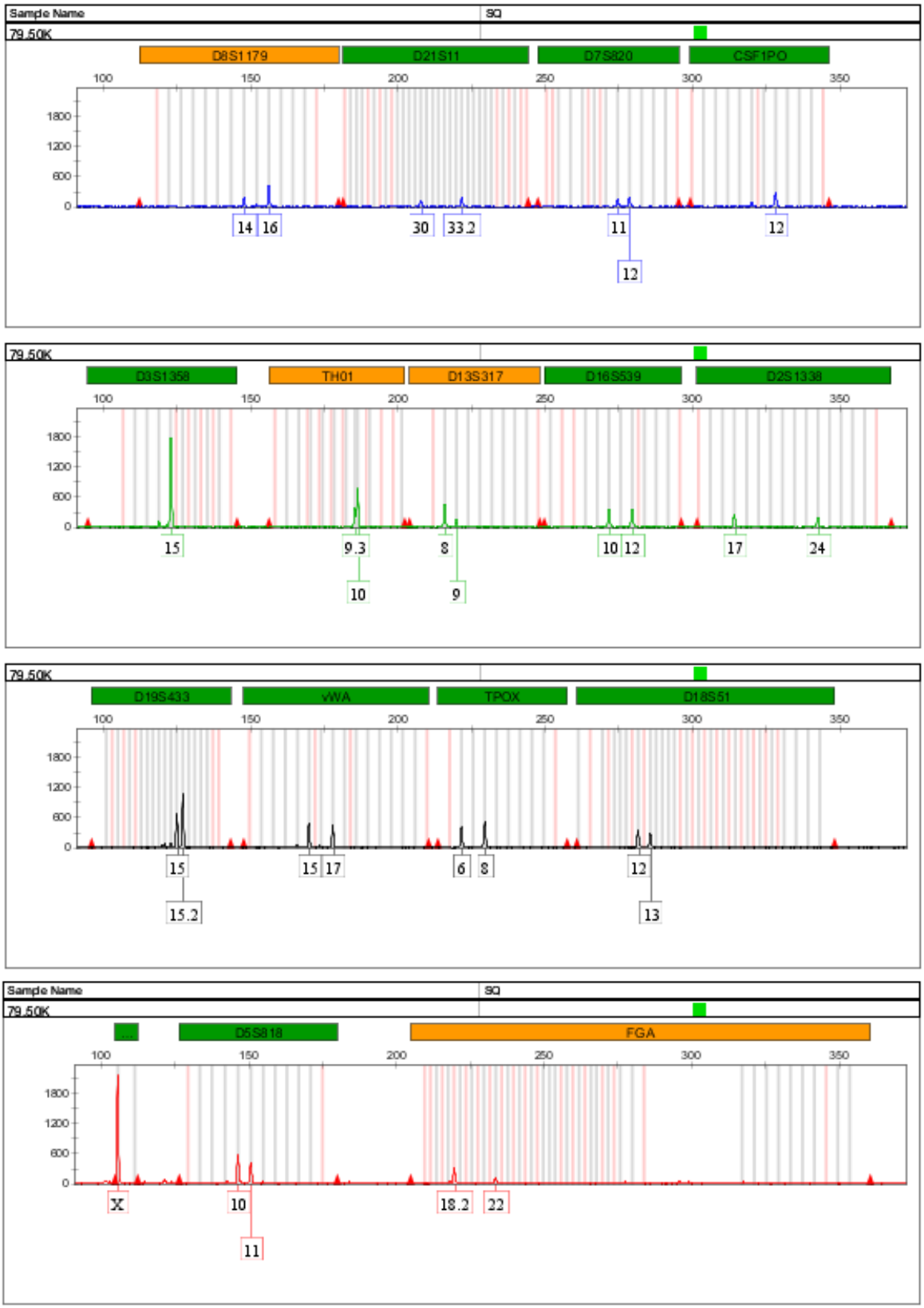


Figure 24. GeneMapper® ID-X electropherogram for untreated sample 79.50K. The y-axis reflects RFUs ranging from 0 to approximately 2,000. Locus CSF1P0 shows no peak for the 10 allele, indicating dropout.

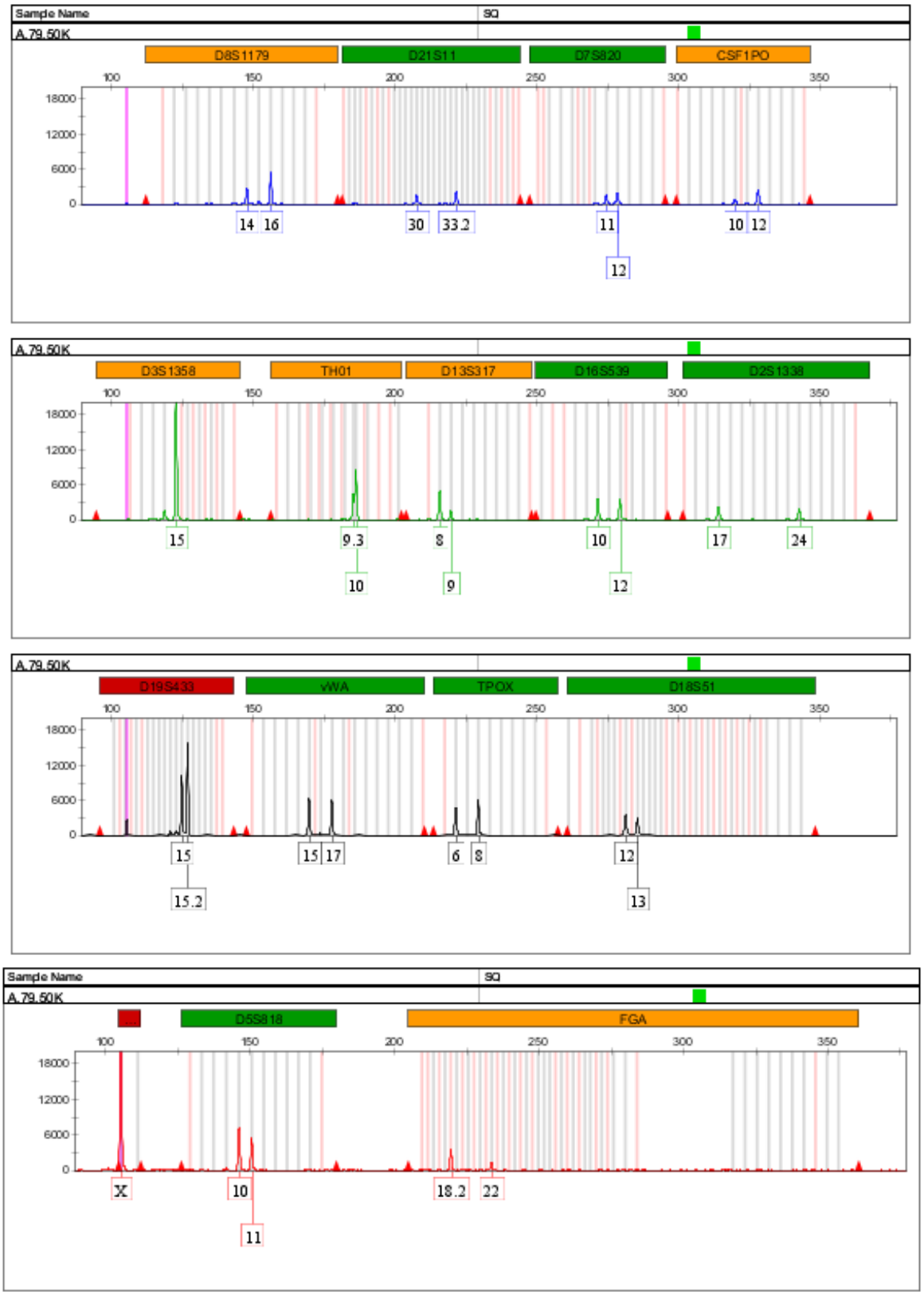


Figure 25. GeneMapper® ID-X electropherogram for Amplicon Rx™ sample 79.50K. The y-axis reflects RFUs ranging from 0 to approximately 20,000. Locus CSF1P0 shows the recovered 10 allele.

Table 1. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 79.10K.

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
79.10K	D8S1179	14	2388	7364	3.1
79.10K	D8S1179	16	2648	8287	3.1
79.10K	D21S11	30	876	2127	2.4
79.10K	D21S11	33.2	942	2348	2.5
79.10K	D7S820	11	598	1452	2.4
79.10K	D7S820	12	509	1209	2.4
79.10K	CSF1PO	10	474	1000	2.1
79.10K	CSF1PO	12	663	1345	2.0
79.10K	D3S1358	15	6841	23146	3.4
79.10K	TH01	9.3	1981	4953	2.5
79.10K	TH01	10	2231	5730	2.6
79.10K	D13S317	8	2361	6143	2.6
79.10K	D13S317	9	1752	4452	2.5
79.10K	D16S539	10	1219	2869	2.4
79.10K	D16S539	12	1068	2421	2.3
79.10K	D2S1338	17	699	1489	2.1
79.10K	D2S1338	24	774	1549	2.0
79.10K	D19S433	15	2586	8939	3.5
79.10K	D19S433	15.2	2507	8725	3.5
79.10K	vWA	15	1511	4725	3.1
79.10K	vWA	17	1505	4502	3.0
79.10K	TPOX	6	1336	3382	2.5
79.10K	TPOX	8	1121	2812	2.5
79.10K	D18S51	12	162	348	2.1
79.10K	D18S51	13	452	1148	2.5
79.10K	D5S818	10	2183	6600	3.0
79.10K	D5S818	11	2190	6268	2.9
79.10K	FGA	18.2	1065	2935	2.8
79.10K	FGA	22	1143	3008	2.6
79.10K	AMEL	X	7578	27244	3.6

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
79.30K	D8S1179	14	1305	12200	9.3
79.30K	D8S1179	16	1494	14252	9.5
79.30K	D21S11	30	841	7058	8.4
79.30K	D21S11	33.2	541	4557	8.4
79.30K	D7S820	11	377	3146	8.3
79.30K	D7S820	12	182	1462	8.0
79.30K	CSF1PO	10	227	1626	7.2
79.30K	CSF1PO	12	410	2945	7.2
79.30K	D3S1358	15	5430	32584	6.0
79.30K	TH01	9.3	1519	12551	8.3
79.30K	TH01	10	1747	14894	8.5
79.30K	D13S317	8	1474	12290	8.3
79.30K	D13S317	9	1375	11327	8.2
79.30K	D16S539	10	1123	8733	7.8
79.30K	D16S539	12	831	6363	7.7
79.30K	D2S1338	17	738	5174	7.0
79.30K	D2S1338	24	472	3219	6.8
79.30K	D19S433	15	2009	21000	10.5
79.30K	D19S433	15.2	2264	23832	10.5
79.30K	vWA	15	1157	11681	10.1
79.30K	vWA	17	666	6503	9.8
79.30K	TPOX	6	919	7907	8.6
79.30K	TPOX	8	736	6092	8.3
79.30K	D18S51	12	484	4152	8.6
79.30K	D18S51	13	482	3934	8.2
79.30K	D5S818	10	1093	10122	9.3
79.30K	D5S818	11	713	6512	9.1
79.30K	FGA	18.2	927	8267	8.9
79.30K	FGA	22	717	5996	8.4
79.30K	AMEL	X	5125	23867	4.7

Table 2. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 79.30K.

The average signal boost provided by Amplicon Rx™ for sample 79.30K was 8.3 times the original peak heights. The average signal boost for sample 79.50K was 11.5 times. The RFU boost provided by Amplicon Rx™ increased as the filter size increased and original peak heights decreased. The RFU boost for the 30K sample was three times that provided to the 10K sample; the boost for the 50K sample was more than four times that provided to the 10K sample.

Table 3. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 79.50K.

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
79.50K	D8S1179	14	201	2680	13.3
79.50K	D8S1179	16	421	5461	13.0
79.50K	D21S11	30	122	1438	11.8
79.50K	D21S11	33.2	200	2201	11.0
79.50K	D7S820	11	142	1501	10.6
79.50K	D7S820	12	176	1907	10.8
79.50K	CSF1PO	-	-	747	-
79.50K	CSF1PO	12	273	2537	9.3
79.50K	D3S1358	15	1768	23566	13.3
79.50K	TH01	9.3	389	4368	11.2
79.50K	TH01	10	774	8551	11.0
79.50K	D13S317	8	462	5024	10.9
79.50K	D13S317	9	140	1454	10.4
79.50K	D16S539	10	361	3544	9.8
79.50K	D16S539	12	350	3464	9.9
79.50K	D2S1338	17	238	2226	9.4
79.50K	D2S1338	24	201	1724	8.6
79.50K	D19S433	15	692	10296	14.9
79.50K	D19S433	15.2	1078	15922	14.8
79.50K	vWA	15	476	6284	13.2
79.50K	vWA	17	460	5924	12.9
79.50K	TPOX	6	411	4922	12.0
79.50K	TPOX	8	521	5957	11.4
79.50K	D18S51	12	347	3634	10.5
79.50K	D18S51	13	276	2963	10.7
79.50K	D5S818	10	580	7173	12.4
79.50K	D5S818	11	432	5398	12.5
79.50K	FGA	18.2	320	3605	11.3
79.50K	FGA	22	132	1296	9.8
79.50K	AMEL	X	2187	27469	12.6

Sample 79.50K had one instance of allele dropout at locus CSF1PO in the untreated sample. The peak height for allele 10 was approximately 80 RFU before treatment, below the minimum detection threshold. After Amplicon Rx™, the peak height was 747 RFU. This allele was recovered after treatment with Amplicon RxTM, meaning that the RFUs met the minimum detection threshold and could be included in the DNA profile.

Table 4. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 80.10K.

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
80.10K	D8S1179	12	1652	24216	14.7
80.10K	D8S1179	14	1426	20625	14.5
80.10K	D21S11	29	855	12372	14.5
80.10K	D21S11	30	736	10658	14.5
80.10K	D7S820	10	460	6824	14.8
80.10K	D7S820	11	279	4186	15.0
80.10K	CSF1PO	10	196	2967	15.1
80.10K	CSF1PO	12	399	5931	14.9
80.10K	D3S1358	15	3444	32631	9.5
80.10K	TH01	6	937	13731	14.7
80.10K	TH01	9	571	8011	14.0
80.10K	D13S317	14	1272	18452	14.5
80.10K	D16S539	11	869	13178	15.2
80.10K	D16S539	12	489	7272	14.9
80.10K	D2S1338	19	359	5402	15.0
80.10K	D19S433	14	928	15129	16.3
80.10K	D19S433	16	773	12560	16.2
80.10K	vWA	16	808	13465	16.7
80.10K	vWA	18	666	10940	16.4
80.10K	TPOX	11	494	8309	16.8
80.10K	TPOX	12	281	4278	15.2
80.10K	D18S51	17	133	2302	17.3
80.10K	D18S51	19	161	2531	15.7
80.10K	D5S818	7	1179	18071	15.3
80.10K	D5S818	11	1038	15349	14.8
80.10K	FGA	20	595	9426	15.8
80.10K	FGA	21	602	10529	17.5
80.10K	AMEL	X	1834	28155	15.4
80.10K	AMEL	Y	1601	24672	15.4

Peak height RFU comparisons of untreated and treated samples from each Amicon® filter for casework sample 80 are presented in Table 4, Table 5, and Table 6. The average signal boost provided by Amplicon Rx for sample 80.10K was 15.2 times the original peak height RFU. The average boost for sample 80.30K was 8.6 times the original peak heights and for sample 80.50K, 6.5 times. For sample 79, the RFU boost decreased as filter size increased; for sample

80 the opposite was true. As filter size increased, so too did RFU boost provided by Amplicon Rx™. The boost provided to 80.10K peak heights was on average twice the boost provided to 80.50K peak heights.

Table 5. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 80.30K.

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
80.30K	D8S1179	12	1753	18573	10.6
80.30K	D8S1179	14	1781	17749	10.0
80.30K	D21S11	29	1374	11660	8.5
80.30K	D21S11	30	1059	8867	8.4
80.30K	D7S820	10	373	2753	7.4
80.30K	D7S820	11	418	3102	7.4
80.30K	CSF1PO	10	371	2436	6.6
80.30K	CSF1PO	12	289	1866	6.5
80.30K	D3S1358	15	3248	32102	9.9
80.30K	TH01	6	1832	15845	8.6
80.30K	TH01	9	551	4513	8.2
80.30K	D13S317	14	1624	12374	7.6
80.30K	D16S539	11	667	4933	7.4
80.30K	D16S539	12	561	3994	7.1
80.30K	D2S1338	19	807	4977	6.2
80.30K	D19S433	14	1400	16652	11.9
80.30K	D19S433	16	1163	13214	11.4
80.30K	vWA	16	851	8666	10.2
80.30K	vWA	18	1390	12268	8.8
80.30K	TPOX	11	792	6126	7.7
80.30K	TPOX	12	771	6006	7.8
80.30K	D18S51	17	167	1143	6.8
80.30K	D18S51	19	148	1045	7.1
80.30K	D5S818	7	1294	13718	10.6
80.30K	D5S818	11	970	9282	9.6
80.30K	FGA	20	718	5498	7.7
80.30K	FGA	21	890	6462	7.3
80.30K	AMEL	X	1912	21751	11.4
80.30K	AMEL	Y	1488	16263	10.9

Table 6. Allele peak heights obtained from Amplicon Rx™ untreated and treated samples from sample 80.50K.

	Marker	Allele	Untreated Sample RFU	Amplicon Rx sample RFU	Signal Boost (times)
80.50K	D8S1179	12	473	4795	10.1
80.50K	D8S1179	14	741	4137	5.6
80.50K	D21S11	29	144	2279	15.8
80.50K	D21S11	30	282	2414	8.6
80.50K	D7S820	10	*	2930	-
80.50K	D7S820	11	287	*	-
80.50K	CSF1PO	10	304	1591	5.2
80.50K	CSF1PO	12	341	820	2.4
80.50K	D3S1358	15	1514	13243	8.7
80.50K	TH01	6	513	1711	3.3
80.50K	TH01	9	1091	3522	3.2
80.50K	D13S317	14	979	5418	5.5
80.50K	D16S539	11	437	1175	2.7
80.50K	D16S539	12	265	1643	6.2
80.50K	D2S1338	19	504	3591	7.1
80.50K	D19S433	14	801	4923	6.1
80.50K	D19S433	16	745	3040	4.1
80.50K	vWA	16	355	5346	15.1
80.50K	vWA	18	830	3151	3.8
80.50K	TPOX	11	237	820	3.5
80.50K	TPOX	12	124	1708	13.8
80.50K	D18S51	17	156	758	4.9
80.50K	D18S51	19	324	2505	7.7
80.50K	D5S818	7	590	2474	4.2
80.50K	D5S818	11	777	2331	3.0
80.50K	FGA	20	288	1365	4.7
80.50K	FGA	21	409	1864	4.6
80.50K	AMEL	X	922	4985	5.4
80.50K	AMEL	Y	857	8258	9.6

CHAPTER IV

CONCLUSIONS

DNA analysis is critical for identification in cases where other traditional methods of identification are insufficient. In the forensic setting, this includes cases of mass disasters (e.g. plane crashes and terrorist attacks) and missing persons cases (9). Bones are a good source of DNA for identification as they protect DNA from degradation and are often the only remaining biological sample that can be tested (5). However, the quality of DNA obtained from bones can often be limited and/or degraded as a result of environmental exposure. With limited DNA present, efficient recovery of DNA is paramount in obtaining complete STR profiles for identification. In this study, I evaluated three different molecular weight cut-off columns to ascertain which is most efficient at retaining amplifiable DNA while filtering small molecular weight inhibitors. Additionally, I evaluated the post-PCR treatment, Amplicon Rx™, and its ability to improve genetic profiles by providing a boost in relative florescence units (RFUs) obtained during capillary electrophoresis. Using an appropriately sized filtration device will aid in the recovery and retention of DNA, while the use of a post-PCR treatment can aid in the quality of results obtained. Together these processes aim to improve STR typing results obtained from current procedures.

The Quantifiler® Duo results show that more DNA is retained with the 10K NMWL Amicon® filter device. This was expected as the filter is designed to retain molecules with

smaller molecular weights. On average, a DNA base pair has a molecular weight of 650 Daltons. Therefore, the 10K NMWL filter should retain fragments greater than 16 base pairs, which would have a molecular weight of 10,400 Daltons. The 50K NMWL filter is only expected to retain DNA fragments greater than approximately 77 base pairs (50,050 Daltons). This difference was expected to result in higher quantification values for samples using the 10K filters than samples using the 50K samples, and the Quantifiler® Duo results for the cadaver bone samples confirmed this. The quantification values for the casework bones follow this same trend; however, the quantification values for these samples were so low that any variation in sample 80 could not be detected.

The Agilent data corroborates the trend seen in the Quantifiler® Duo data. The highest peak heights for the 10K samples average 500 RFUs, the highest peak heights for the 30K samples average 300 RFUs, and the highest peak heights average approximately 50 RFUs for the 50K samples. DNA quantity retained by the 10K filter increased 10 fold compared to the 50K filter. The same trend is seen in the Agilent peak areas and on the Agilent gels. This increase in DNA retention is especially significant when considering DNA extraction from bone samples, which have initially low levels of DNA present.

The possible loss of DNA fragments due to the molecular weight cut off of the Amicon® filter devices was also evaluated. Overall, the Agilent results did not show a loss of DNA fragments less than 1,000 base pairs in size as hypothesized, but rather showed the presence of DNA fragments in all samples ranging from 50 to more than 7,000 base pairs. The greatest concentration in fragments was seen at approximately 150-200 base pairs. Fragments 150 base pairs in size have a molecular weight of approximately 97,500. The Amicon® filter device manufacturer recommends using a filter at least two times smaller than the molecule you are

concentrating. Fragments of MW 97,500 would fall beneath the recommended cut off of the 50K filter, and it is likely that more of these 150 base pair DNA fragments are being filtered out. This correlates to the lighter band at 150-200 base pairs and to the lower peak FU on the electropherograms for the 50K samples.

It was expected that the Amicon® 10K NMWL filter device would retain more DNA fragments but that it might also retain small molecular weight inhibitors that would interfere with analysis, outweighing any benefit provided by retaining the smaller DNA fragments. Cycle thresholds (Ct) of the internal positive control (IPC) were analyzed to assess possible inhibition of the samples and compare the presence of inhibitors among the filters. Weak amplification of both the IPC and the sample, which results in high Ct values, could indicate the presence of inhibitors. There was so significant shift in the IPC Cts in any of the samples, indicating that no inhibitors were detected. These were cadaver bone samples, so many of the typical inhibitors associated with bone samples, such as soil components like humic acid, and other environmental inhibitors, were not present. Other inherent inhibitors, such as calcium ions, coextract with DNA and were expected to be present. It is possible that even the smallest filter is not small enough to retain common PCR inhibitors. Alternatively, it is possible that inhibitors were present in the samples but were not detected by Quantifiler® Duo. There is some evidence of this occurrence with bone samples in particular (10).

To determine the quality of the DNA profile obtained, peak height RFUs were evaluated and compared among the samples. Cadaver samples were normalized to 1 ng/μL prior to amplification and capillary electrophoresis and despite this normalization, peak heights varied widely among the samples. Peak heights were inversely related to filter size; as filter size increased, peak height averages decreased. Samples from the 10K filter devices consistently

generated profiles with higher peak heights, while samples from the 50K filter devices generated the lowest peak heights of the three filters. These results may indicate the presence of more DNA in the 10K samples due to the smaller filter size, which led to retention of small DNA fragments and higher peak height averages, particularly of the smaller alleles.

For casework samples, this was true in two of the three. Samples 73 and 79 followed the same trend: higher peaks in the 10K samples, lower peaks in the 50K, with the 30K samples falling somewhere in the middle. However, casework sample 80.30K generated the highest peak heights among the three filters for that sample. The quantification values for casework sample 80 were very low for all three Amicon® filters and no variation could be detected. The resulting peak heights could be due to variation in input DNA or differences in amplification efficiency. The difference between the 10K and 30K was negligible, but again the 50K sample generated much lower peak heights.

The number of reportable alleles was compared among the Amicon® filters and samples to determine if there was a correlation between filter and DNA profile quality. The only cadaver bone that exhibited any allele dropout was the left tibia, which was the bone that had the lowest quantification values. There was dropout in every replicate; however, the 10K sample generated more alleles than the 30K and 50K samples in each case. The first 50K replicate had five alleles dropout, the most of any sample. In the casework samples, there were two instances of apparent allele dropout, both in the 50K samples. These results indicate that the use of a 50K filter could significantly compromise the quality of the DNA profile obtained.

Overall, the smallest Amicon® filter device, the 10K NMWL, outperformed the other two filters, the 30K and 50K NMWL, in terms of DNA retention, allele peak heights, and DNA profile quality. The 10K filters had higher quantification values, higher peak heights, and more

reportable alleles than the 30K and 50K filters. These factors are especially important when working with bone samples, which are notoriously difficult sources of DNA. These samples are often degraded and inhibited, both factors that affect the resulting profile. Retaining as much DNA as possible and generating more peak heights that meet and exceed thresholds is vitally important when working with these samples. The Amicon® 10K NMWL filter appears to be the best choice when considering these data and circumstances.

Amplicon Rx™, a post-PCR clean-up treatment, was evaluated as another option for improving profiles obtained from bone samples. Amplicon Rx™ is a product that seeks to improve DNA profiles by concentrating the reaction and reducing the amount of molecules competing with DNA amplicons for injection into the capillary for capillary electrophoresis. Amplicon Rx™ clearly provided an RFU boost to allele peak heights, with averages ranging from 2.7 to 15.2 fold. Amplicon Rx™ also recovered one allele that dropped out in the original sample, casework sample 79.50K. These data indicate that this product is capable of providing a significant RFU boost and may be able to boost allele peak heights that fail to meet the minimum detection threshold to a height above the threshold so that they may be included in data analysis.

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