Evaluating the Use of DNA and RNA Degradation for Estimating the Post-Mortem Interval

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Investigators:

Arpad A. Vass, PhD
Adjunct Research Associate/Joint Faculty Oak Ridge National Laboratory
University of Tennessee
Knoxville, TN
Phone 865-574-0686
Email: vassaa@ornl.gov

Rachel I. Fleming, PhD
Post-Doctoral Senior Scientist
Institute of Environmental Science and Research
Hampstead Road
Auckland 1025
New Zealand
Phone: 649 845 1736
Email: Rachel.Fleming@esr.cri.nz

SallyAnn Harbison, PhD
Technical Leader and Science Leader
Forensic Biology Team
Institute of Environmental Science and Research
Hampstead Road
Auckland 1025
New Zealand
Phone 649 815 3670
Email: Sallyann.Harbison@esr.cri.nz

James M. Curran, PhD
Associate Professor
Department of Statistics
The University of Auckland
Private Bag 92019
Auckland 1142
New Zealand
Phone 649 3737599 x86893
Email: curran@stat.auckland.ac.nz

Eletra Williams
Forensic Biology Team
Institute of Environmental Science and Research and Department of Chemistry
University of Auckland
New Zealand

Abstract

Estimating the post-mortem interval (PMI) is difficult due to the many factors that influence the decomposition process. Tissues such as nails, teeth and bones are more resilient to environmental factors. Measuring the rate of degradation of nucleic acids in these tissues could provide a method for estimating the PMI for longer time intervals, from days and weeks, through to months and years. These time intervals have not been studied before in a systematic manner using human tissues. Our initial focus has been on nails. Nails are hard tissues, relatively easy to sample and until now, have been under-utilized as a tissue that has forensic value. We have investigated the stability of nucleic acids in nails to determine if both DNA and RNA can be co-extracted in levels suitable for PCR analysis. Furthermore, multiple nail samples can be taken from the same individual so that measuring changes in nucleic acid degradation can be undertaken where there are a minimal number of external variables so that the nucleic acid degradation is a more likely representation of the PMI.

Using different methods for extracting DNA and RNA from nails, we have found that by modifying the Promega DNA IQ™ method, both DNA and RNA are efficiently extracted. This modified method has been implemented into operational forensic casework at ESR and nails are now being utilized in more human identification cases.

We have developed multiplex PCR assays to measure the rate of degradation of messenger RNA (mRNA), ribosomal RNA (rRNA) and DNA. Using nails placed in different environmental conditions (air, soil and water), we have found that nails are protected from other environmental factors and that nucleic acids (both DNA and RNA) can be amplified from samples left submerged in water or placed in soil for 120 days (1043 accumulated degree days). Nails from human cadavers have been collected, and the DNA and RNA co-

extracted. Using our multiplex PCR assays, we have shown that DNA and RNA can be co-extracted from cadaver nails with a PMI up to 20,925 ADDs.

Using statistical analyses, some of the nucleic acid amplified fragments (from DNA and mRNA) look promising to use for deriving a statistical model for estimating the PMI.

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Executive Summary

Although the post-mortem interval (PMI) is important in the investigation of criminal cases, it is still difficult to estimate post-mortem intervals ranging from weeks to years. This is because decomposition is heavily influenced by taphonomic factors such as environmental conditions (moisture, temperature, ecosystem, insects, animals and season), the circumstances of the death and the location of the body, indicating that the stage of decomposition of a body is not always a reliable PMI indicator. Individual tissues decompose at different rates which add to the difficulties of determining the PMI. While there have been studies looking at several of these variables, only a few have investigated longer post-mortem intervals.

There is a lack of research in estimating the PMI over longer time intervals (days through to weeks and months). The purpose of this project is to provide proof-of-concept that nucleic acid degradation can be utilized in the estimation of the PMI over longer time intervals. Using tissues that are less influenced by environmental factors, such as nail and bone material, the degradation of nucleic acids are being used to derive a model that can be used for estimating the PMI.

A unique aspect of this research is the use of tissues where multiple samples from the same individual human cadaver can be used. This means that the same tissue type from the same individual from the same environment can be used, reducing the number of variables influencing the decomposition and degradation of nucleic acids present in the tissue. As a result, the measurement of nucleic acid degradation will likely closely reflect the true PMI, free from confounding factors. To achieve this, we have focused on the use of nail material, including some preliminary work using rib bone. These tissues were chosen as they are slower to degrade than other tissues such as skin or muscle. The use of nails and rib bones,

combined with multiple sampling from the same individual, will further aid in the development of accurate PMI indicators for these longer time intervals.

While the use of animal models is widespread, there are problems associated with the ability to correlate what happens in an animal to what happens in a human. A further problem identified with using animals instead of humans in PMI studies, particularly with animals in the laboratory (mice, rats and rabbits) is that they are kept in a relatively sterile environment. Therefore post-mortem nucleic acid degradation is not representative of what happens in human post-mortem tissue due to the differences in exogenous nucleases present.

The rate of RNA degradation has not previously been studied in a systematic manner using human tissues over a long post-mortem interval. A number of groups have shown that RNA can be extracted from post-mortem tissues in various animals, including humans. Furthermore the research of our group based at ESR Ltd, alongside that of other groups worldwide, have shown that it is possible to extract RNA from very small volumes of body fluids found at crime scenes.

Post-mortem RNA degradation in human, rat, rabbit and cattle post-mortem tissues has been studied, however the results were inconsistent with significant differences seen in the rate of RNA degradation when tissues were sampled at the same post-mortem times. This can be attributed to the use of tissues that are rapidly degraded, focusing on a short post-mortem interval and most importantly, the use and comparison of tissues originating from different animal species. This highlights the importance of the inclusion of appropriate material for research of this kind, and the need for the use of human tissue in the production of meaningful forensic research applicable to casework situations.

The goals of this project are to determine which tissue is the most appropriate for use in the estimation of PMI using nucleic acid degradation and to investigate an extended range of PMI, extending to months.

The goal of this project is being achieved through the following objectives:

1. Adapt and develop the Promega DNA IQ™ method to successfully isolate RNA and DNA from sample tissues.
2. Identify candidate markers for both RNA and DNA degradation assays that differ in degradation pattern and/or frequency.
3. Use muscle samples to test and refine degradation assays.
4. Apply the RNA and DNA assays to samples of nails, ribs and teeth* over a several month time course.
5. Evaluate the success of this procedure.

* - teeth are no longer being evaluated for this project

Our initial approach was to study the degradation of nucleic acids over longer time intervals - from months to years. While muscle tissue was going to be initially used for establishing the co-extraction of DNA and RNA, the concentrations of both types of nucleic acids was low and not suitable for PCR assays. The most likely explanation for this is that the muscle tissue had degraded to a level where the nucleic acids were too damaged to be useful. Due to the low concentrations of DNA and RNA and that muscle tissue is not suitable for the time intervals we are studying (weeks and months), no further work using muscle tissue was performed. Therefore objective three using muscle tissue was not continued. Instead, nail material was substituted to test and refine the degradation assays to address this objective.

Given the reduced timeline of this project we have also had to decrease the time interval under study (to weeks) and reduced the number of tissues that being studied, focusing on nail material with some initial work on rib bones. Our reasons for this reduction in the number of tissues include:

1. We have successfully adapted and developed the method for the co-extraction of RNA and DNA. This has been shown to be ideal for nails, therefore we did not need to further evaluate other tissue samples for this part of the work.
2. Muscle tissue (specifically the nucleic acids in muscle) is not sufficiently stable over the time intervals focused on in this project, so this tissue was not used in any further degradation studies.
3. Nails have proven to be very successful in terms of our aim of finding the most relevant tissues to study.
4. Technical difficulties in obtaining teeth and rib bone samples, combined with the short duration of this study did not allow for an accurate evaluation of the degradation of nucleic acids in these tissues.

A critical aspect of assessing nucleic acid degradation is to recover the nucleic acids (both DNA and RNA) without any further fragmentation. We have previously shown that the DNA IQ™ extraction system can be successfully adapted to allow the co-extraction of RNA and DNA without compromising either. An important practical aspect of this work is the further adaptation of this method to co-extract RNA and DNA from tissue samples such as those described herein (nails).

This was achieved using simple and efficient alterations to the nucleic acid co-extraction protocol. Traditionally, nail material is incubated with some form of a lysis buffer for an

extended period of time to facilitate the extraction of DNA material. Unfortunately, this extended time interval is both temporally costly and detrimental to the quality of RNA co-extracted. The lysis incubation time has been reduced to 40 minutes total, with the nail material fragmented to speed digestion, as well as the inclusion of high concentrations of dithiothreitol (DTT).

Using fresh nail clippings processed as per the co-extraction protocol we have successfully amplified keratin mRNAs, (keratins 85 and 86), 18S rRNA and DNA transcripts from the nucleic acid extracted fractions. PCR-based techniques, including reverse-transcriptase PCR have been used to develop RNA and DNA based assays that will measure the rate of decay of the respective nucleic acids. This involved amplifying fragments of varying length (as a measure of the quality of the RNA and DNA).

Our approach to developing the method for measuring nucleic acid degradation was to collect nail clippings and placing these clippings in different environmental conditions. Over a defined time period, nails were collected and the RNA and DNA extracted. The amount of DNA and RNA present in the nail material was quantified using real time PCR (DNA) or reverse transcriptase real time PCR (RNA). Using the PCR multiplexes we developed, we have been able to amplify all the targeted nucleic acids (mRNA, rRNA and DNA). Furthermore, the mRNA transcripts amplified are specific to nail material and add a level of reassurance and control against contamination.

What has been surprising is the overall stability of the nucleic acids found in nail material. When nail clippings are buried in moist soil, and submerged in water for 120 days (1043 ADD), we are able to amplify mRNA, rRNA and DNA with amplicons around 400bp still

being detected in the extracted samples. There was a noticeable trend in the peak heights from the PCR assays. The larger mRNA amplicons in these nail clippings exposed to environmental pressures were significantly reduced compared to those seen in the time 0 samples, and the samples left exposed to air in the lab for the same extended time intervals.

Also surprising is the concentration of RNA and DNA being extracted from nail material (picogram and nanogram concentration levels respectively). With the development of the modified extraction process described above allowing for the co-extraction DNA and RNA, sufficient amounts of both nucleic acids are usually extracted from portions of nails to enable all necessary downstream processing. This method has now been implemented into operational forensic casework for the identification of individuals in numerous cases, indicating the value of these findings to real-world forensic scenarios.

We have also investigated other factors that may have some influence on the stability of nails such as is there any difference in the rate of degradation of nucleic acids between children's and adult nails, and is there any difference between fingernails and toenails. Children's nails grow quicker than adults and are softer, therefore they have the potential to degrade more rapidly. Research has shown that toenails grow slower than fingernails and are thicker. So toenails may be less prone to degradation. Toenails spend the majority of their time in an enclosed, humid environment (inside socks and/or shoes) that has the potential to cause a quickening of nucleic acid degradation compared to fingernail nucleic acids. Alternatively, the increased environmental contaminants that fingernails are exposed to in everyday life, compared to toenails, could also mean increased nucleic acid degradation. Our results indicate that there is no difference in the amount of nucleic acids extracted or in the rate of degradation of nucleic acids between fingernail and toenails when the weight difference

between the larger toenails is taken into account. There are differences in stability between nails from the small finger/toe which degrade more rapidly than the larger nails.

As we have developed an efficient and effective method for measuring the degradation of nucleic acids, this method has been used on aged samples collected from human cadavers located at the Anthropological Research Facility at the University of Tennessee. We have amplified nucleic acids from human cadaver nails up to 1310 days PMI (20,295 ADD).

We have obtained a small number (4) of rib samples and have assessed whether ribs can be used for PMI estimation in this manner, with a methodology for the co-extraction of DNA and RNA from bone material being developed in a similar manner to the nail DNA and RNA co-extraction. This co-extraction of nucleic acids in bone material is more difficult due to the lower levels of DNA and RNA in bone compared to the nail material, as well as being complicated by the components of bone that may interfere with genetic analysis due to the structure of the bone. We were able to extract DNA from the rib samples. We were not able amplify mRNA or rRNA from the rib samples and this could be due to the co-extraction method needing further refinement or that the mRNA and rRNA are too degraded to be amplified in our PCR assays (although we were able to extract RNA from nail samples collected during the same time period).

Thus far, the success of this project and economic applicability has been clearly demonstrated as the method developed to co-extract DNA and RNA from nails has been validated and implemented into operational forensic casework and is being used in cases requiring human individual identification. This further means that one of the overall aims of this research, to develop methods and techniques that can be introduced into operational forensic casework,

has already been achieved. The initial results from this pilot (proof-of-concept) study indicate much promise for the use of mRNA, rRNA and DNA extracted from nail material in the development of a novel method of determining the post-mortem interval for extended time intervals.

Introduction

Knowing when a person died is a fundamental question in forensic science. To date, there are a number of methods that provide estimates for the PMI in pre-skeletonized remains such as volatile fatty acids [1], body temperature [2, 3], analyte concentration [4], insect colonization [5] and the state of decomposition of the body [6]. For longer time intervals or when there are only skeletal remains there is are very few methods available for determining the time since death, which is problematic as this can be of crucial importance in determining the circumstances surrounding crimes.

The difficulty with determining the PMI arises due to the number of variables that affect the rate at which a body decomposes such as the environmental conditions and the differential rates at which individual organs decompose [7]. While there have been studies looking at one or two variables, only a few have investigated longer post-mortem intervals and few have considered the influence of multiple factors (temperature, size, age, manner of death) [7 - 9].

While DNA has been previously investigated in terms of environmental degradation in nails, teeth and bone [10 – 13] and on blood placed on fabrics that were buried in soil [14], no systematic study using the same tissue from the same individual has been used to correlate the degradation of DNA.

To date, there has been no study on RNA in nails, and it is not even known if RNA could be extracted from nails. Nails have been found to be stable after death with DNA being successfully extracted and amplified thereby providing a good source of nucleic acids [11, 12]. However, the rate of degradation of DNA from nails has not been investigated, and given the stability of DNA, there is potential for a new method for estimating the PMI.

Furthermore, although RNA transcripts in nail material have not been studied before, the stability of DNA in nails and the stability of nails in the environment also indicate there is potential for nail RNA to also be used in the estimation of the PMI.

In order to correlate samples from different cadavers over time and from varying environmental conditions, Accumulated Degree Days (ADDs) were calculated. Accumulated Degree Days, as described by Edwards et al. [13], have typically been used for PMI determinations and are determined by taking the sum of the average daily temperatures (in degrees Celsius) for however long the corpse has been decomposing.

Post-mortem RNA degradation has been studied in human and animal post-mortem tissues with inconsistent results with differences in the rate of RNA degradation at the same post-mortem times [14-16]. This can be attributed to using tissues that are rapidly degraded, using a short post-mortem interval and more importantly, the differences in animal species [16]; therefore it is important to study the appropriate human tissues.

While the use of animal models is widespread, there are problems associated with the ability to correlate what happens in an animal to what happens in a human. A further problem identified with using animals instead of humans in PMI studies, particularly with animals in the laboratory (mice, rats and rabbits) is that they are kept in a relatively sterile environment. This is not representative of what happens in human post-mortem tissue due to the differences in exogenous nucleases present [12].

The value of co-extraction procedures (through the inclusion of RNA) allows you to identify the origin of the body fluids [17]. RNA has not previously been used for identification purposes because of its perceived instability which has been shown to be incorrect.

Both messenger RNA (mRNA) and ribosomal RNA (rRNA) have been shown to degrade at different rates [18-20]. Therefore, successful analysis of post-mortem RNA levels not only depends on the post-mortem interval or the extraction method used, but also on the different RNA transcripts investigated. Few studies have attempted to use the correlation of DNA and RNA degradation and no studies have investigated longer time periods for using RNA (and DNA) for estimating the PMI.

In this study, we investigated the rate of degradation of mRNA, rRNA and DNA in nails over time from human cadavers located at the University of Tennessee's Forensic Anthropology Research Center (FARC). In particular, we have investigated the differential rates of decay of different mRNA transcripts (keratins 31, 34, 85 and 86), 18S rRNA and DNA from nail clippings placed in different environmental conditions over a period of time. We have focused on longer time intervals after death, from days and weeks, through to months. These time intervals have not been studied in relation to the degradation of nucleic acids in a systematic manner in human cadavers before. Using this data we will derive a statistical linear model for the estimation of the PMI.

II. Methods

Sample Collection

Cadaver Nails

Nails from human cadavers (n=14) were collected from the Anthropological Research Facility at the University of Tennessee. Nails were collected by surgical removal or as the decomposition progressed, the nails fall from the hand or foot and were recovered from the soil surface or from within the decompositional matrix (Figures 1-3). The length of the post-mortem interval in days and ADDs for cadavers whose nails were used in this study is shown in Table 1.

Nails were washed to remove exogenous DNA and RNA. This was done by scrubbing the nails in ethanol using a soft toothbrush that had been cleaned prior to use to prevent contamination between samples. The nails were then rinsed in water and wiped with a tissue to remove any remaining liquid, after which the nails were weighed.

Nail samples (in the form of clippings) were cut from the nail, usually approximately 10mm x 2-3mm (Fig. 4). To help with the lysis of nail material, the nail clippings were cut into small pieces using a sterile disposable scalpel. The resulting fragments were approximately 1 x 2-3mm in size (average weight of nail material used was 0.03g).

Fresh Nails (time 0 days, 0 ADDs)

Nail samples from 35 subjects were collected at time zero. These samples were approximately 10mm x 1-2mm. To help with the lysis of nail material, the nail clippings were cut into small pieces using a sterile disposable scalpel. The resulting fragments were approximately 1 x 1-2mm in size (average weight of nail material used was 0.01g).

Rib Samples

In addition to nail samples, four rib samples were collected from two cadavers at the same time the nails samples were collected. These rib samples came from cadavers 11 and 12 (Table 1). Samples of rib bone, approximately 1.5 cm long were removed for the DNA and RNA co-extraction.

Environmental Studies

Three environmental conditions were used; open to air, immersed in water and buried in moist soil. The soil was collected locally from Mt Albert, Auckland, New Zealand and was a porous/volcanic type soil. The water and soil samples were placed outside in a shaded, damp area from July through to October which covered winter and spring seasons in New Zealand (Fig. 5). Nail clippings from three individuals were placed in these conditions for approximately 1, 40, 80 and 120 days. The temperature was measured and the accumulated degree days calculated (Table 2). Control samples at 0 days (fresh) were used for all environmental studies. The nail clippings (in quadruplicate while duplicate samples were used in the extraction and analysis) were placed in the environments as soon as they were collected. The time point at which the nails were placed within the environment was taken as time 0.

Table 1: Length of post-mortem interval and the corresponding accumulated degree days of human cadavers that nail samples were collected from.

Cadaver	Post-Mortem Interval (days) when nail samples were collected	Accumulated Degree Days when nail samples were collected
1	389	5699
2	387	5663
3	48	1164
4	7	173
5	15 24	389 611
6	1310	20925
7	276	4901
8	162	4237
9	20 23 26 29	335 378 415 441
10	5 8 11 14 16	73 111 136 165 184
11*	189 196	4237 4313
12*	189 196	3964 4040
13	54 57 60 63 66 68 72	362 405 443 468 497 516 563
14	3 6 9 12 15 17 21	42 85 123 148 177 196 243

* - rib samples also collected

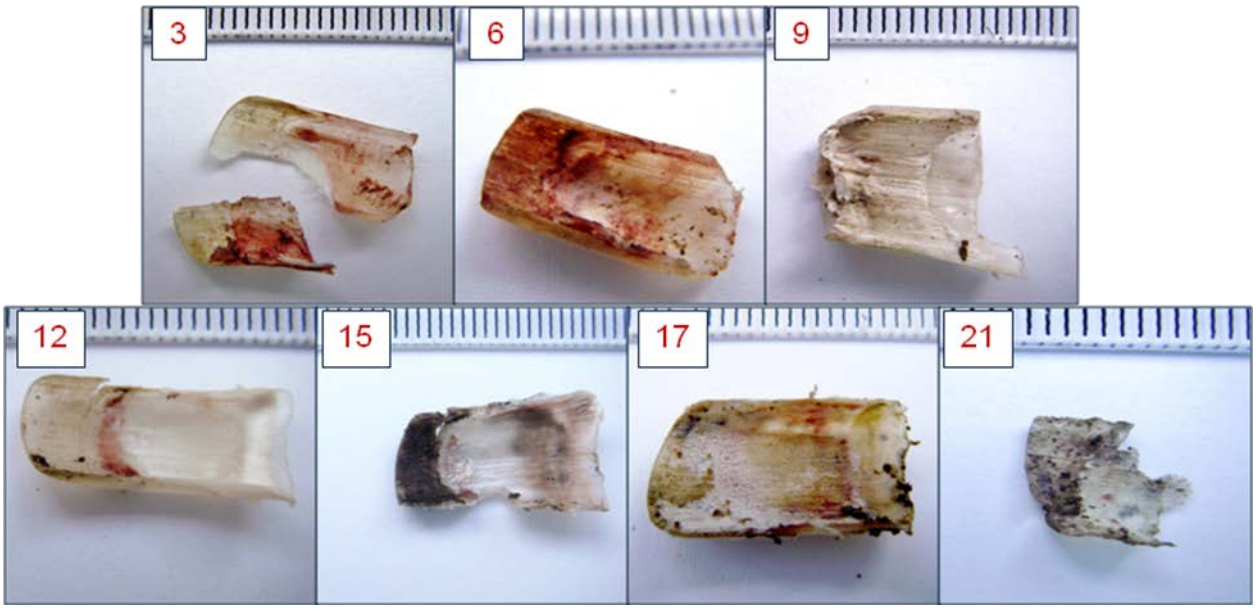


Figure 1: Cadaver 14 fingernails collected from days 3-21 PMI (42-243 ADD).

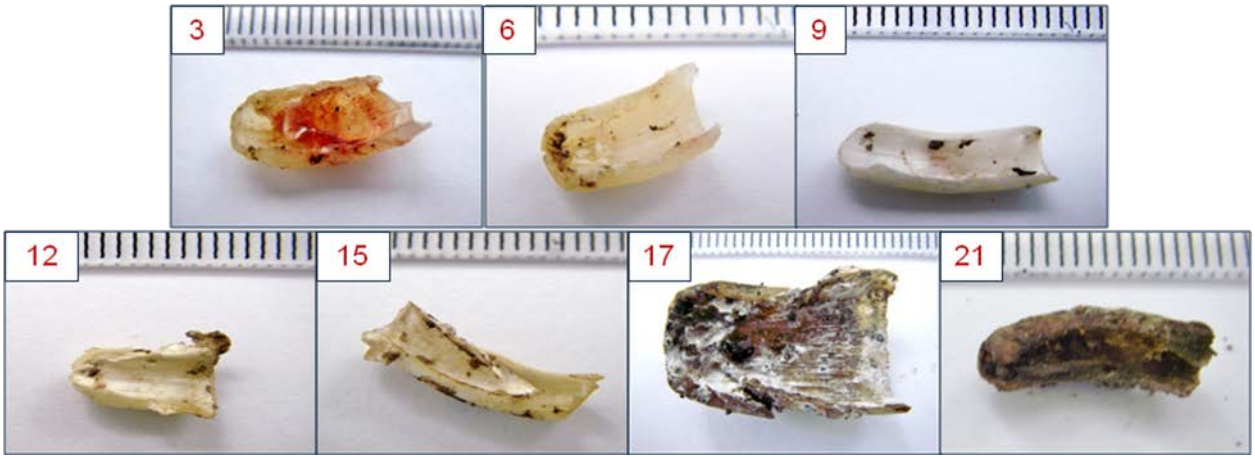


Figure 2: Cadaver 14 toenails collected from days 3-21 PMI (42-243 ADD).

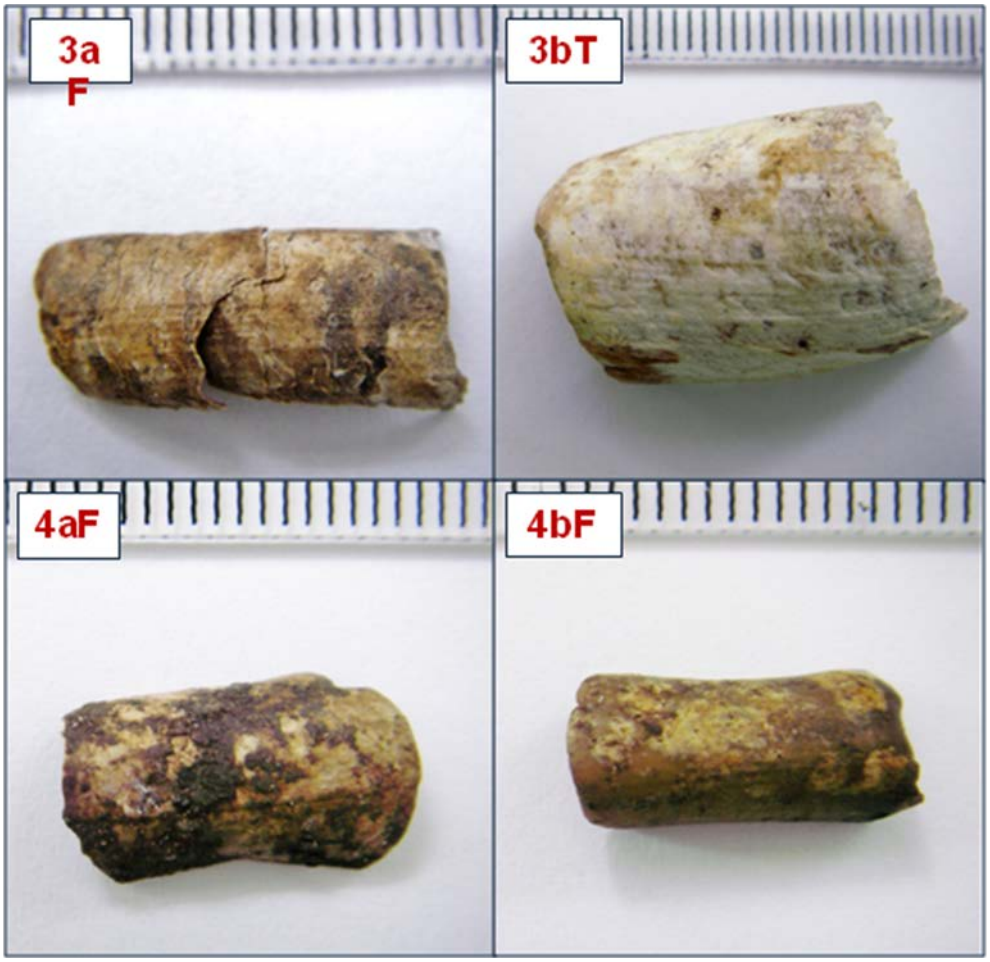


Figure 3: Cadaver nails at 189 days PMI (3aF 4237 ADD and 4aF 3964 ADD) and 196 days PMI (3bF 4313 ADD and 4bF 4040 ADD)

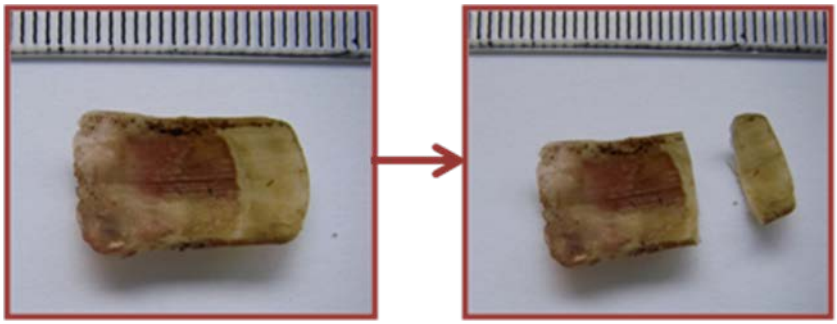


Figure 4: Cadaver nail (19 days PMI, 322 ADD) showing the proximal end (approximately 3mm) cut from the tip and used for the co-extraction of DNA and RNA. Scale shown is in millimeters.

Figure 5: Experimental set-up of environmental samples where nail clippings were placed exposed to the air, in soil and in water. Photos are shown approximately 80 days after samples were placed.

Table 2: Time periods that nail clippings were left in the environment and the corresponding accumulated degree days. Nails were placed at different times so there is more than value for each time point.

Number of days that nails were in the environment	Accumulated Degree Days nails were in the environment
2	18.25 17.4 15.25 22.1
6	64.65
12	104.95
21 22	137.3 129.1
41 41 41 42 42 42	309.25 285 309.25 305.3 276.8 286.55
82 84	596.55 644.2
119	1007.9 1034.8 1043.5

Extraction of DNA and RNA

Two kits were trialed for the co-extraction of DNA and RNA from nail material. The two Promega kits were the DNA IQ™ small sample casework kit and the DNA IQ™ hair and tissue kit. Modifications to the methods included changing the incubation time, temperature of lysis and increasing the dithiothreitol (DTT) concentration.

Two kits were also trialed for the purification of RNA, these kits were the Zymo Research Mini RNA Isolation Kit™ (Ngaio Diagnostics) and the Zymo Clean and Concentrate Kit™. Post-purification, it is necessary to convert the RNA extracted to cDNA that may be used in quantification and amplification. This was trialed using two cDNA synthesis kits, Superscript III First Strand Synthesis kit (Life Technologies) and the High Capacity cDNA Synthesis Kit (Life Technologies) following the manufacturer's instructions and using the maximum volume of RNA as stated for each kit in a 20µl cDNA reaction. Negative controls were included for each RNA sample (no reverse transcriptase). The cDNA was either used immediately or stored at –20°C until required.

Bone method

Bone material was collected (from ribs) and pulverized using the SPEX 6850 freezer mill. The pulverized bone samples were transferred to 15mL tubes where they were incubated with bone extraction buffer (Promega) followed by the DNA IQ™ small sample casework kit extraction method using an increased DTT concentration (final concentration 100 µM). RNA was extracted using the Zymo Research Mini RNA Isolation Kit™ (Ngaio Diagnostics).

DNase Treatment

The RNA fraction was treated with DNase using TURBO DNA-*free*™ (Ambion®) according to the manufacturer's instructions. The supernatant was removed and placed into a clean RNase-free tube and either used immediately or stored at –20°C until required.

Primer Design

Primers for keratin 85 and 86 (GenBank references NM_002283.3 and NM_002284.3 respectively), 18S rRNA (GenBank reference XO3205) and genomic DNA sequences for keratin 31 (GenBank reference NT_010783) and keratin 86 (GenBank reference NC_000012.11) were designed using Primer Express V3 (Applied Biosystems) and are listed in Table 3.

Quantification of Nucleic Acids

Quantification of RNA

To quantify the amount of nail RNA (both mRNA and rRNA) extracted, a real-time quantification assay was developed. To determine a suitable mRNA marker to use for a standard curve, TaqMan gene expression plates were used. Nail RNA was extracted from both fresh nails and nails placed in water for 10 days and cDNA synthesized as described above using the High Capacity RNA-to-cDNA kit. Ten microlitres of Universal TaqMan Buffer (Applied Biosystems) was added to each well with 1µl of cDNA added to each set of 32 genes. This was repeated with each cDNA sample (fresh nail, water nail and skin). The TaqMan plates were amplified using an Applied Biosystems 7500 real-time PCR machine with the following conditions; 50ºC for 10 minutes, 95ºC for 2 minutes followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 60 seconds.

Total human control RNA (Applied Biosystems) was used to synthesize cDNA (using 400ng of RNA in total with a final volume of 10ul) for generating the standard curve (for both 18S rRNA and MT-ATP6). This stock cDNA was serially diluted using sterile water by one half to give final concentrations of 4, 2, 1, 0.5, 0.25, 0.125, 0.0625 and 0.03125 ng/µl of cDNA. The cDNA quantification used two RNA transcripts; the 18S rRNA and the mitochondrial ATPase-6 (MT-ATP6) mRNA so that both mRNA and rRNA could be quantified before amplification. Primers and probes specific to these transcripts were used in the real-time PCR reaction, with 0.3µl (18S) or 1µl (MT-ATPase6) total of the primer and probe solution added to each sample and standard for the reaction. Final concentrations of probes and primers were 1µM for the 18S rRNA primers and probe and 900nM for the MT-ATPase 6 probe and 200nM for the primers. This was combined with 10µl Universal Master Mix, 1ul of either standard or sample per well and made up to 20µl with sterile water. PCR assays were run on the 7500 real-time PCR machine using the following conditions 50ºC for 10 minutes, 95ºC for 2 minutes followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 60 seconds.

Concentrations of mRNA and rRNA were determined based on the Ct value compared to the standard curve.

Quantification of DNA

The DNA concentration was measured using the Quantifiler™ system (Applied Biosystems), using an ABI 7500 real time PCR machine. A total of 1µl of DNA was used in a 12.5µl reaction, according to the manufacturer's instructions.

Amplification

Optimization of PCR Amplifications

Optimization of the PCR amplifications for the DNA, mRNA and 18S rRNA assays involved adjusting the primer and template concentrations and the annealing temperatures. Also the DNA assay for the keratin 31 locus was split into singleplex reactions due to the different optimal annealing temperatures of the forward primers.

The final primer concentrations for the assays are shown in Table 3.

Table 3: The primer sequences, final concentrations and annealing temperatures for the primers used for measuring the degradation of DNA, rRNA and mRNA in nail (and rib) samples.

Assay	Primer Sequence	Final Primer Concentration (uM)	Annealing temperature (˚C)	Size of expected amplicon (bp)
KRT85 mRNA				
forward 1	aggaggtgctggagggcta	0.03125		128
forward 2	tggaggccctggtggag	0.03125	58	
reverse	FAM-gccgagacctgaacatggac	0.03125	58	264
KRT86 mRNA				
forward	HEX-agggagtaccaggaggtgatgaa	0.0625		183
reverse 1	caacaggggcagtagtggagg	0.0625	58	340
reverse 2	tgacggcgacaggcg	0.125	58	
18S rRNA				
forward	HEX-cgttcttagttggtggagcgat	0.03125		
reverse 1	gccacccgagattgagca	0.03125	58	146
reverse 2	ccagtaagtgcgggtcataagc	0.0625	58	326
DNA keratin 86				
forward 1	ggggtctgtctggctggg	0.0625	58	130
Forward 2	atcacacggggctcttgg	0.0625	58	294
Forward 3	gggaagctattgggagggaa	0.0625	58	450
reverse	HEX - accacagtcctctcatccgg	0.0625		
DNA keratin 31*				
forward 1	ttgcacagggtcagctcatc	0.0625	68	500
forward 2	actttctgtaatgaataggccc	0.0625	58	320
reverse	FAM- ccaagtctgagaatgccagg	0.0625		

*Run as singleplex PCR assays due to different optimized annealing temperatures.

The final optimized concentrations of nucleic acids for the PCR assays are: 3pg for the mRNA assays, 5pg for the 18S rRNA assay and 0.5 ng DNA for the DNA PCR assays.

Final Amplifications

The PCR assay for each nucleic acid (mRNA, rRNA and DNA) contained 2.5µl of the respective primer combinations, 12.5µl Qiagen Master Mix, 3 or 5pg for the mRNA and rRNA assays respectively and 0.5ng DNA with the volume made up to 25µl with sterile water. Amplification conditions were 15 minutes at 95°C followed by 35 cycles of 95°C for 90 seconds, 58°C for 3 minutes and 72°C for 90 seconds and a final step of 72°C for 45 minutes. For the D31F1 amplification, the annealing temperature was changed to 68°C but all other conditions were as described above.

Amplification products were run on 3130 genetic analyzers (Applied Biosystems) using GS-500 size standards and analysed using GeneMapperID V3.2 (Applied Biosystems).

Statistical Analysis

Statistical interpretation using a multiple regression approach was used to derive the relationships between the age of the samples, the relative peak heights and the relationships within and between individuals including any differences between fingernails and toenails. Based on this information, equation(s) will be derived that will be used to estimate the time since death based on the relative rates of decay of RNA and DNA.

III. Results

Nail Tissue

Lysis of nail material is difficult due to the keratins that make up the nail material. Keratins are resistant to pepsin or trypsin protease digestion as well as being insoluble to weak acids, alkalines, water, organic solvents and aqueous salt solutions [21]. Keratins are soluble in strong denaturing agents such as urea, reducing agents such as dithiothreitol and specialized proteinases such as proteinase K.

Due to the nature of nail material (and bone and teeth), the co-extraction method we had previously developed for body fluids (blood, semen and saliva) needed to be slightly modified. The lysis buffer step was increased to 40 minutes (from the recommended 30 minutes), with 15 µl DTT (0.4M) added per sample at 0 and 20 minutes of the incubation, where the traditional protocol calls for only 2.5µl DTT (0.4M) per sample added at the start of the incubation. The nail material became gelatinous after the lysis step and would break into small pieces that interfered with the magnetic resin. Using a spin basket promoted the fractionation of the gelatinous nail remnants and so was not used to separate the waste nail material, but instead the supernatant was removed from the solution. This was possible without the transference of any contaminant nail waste with careful action and minimal agitation of the gelatinous fragments.

The Mini RNA Isolation Kit™ (Ngaio Diagnostics) was chosen for use over the Zymo Clean and Concentrate Kit™ as it contained a wider spin filter than the Clean and Concentrate Kit, whose narrow filter tended to become blocked by the presence of any remnant nail fractions.

The average weight of nail clippings was 0.01g and the average concentration of DNA extracted was 1.5ng/µl. With the cDNA quantification method, the average concentration from nail clippings was 2.6pg/µl for mRNA and slightly higher for 18S rRNA.

mRNA PCR Assays

Based on the morphology and biochemical properties of nails, keratins were chosen as the mRNA transcripts for use in our assay. There is a family of keratin proteins that are specific to hair and nails, and these were chosen for use as the mRNA transcripts. These mRNA transcripts are not present in skin (results not shown) so that the mRNA result are from the nail RNA material in question. Four keratins were initially chosen for use, based on their specificity to the nail plate, namely keratins 31, 34, 85 and 86. Primers were designed for each keratin, to amplify target sequences ranging from approximately 120-500bp in length. Initially the larger amplicons were designed to be around 250bp, but as a result of the stability of the RNA, further primers were designed to amplify larger amplicons. Keratins 31 and 34 were dropped for the mRNA assays during the optimization work due to difficulties with optimizing the primer concentrations and annealing temperatures.

The mRNA degradation from nail clippings placed in the three different environments is evident by the gradual decrease in peak height or the absence of the larger amplicons compared to the shorter amplicons. Figure 6 shows the results of the amplification of keratin 86 mRNA from nails exposed to the three different environmental conditions after 41 days (309.25 ADD). As can be seen in this figure, the larger amplicon (264bp) has a lower peak height from samples placed in soil or water indicating that these environments degrade the mRNA at a faster rate than samples left in the air. This is not unexpected as these types of environments are harsh and will have a greater influence on tissues and nucleic acids. We

did not know how much of an effect environmental factors would have on mRNA in nails. From these results, while mRNA is stable in nails, degradation does occur when nails are in different environments. While degradation did occur, the fact that mRNA could be isolated and amplified from nails submerged in water and placed in soil for over 120 days (ADD over 1007) indicates that nucleic acids in nails are stable over time.

It should be noted that not all nails produced mRNA results. While most fingernail samples gave results, the lack of mRNA results could be a result of degradation or not enough amplifiable mRNA extracted. There are variables in the extraction of the RNA where RNA may be lost. In addition, only small pieces of nails were used, and results may be obtained by using a larger nail sample. However, larger nail sizes are more difficult to lyse and so further refinement of the co-extraction method may be necessary such as performing the lysis and extraction in larger volumes of buffer.

Similarly to fingernails, not all toenails gave mRNA results. Toenails were at time 0 and at 40 days (ADD ranging from 305-840) from the three different environments. No toenails produced results for the longer keratin 86 mRNA fragment (340bp) but all toenails placed in soil for 40 days (ADD 305-309) amplified the keratin 85 long mRNA fragment (264bp). Nail samples placed in water, as expected, did not have the keratin 86 long fragment amplified and two out of the four toenails amplified the keratin 85 long fragment. Toenails were a small sample set but based on these environmental results there was little difference between fingernail and toenail samples.

To investigate the stability of mRNA in nails further, cadaver nails with different PMIs (Table 1) were amplified with the keratin mRNA primers (Figure 7). From this figure, the

large amplicon from keratin 85 mRNA (264bp) has only just been detected in the sample with an ADD of 305 whereas it is absent in the sample with an ADD of 5699. The absence of this peak indicates that the mRNA has degraded to a point where this size amplicon is no longer amplifiable. The pattern of this degradation was evaluated statistically (described further on).

The results from the cadaver nails are extremely promising as mRNA was able to be amplified from cadaver nails with PMIs of over 380 days (over 5600 ADD). No mRNA results were obtained from a cadaver nail with a PMI of 1310 days (20925 ADD), where this sample had been buried for quite some time.

No mRNA results were obtained from the rib samples. This could be due to the low level of RNA contained in bone samples, or that the method for co-extracting the RNA from bone samples needs further refining. We did not have many rib samples and so optimization of the co-extraction method was not possible.

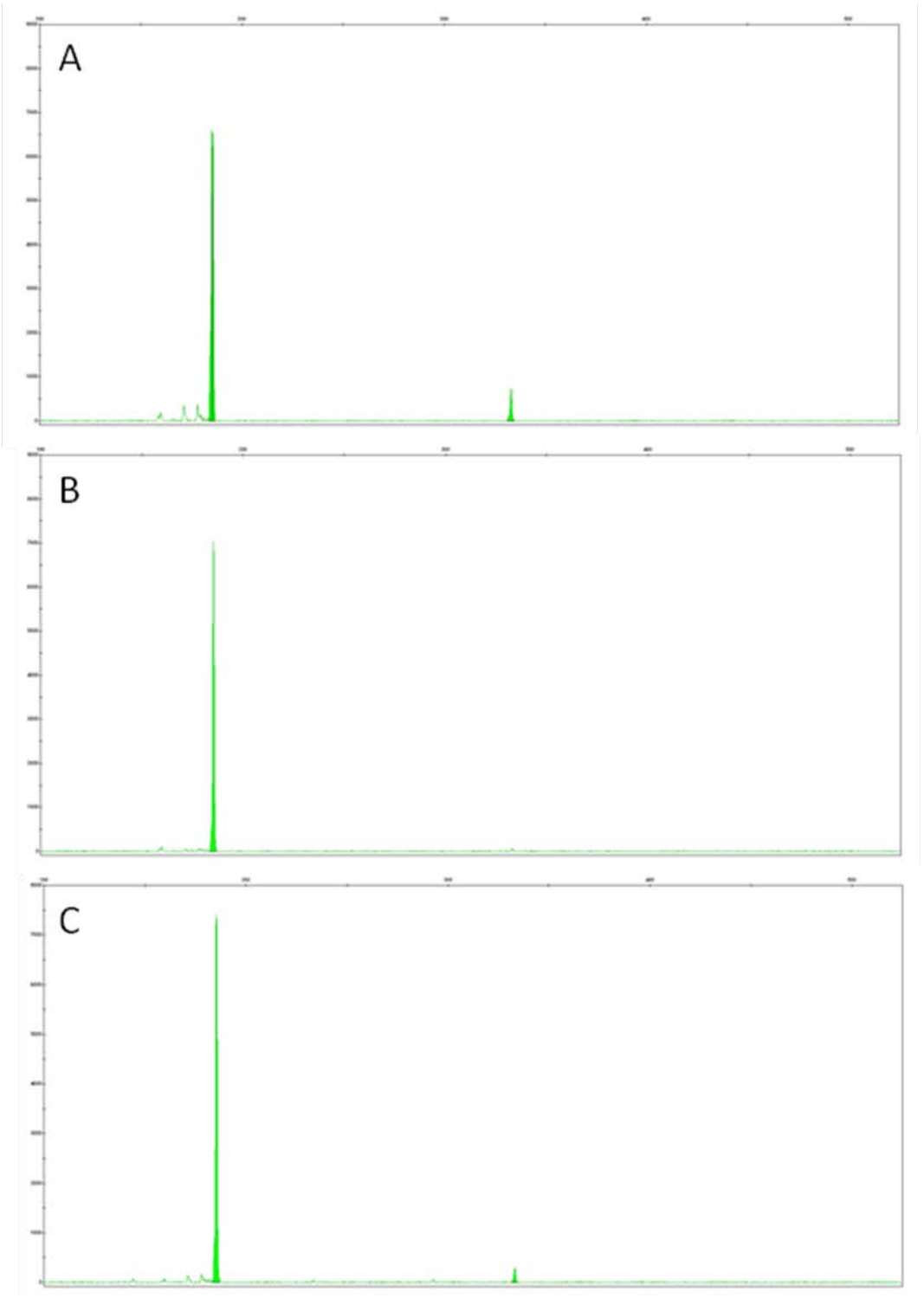


Figure 6: Electropherograms of the keratin 86 mRNA transcript amplified from nail clippings left in soil, in water and from human cadaver nail samples with similar ADD values. Amplicon sizes are 183 and 340bp. A: Nail sample in soil, 309 ADD; B: Nail

sample submerged in water, 309 ADD and C: Nail sample from human cadaver, 305 ADD. Scale is 8000 rfu.

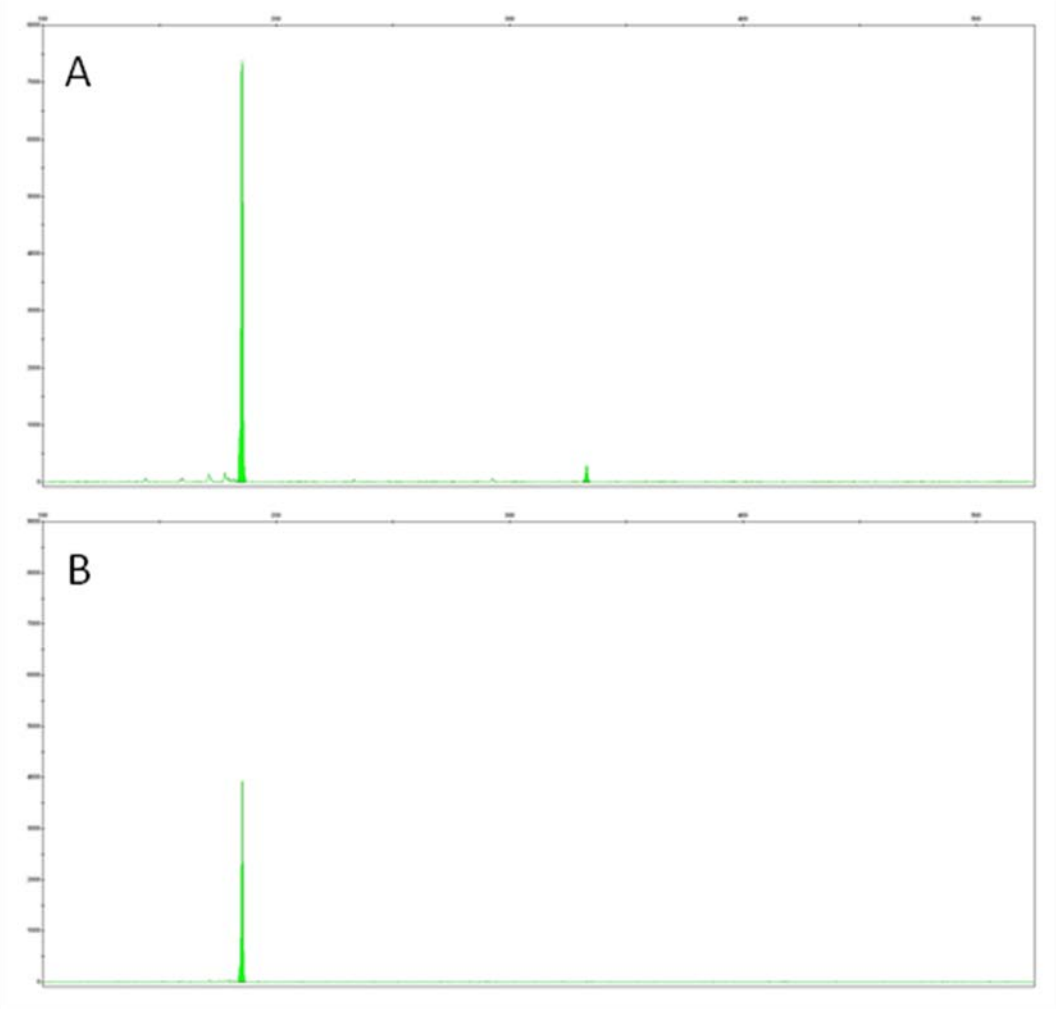


Figure 7: Electropherograms of the keratin 86 mRNA transcript amplified from two human cadavers with different post-mortem intervals. Amplicon sizes are 183 and 340bp. A: 305 ADD and B: 5699 ADD. Scale is at 8000 rfu.

18S rRNA Assays

Primers for amplifying the 18S rRNA were designed for two transcripts of 146 and 326bp in length (Fig. 8). The 18S rRNA was used as it is a different type of nucleic acid compared to mRNA and it is present in all cells and may degrade at a different rate than the keratin mRNA transcripts.

Figure 8 shows the results of amplifying the 18S rRNA from nail samples submerged in water for different periods of time (1 to 119 days, or from 17 to 1043 ADD). As can be seen in this figure, the larger of the amplicons has a much smaller peak height with the sample with the longest ADD indicating some degradation has occurred. There is little difference in the peak height of the larger amplicon between the samples with ADD values of 17 and 287.

Figure 9 shows the results of the amplification of the 18S rRNA from different human cadavers with different PMIs (ranging from 17 to 389 days or 305 to 5699 ADD). As can be seen in this figure, the two 18S rRNA amplicons were amplified from the cadaver nails although the sample with the longer PMI had a small 18s rRNA at 326bp. This would indicate that the 18s rRNA is stable and can still be detected over longer time periods in cadaver nail samples.

What was most interesting was the result from the cadaver with the longest PMI (1310 days or 20925 ADD) as this still had both amplicons present, although at lower rfu values (Figure 10). This cadaver was on the surface before being buried in soil for quite some time so comparing this sample with our longest environmental soil sample shows that the 18S rRNA is stable over long periods of time. Both the amplicons for the 18S rRNA were obtained from all nail samples submerged in water (results not shown). These results indicate that the 18S rRNA is stable in nails over long PMIs and that rRNA is more stable than mRNA in nails (which was expected).

The rib samples gave small 18S rRNA peaks (below 66 rfu) with the smaller amplicon (146bp) with no amplicon detected with the larger fragment (326bp) (results not shown). There also was no 18S rRNA detected in the cDNA quantitation assay.

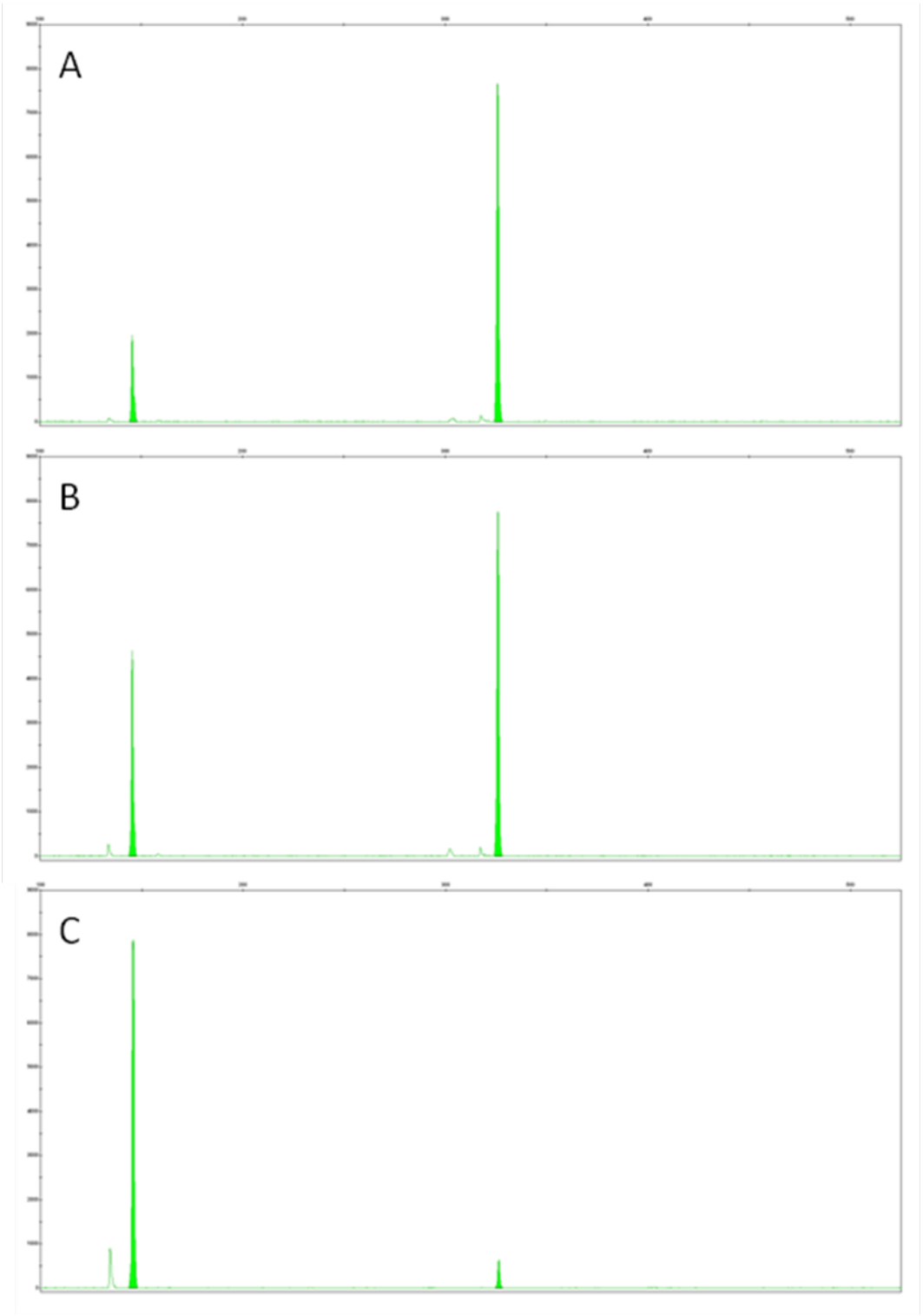


Figure 8: Electropherograms of the 18S rRNA amplification from nail samples submerged in water for different periods of time. The amplicon sizes are 146 and 326bp. A: 17 ADD; B: 287 ADD and C: 1043 ADD. Scale is 8000 rfu.

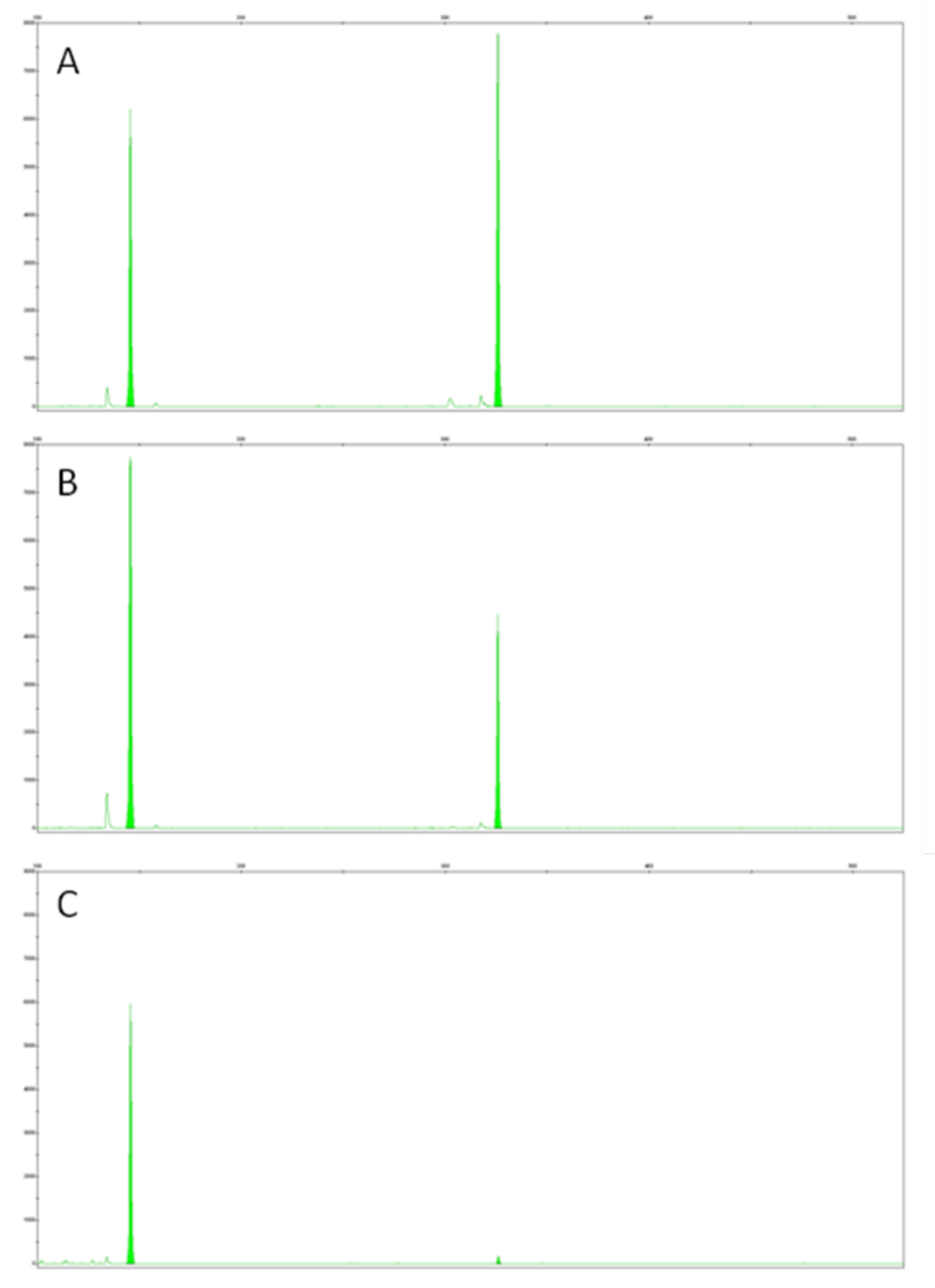


Figure 9: Electropherograms of the 18S rRNA amplification from different human cadavers with different post-mortem intervals. The amplicon sizes are 146 and 326bp. A: 17 days (305 ADD); B: 48 days (1164 ADD) and C: 389 days (5699 ADD). The scale is at 8000 rfu.

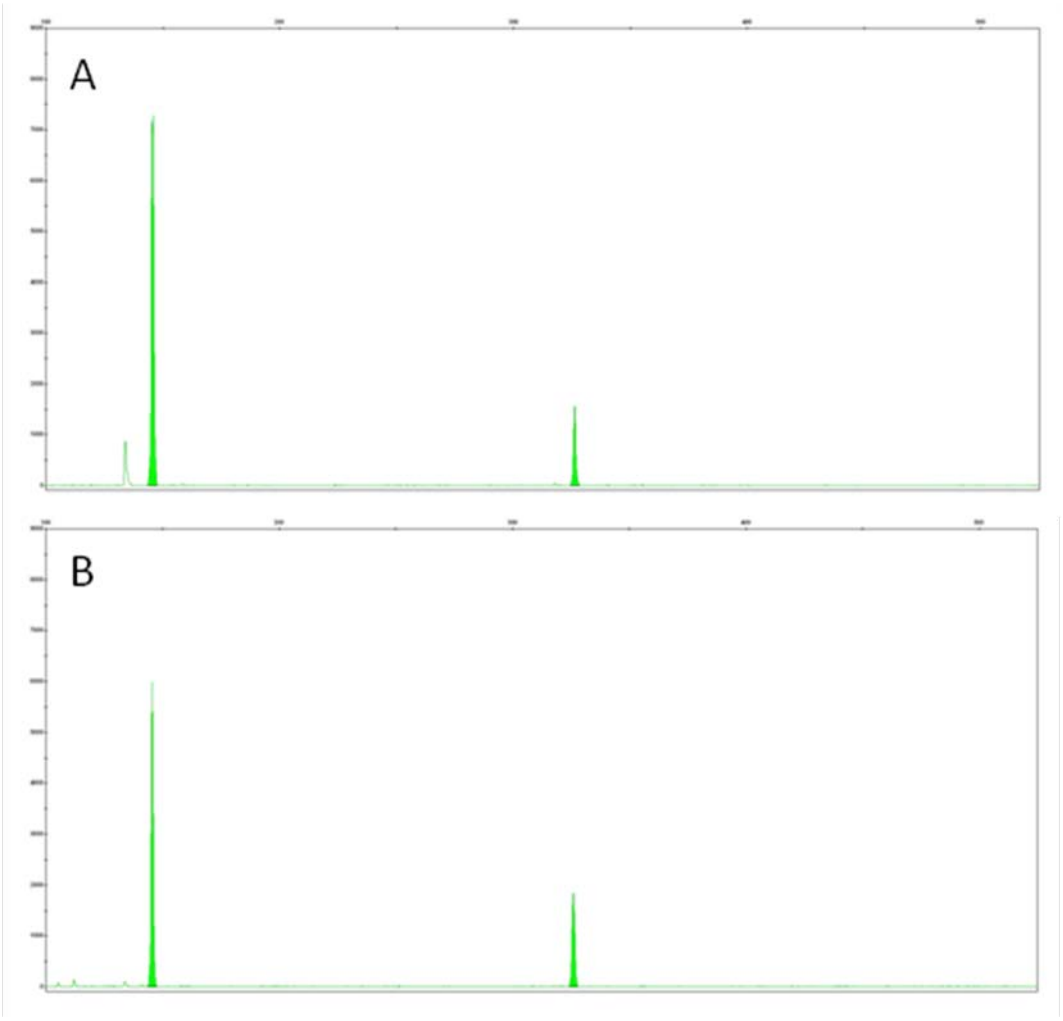


Figure 10: Electropherograms of the 18S rRNA amplified from two nail sources showing the stability of the 18S rRNA. A: Nail placed in soil for 120 days (1034 ADD) and B: Cadaver nail with a PMI of 1310 days (20925 ADD). Scale is 8000 rfu.

DNA Assays

Genomic DNA is expected to be more stable than RNA and DNA was amplified using the keratin 31 and 86 genomic sequences (Fig. 11).

An example of the time-wise degradation of DNA from nails is shown in Figure 11. In this figure (nail samples in soil with increasing time periods) the largest amplicon (450bp) displays a decreasing peak height Similarly in Figures 12 and 13 with the DNA amplified

from nails from the same cadaver taken at different PMI periods, both keratin DNA loci show a decrease in peak height over time with the larger amplicons.

There were mixed results when comparing fingernail and toenail DNA degradation patterns. Taking fingernails and toenails from the same cadaver at the same time point did not show any difference in DNA degradation (Figure 14). However, in other cases there were differences (Figure 15) where the DNA from ribs were also compared to the fingernail and toenails. This result could indicate that there are differences depending on what nail (i.e. thumb or small finger) is used. For example the small finger or toe nail appeared to degrade quicker in the environment while the thumb and big toe nails were more resilient to environmental factors. However, in some cases the toe nails were very hard and difficult to cut and lyse which may have affected the efficiency of the DNA and RNA co-extraction.

All nucleic acids (DNA, rRNA and mRNA) were successfully extracted and amplified from children's nails that had been exposed to air for 20 days (137 ADD, Fig. 16). There were not enough samples for any statistical analysis of nails from children compared to adult nails.

Modifying the DNA IQ™ method has resulted in the successful co-extraction of DNA and RNA from nail clippings. This method has been validated and implemented into operational casework at ESR where nails have been used for the identification of individuals. This work has demonstrated that nails are a good source of nucleic acids for forensic casework.

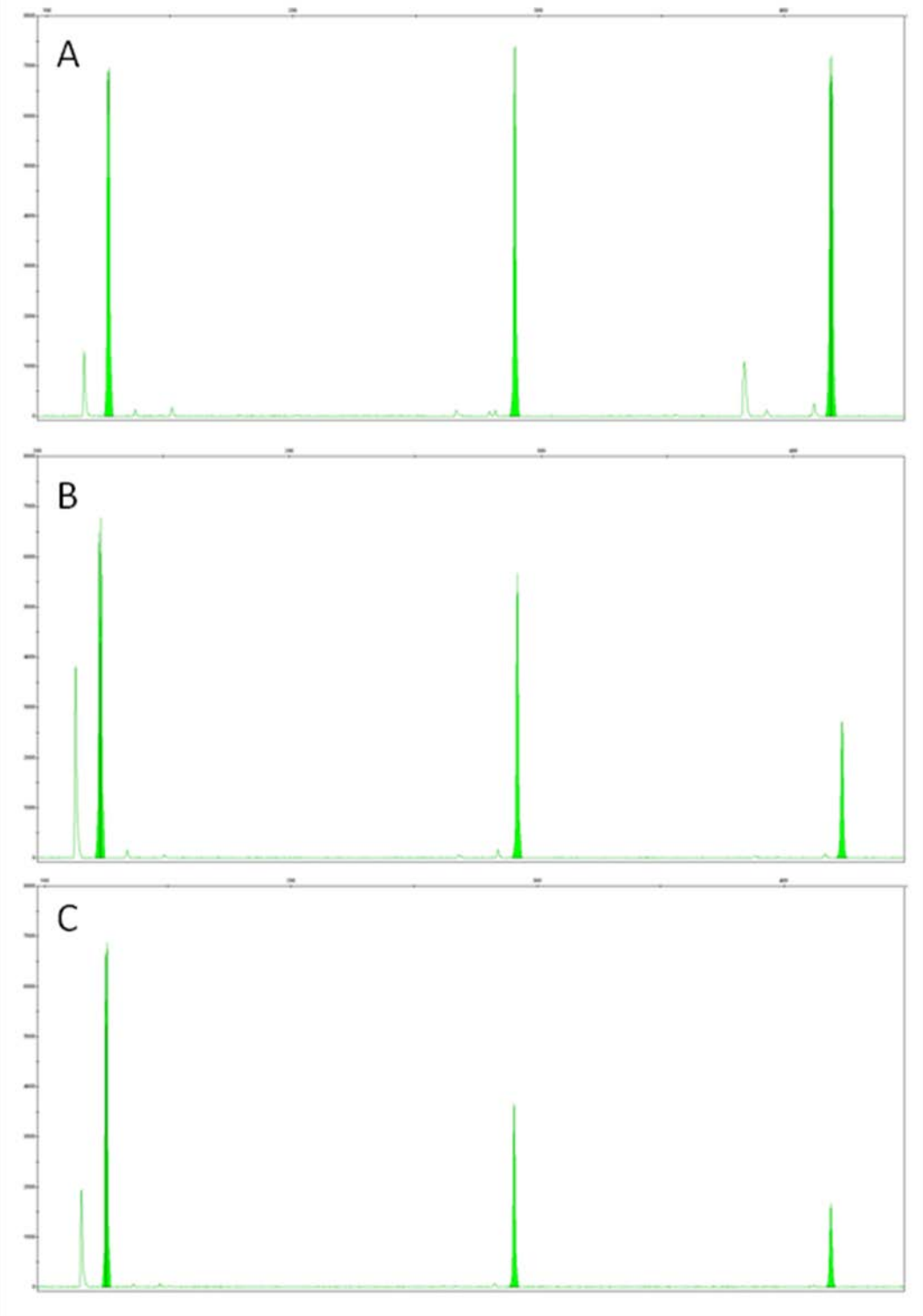


Figure 11: Electropherograms from environmental samples amplified using the DNA keratin 86 primers. Nails were placed in soil for A: 1 days (22 ADD); B 40 days (276 ADD) and C: 120 days (1043 ADD). The amplicon sizes are 130, 294 and 450bp. The scale is 8000 rfu.

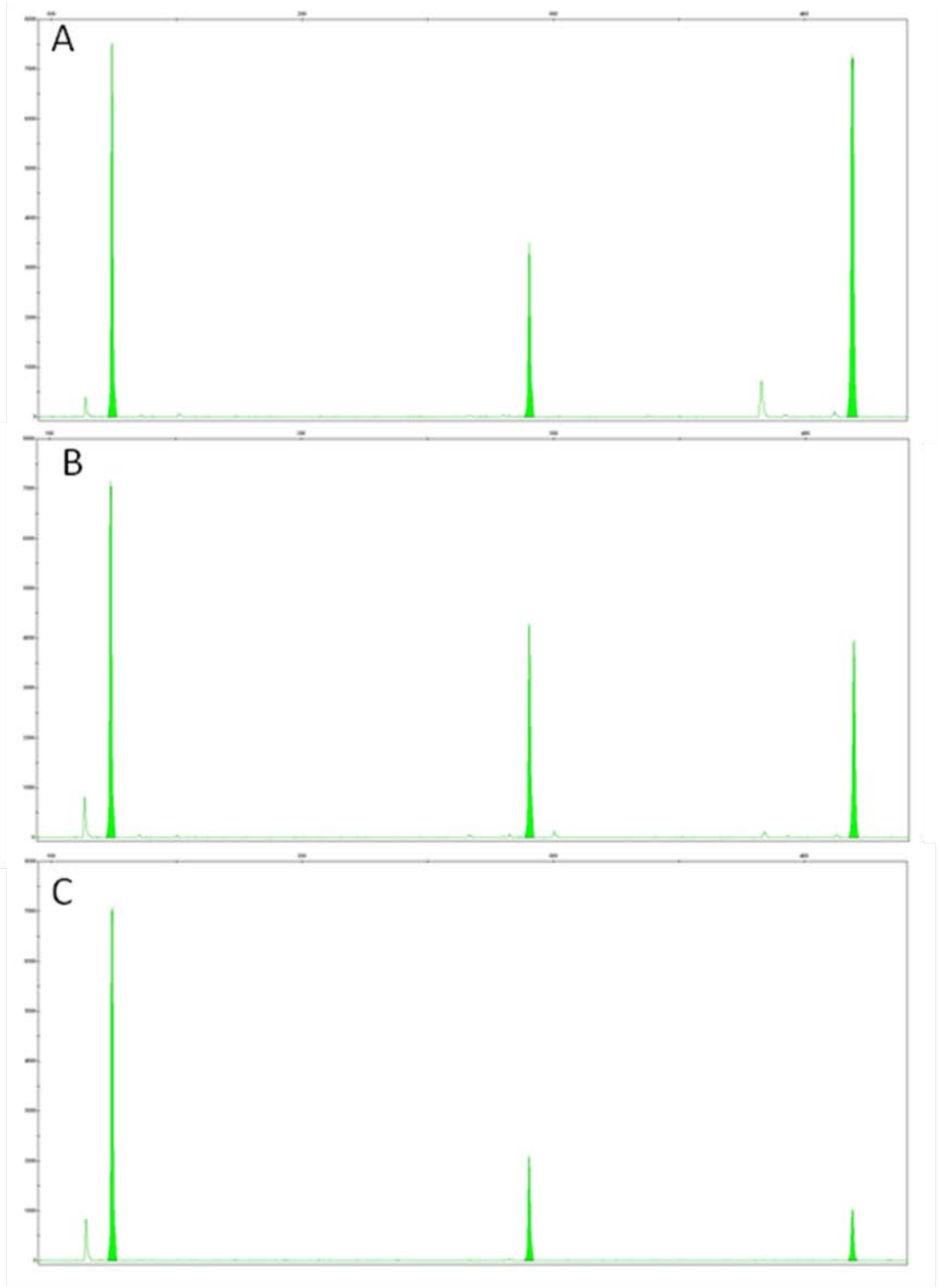


Figure 12: Electropherograms showing the DNA keratin 86 loci being amplified from nails collected from the same cadaver over a period of time. The amplicon sizes are 130, 294 and 450 bp. A: 54 days (362 ADD); B: 60 days (445 ADD) and C: 66 days (497 ADD). Scale is at 8000 rfu.

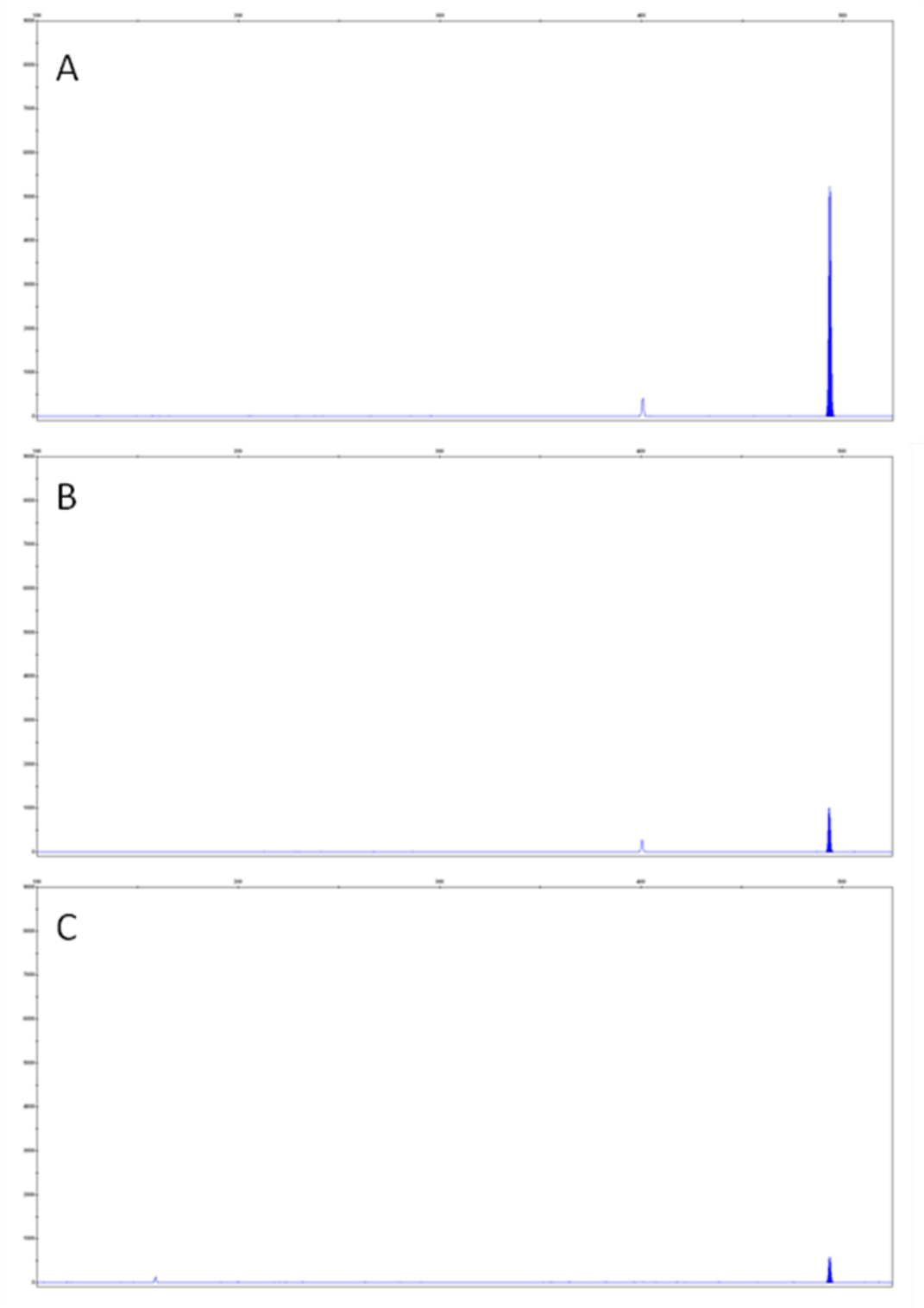


Figure 13: Electropherograms showing the DNA keratin 31 locus being amplified from cadaver nails from the same cadaver with ADD values of A: 362, B: 445 and C: 497. Scale is at 8000 rfu. The peak is at 500 bp.

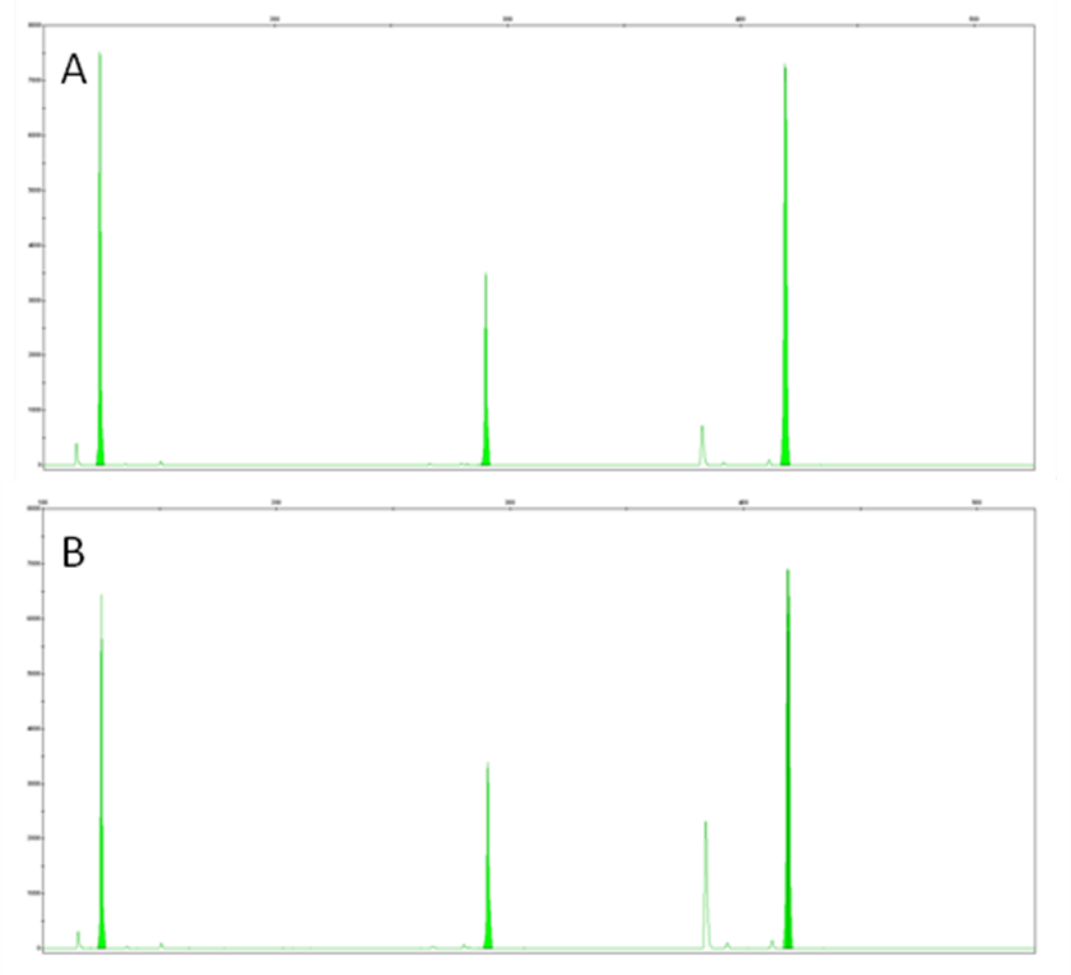


Figure 14: Electropherograms of the DNA keratin 86 locus amplified from a finger and toe nail from the same cadaver at the same time with a PMI of 17 days (305 ADD). The amplicon sizes are 130, 294 and 450 bp. A: Fingernail B: Toenail. Scale is 8000 rfu.

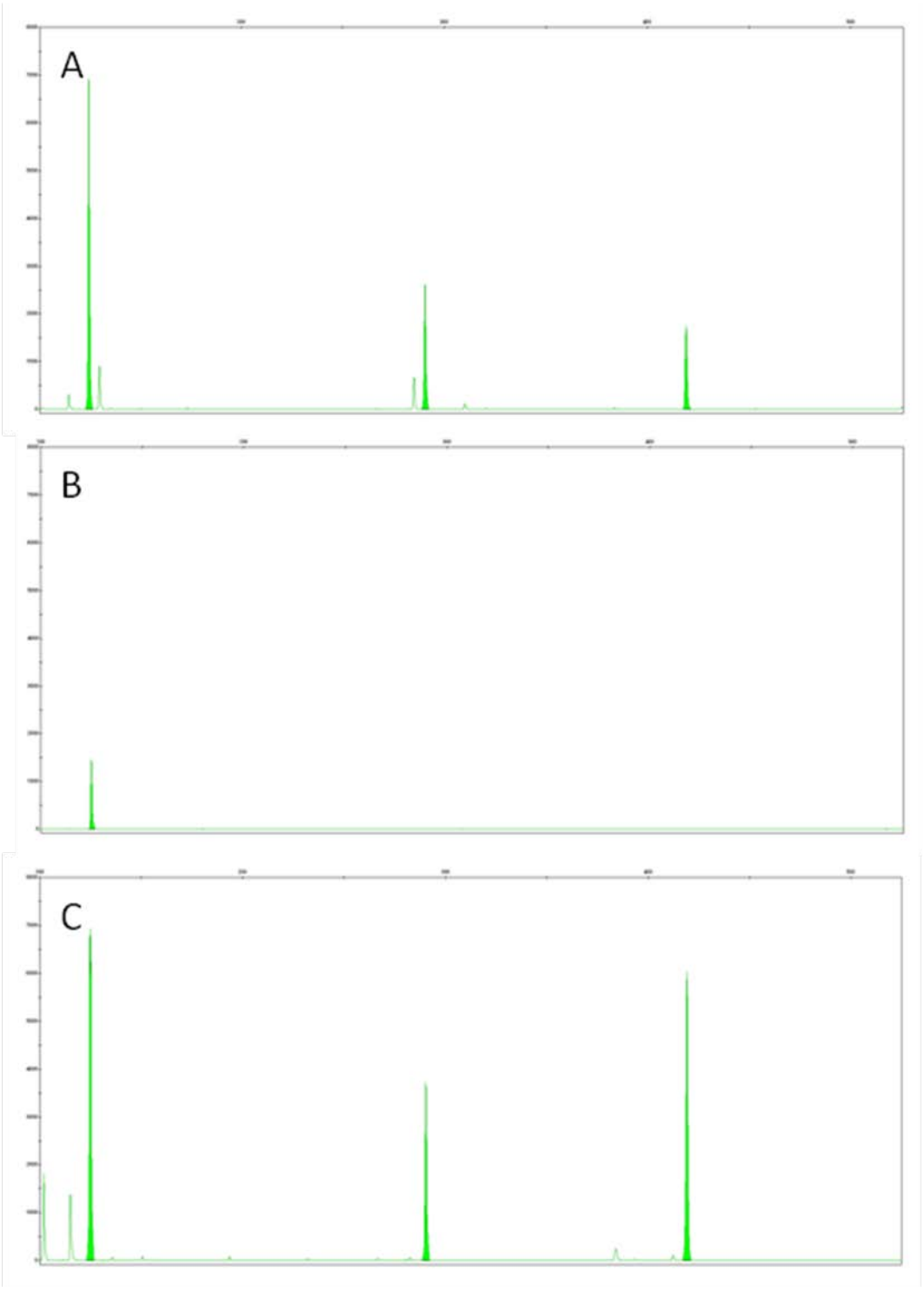


Figure 15: Electropherograms showing the DNA keratin 86 locus amplified from a rib, fingernail and toenail collected from the same cadaver within a similar timeframe. Amplicon sizes are 130, 294 and 450 bp. A: rib sample (189 days, ADD 4237); B: Fingernail (189 days, ADD 4237) and C: Toenail (196 days, ADD 4313). Scale is 8000 rfu.

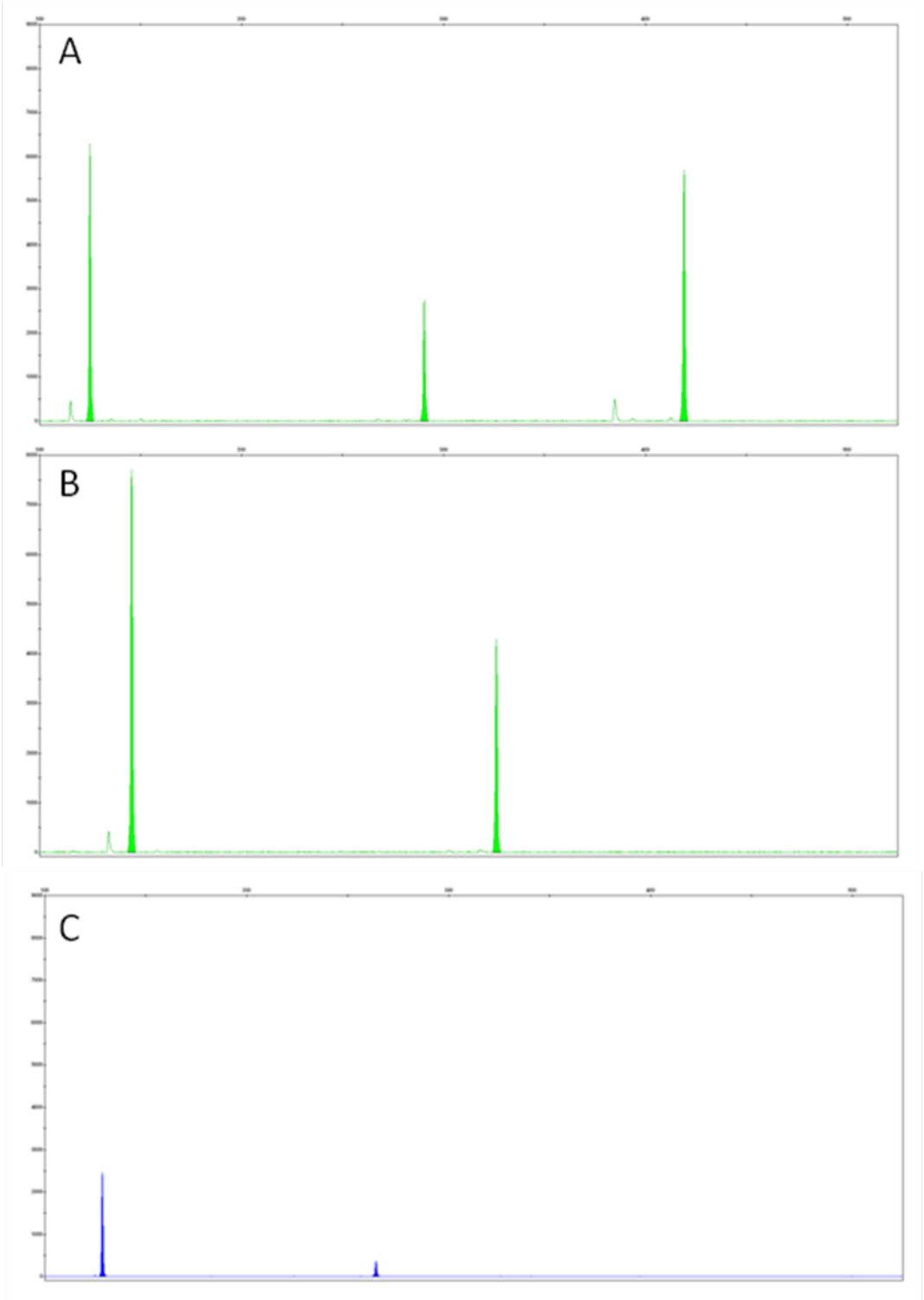


Figure 16: Electropherograms of DNA and RNA amplified from a child's nail samples. These samples were left exposed to air for 20 days (137 ADD). A: Keratin 86 DNA, amplicons at 130, 294 and 450bp; B: 18S rRNA, amplicons at 146 and 326bp and C: Keratin 85 mRNA, amplicons at 126 and 264bp. Scale is 8000 rfu.

Other Tissues

Lysis of muscle tissue using the DNA IQ™ small sample casework kit resulted in low levels of DNA and RNA being detected. For example, using the real-time 18S rRNA kit for determining the concentration of rRNA, results from muscle tissue ranged from 0.1-1.0 pg/µl. This concentration of RNA was too low to perform accurate PCR assays for short-term degradation studies as the absence or low levels of peaks detected in the assays could not be confirmed as being the result of degradation or because there was not enough starting material to be properly amplified. Due to these results, muscle tissue was not used in any further studies.

Objective three of this project was to use muscle samples to test and refine degradation assays. Based on these results, the time intervals that this project is focusing on, and the success of the DNA and RNA co-extraction from nails, it was decided that muscle tissue was not suitable for these degradation studies and was not pursued. Therefore, objective three was not completed using muscle tissue, but was completed by replacing muscle tissue with nail samples to test and refine the PCR degradation assays.

Samples of bone material are being used to develop a method for co-extracting the DNA and RNA from bone samples. Presently DNA is recovered by using a freezer mill to pulverize the bone samples, followed by nucleic acid extraction using the Promega bone extraction buffer and the DNA IQ™ method. Currently there are difficulties with recovering the RNA from the bone material using both the Zymo Research Mini RNA Isolation Kit™ and the Zymo Clean and Concentrate Kit™. These kits do not appear to be able to recover sufficient levels of RNA extracted from bone samples. Other methods for recovering the RNA are being investigated including an ethanol precipitation method. While the RNA co-extraction

method is being developed, the DNA PCR degradation assays are being optimized. Collection of rib samples from human cadavers is currently underway. These samples will be used to measure the rate of degradation of the nucleic acids. As DNA can be extracted from rib samples, the PCR degradation assays for DNA will be used to measure the rate of degradation and to determine if we can statistically correlate the rate of DNA degradation to the PMI. Further work is required to develop the RNA co-extraction method, and RNA PCR degradation assays will be performed once this method has been developed and optimized.

Statistical Analysis

The data collected in this project consisted of measuring DNA and RNA degradation through amplifying different sized fragments from nails exposed to a variety of environmental conditions (exposed to air, buried in soil and submerged in water) for different periods of time. The amplification products were measured through peak heights in relative florescent units (rfu).

Plotting the data of peak height against the log of accumulated degree days of each PCR amplicon exposed to the environment provides initial information as to whether that amplicon (fragment) is stable (not degraded) throughout the time course of the experiment. An example of this is shown in Figure 17, where the smallest fragment from the keratin DNA 86 assay (130 bp) is plotted against time and separated into the three different environmental conditions. Both the air and soil graphs (Figure 17: A and B) show that this amplified fragment is stable in these conditions, which is evident by the relatively straight line of the data. While there are some outliers, the general trend can be seen. These results mean that this PCR fragment will not be useful for measuring degradation as it was not degraded in our study.

In contrast, plotting the three keratin 86 DNA fragments amplified from nails buried in soil against the log of the accumulated degree days show different trends (Fig. 18). The largest DNA fragment (450 bp) has a downward trend, indicating that this fragment is unstable (degraded) over time. The medium fragment data is quite scattered with a slight downward trend. The data from the smallest fragment is in a relative straight line indicating that it is stable (not degraded) in these conditions over the time course studied and therefore would not be of much use. The result for the keratin 86 DNA large fragment is promising for looking at a relationship between DNA degradation and PMI.

To further explore these potential trends, linear regression was carried out with ADD and peak height (rfu) for the keratin 86 long and medium fragments in the separate environments (Tables 4 and 5).

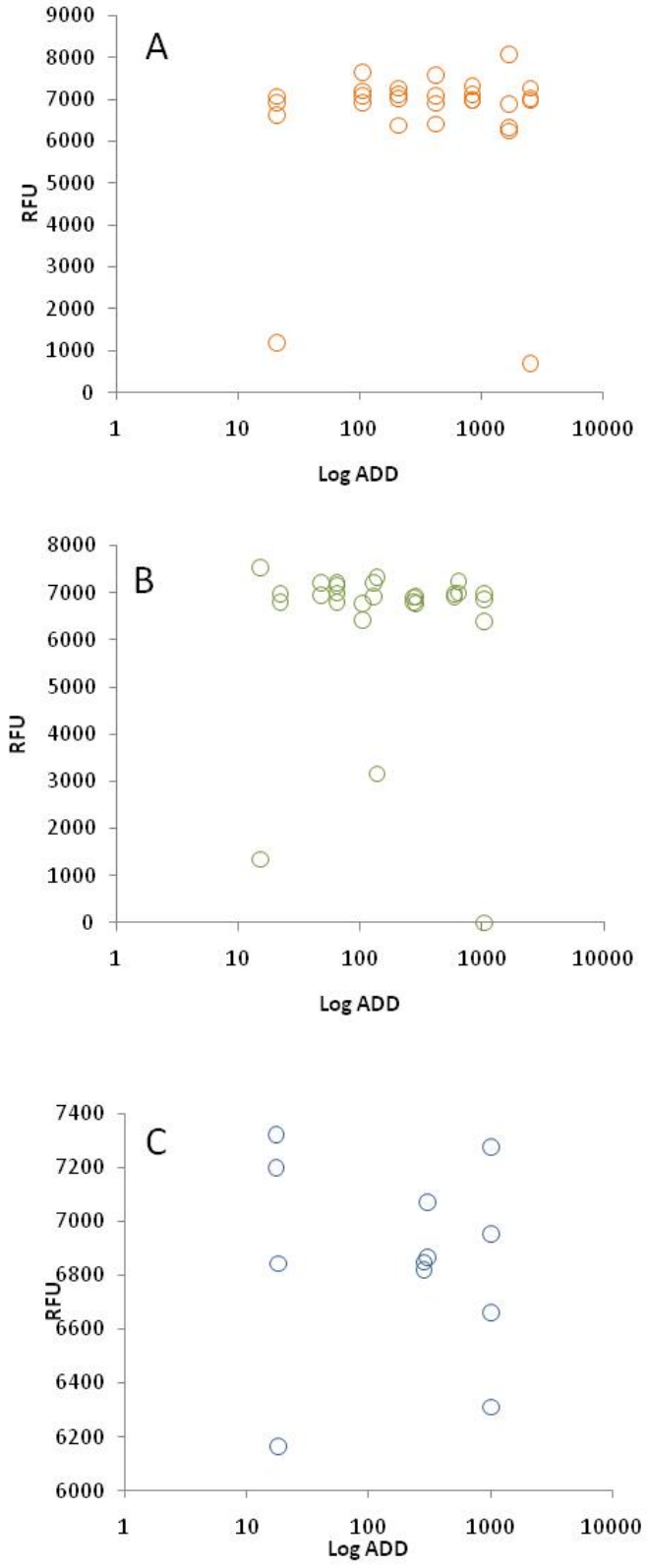
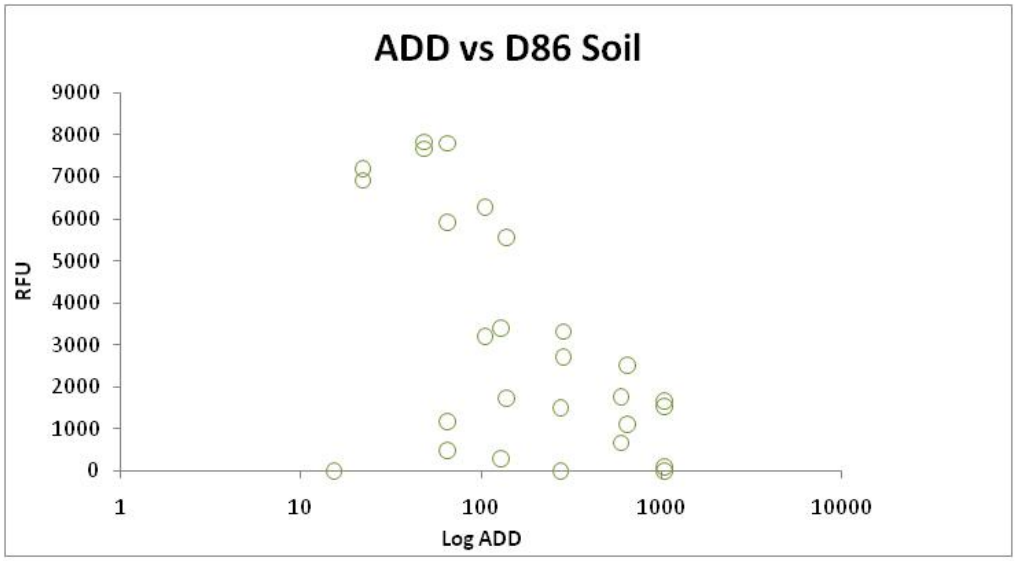
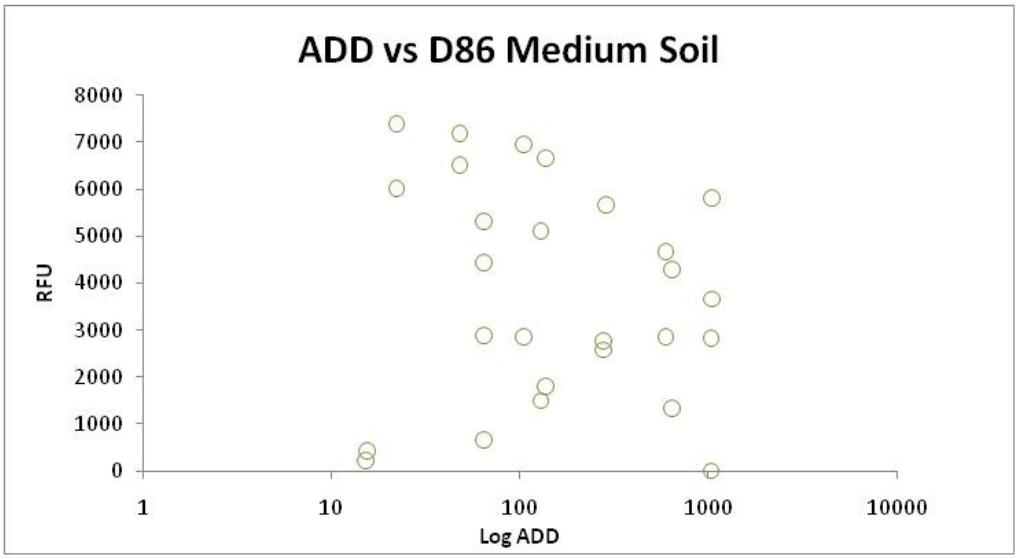


Figure 17: Graphs of the log of accumulated degree days vs peak height for the keratin DNA 86 small fragment (130 bp) from nails placed in different environments. A: Air; B: Soil and C: Water.





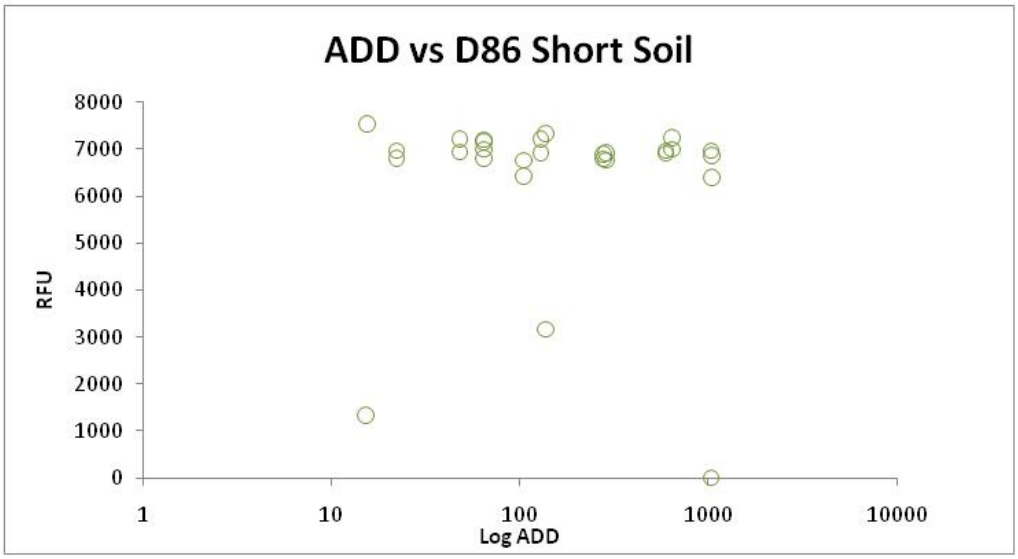


Figure 18: Graphs of the log of accumulated degree days (ADD) and peak height (rfu) of keratin 86 DNA fragments amplified from nails buried in soil for different periods of time. A: Long fragment (450 bp); B: Medium fragment (294 bp) and C: Short fragment (130 bp).

Table 4: Regression statistics for the keratin 86 DNA long and medium fragments amplified from nails buried in soil over different time periods.

Regression Statistics	Keratin 86 DNA long fragment	Keratin 86 medium long fragment
Multiple R	0.46399918	0.18036577
R^2	0.21529524	0.03253181
Adjusted R^2	0.18511429	-0.0046785
Standard Error	322.308742	357.879885
Observations	28	28

The R^2 value for the keratin 86 DNA long and medium fragments suggests that only approximately 21% and 3% respectively of the variation is accounted for by the accumulated degree days.

Table 5: Regression statistics for the keratin 86 DNA long and medium fragments amplified from nails buried in soil over different time periods.

	Keratin 86 DNA long fragment		Keratin 86 medium long fragment	
	Intercept	X Variable 1	Intercept	X Variable 1
Coefficients	495.065409	-0.0598059	428.033296	-0.0281949
Standard Error	89.70725	0.022392	134.6489	0.030154
t-Stat	5.518678	-2.67086	3.178884	-0.93502
P-value	8.6E-06	0.012876	0.003796	0.35838

The P-value for the X variable is <0.05 (0.012), suggesting that for the keratin 86 DNA long fragment buried in soil there is a significant relationship between ADD and peak height (rfu), with the rfu decreasing as ADD increases (shown by the negative coefficient value for the x

variable). For the keratin 86 DNA medium fragment, the p-value is high, therefore no significant relationship has been observed.

From initial ANOVA analyses, there is evidence that both days and environment on the DNA and RNA fragments. Tables 6 and 7 show the ANOVA results for fragments that are susceptible to time only or are susceptible to both time and environment respectively. ANOVA also confirms that keratin DNA 86 short fragment is not susceptible to time or the environment (it is stable). Further analysis is required to investigate whether accumulated degree days or days (without taking into account the temperature) gives a similar response.

Table 6: ANOVA analysis of amplified DNA and RNA fragments that are susceptible to time in the environment.

	Keratin DNA 86 long fragment					Keratin 86 mRNA short fragment			
	Df	Sum Sq.	Mean Sq.	F value	Pr (>F)	Sum Sq.	Mean Sq.	F value	Pr (>F)
Days	1	71442560	71442560	15.5608	0.000265	62740983	62740983	7.7028	0.007889
Env.	2	15480242	77401210	16.8587	024e-06	8349293	4174647	0.5125	0.602290
Donor	15	12454295	8302863	1.8084	0.062016	11269334 0	7512889	0.9224 0	546576
Days Env	2	6846049	3423025	0.7456	0.479993	25524452	12762226	1.5668	0.219410
Residuals	47	21578541	4591179			38282619	8145238		

Table 7: ANOVA analysis of amplified DNA and RNA fragments that are susceptible to the environment.

	Keratin DNA 31 long fragment					Keratin 86 mRNA long fragment			
	Df	Sum Sq.	Mean Sq.	F value	Pr (>F)	Sum Sq.	Mean Sq.	F value	Pr (>F)
Days	1	1033316	1033316	0.3379	0.5638128	168530	168530	1.7453	0.1929
Env.	2	60642344	30321172	9.9156	0.0002554	597769	298885	3.0952	0.0546
Donor	15	71481901	4765460	1.5584	0.1233410	967807	64520	0.6682	0.8013
Days Env	2	5929365	2964682	0.9695	0.3867237	68341	34170	0.3539	0.7038
Residuals	47	143722134	3057918			4538448	96563		

IV. Conclusions

The goals of this project were to determine what tissue(s) is the most appropriate for use in the estimation of PMI using nucleic acid degradation and to investigate an extended range of PMI,(to months). Our initial focus has been on nails and this tissue has demonstrated to be an appropriate tissue for studying the degradation of nucleic acids over a defined time period.

One objective of this project was to modify the DNA IQ method so that DNA and RNA can be co-extracted from nail material. This objective has been successfully achieved. This method has been validated and implemented into operational casework at ESR where nails have been used for the identification of individuals. This work has demonstrated that nails are a good source of nucleic acids for forensic casework.

Candidate markers (keratin mRNAs, 18S rRNA and DNA) have been identified and successfully amplified from nail material, including nails left in different environmental conditions. After 40 days all nucleic acids were amplified, and we had to increase the size of amplicons detected due to the surprising stability of RNA in nails. The identification of these transcripts has satisfied another objective of this project.

Using nail samples has demonstrated our proof-of-concept that the time-wise degradation of nucleic acids can be measured. After the longer time intervals of our environmental trials (up to 4 months), statistical analysis has shown that some amplified fragments show promise as markers for measuring the degradation of nucleic acids that are not affected by environmental conditions. Further analysis is required to develop equation(s) that can be used with our cadaver nail data to estimate the PMI and if this value correlates with the actual PMI of the cadavers.

The degradation of nucleic acids appears to be happening in a time-wise manner. What has been evident with this project is the relative stability of both RNA and DNA in nail samples. The results to date show great potential for providing the evidence-based results we require for estimating the PMI.

Implications for Policy and Practice

This project addresses one of the most difficult, but crucial problems in criminal casework, estimating the PMI. The use of improved extraction methods and molecular biology techniques has improved the sensitivity and detection of small samples over recent times. These techniques are available in the majority of forensic biology laboratories in the US and will enable quick uptake of this research into casework. The modified method for co-

extracting the DNA and RNA from nail material has been validated and implemented into the forensic biology laboratory at ESR Ltd (New Zealand), and can be quickly and easily validated and implemented into forensic laboratories throughout the US.

The successful outcome of this research will have a significant impact on the estimation of the post-mortem interval in crime scene investigations in the United States and world-wide. Utilization of these new methods will allow many forensic institutions throughout the US and beyond to estimate the PMI more accurately. The results to-date show great promise in being able to deliver the overall goal of this project – developing a method for estimating the PMI over longer time periods. This will allow many forensic institutions to more accurately estimate the PMI which will be a major achievement.

Implication for Further Research

This project has been successful demonstrating the proof-of-concept that nucleic acid degradation can be measured in nails, a tissue that can be sampled multiple times from the same individual. This reduces the number of factors that could influence the rate of nucleic degradation and provide a more accurate method for estimating the PMI. Further work should include using blind samples, as well as testing the model out on nail samples from human cadavers from different areas of the US, to see if and how different environments affect nucleic acid degradation in nails.

Further work should focus on using teeth and rib bones in a similar manner to the nails used in this research as these tissues can be used over extended periods of time (years). As these samples can be taken from the same human cadaver over a time period, the development of multiplex PCR assays to measure the degradation of nucleic acids in a manner to what has

been developed in this project, will together provide an accurate method for estimating the PMI.

V. References

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VI. Dissemination of Research Findings

The research findings of this project, to date, have been presented at the NIJ conference (June 2011, Washington DC) and will be presented at the 22nd International Society for Human Identification conference (October 3-6th, Maryland, US) and an abstract has been submitted for presentation at the annual meeting of the American Academy of Forensic Sciences (February 2011, Atlanta, GA, USA). This work may also be presented at the Australian and New Zealand Forensic Science Society conference in Hobart, Australia in September 2012. These findings will also be submitted to a peer-reviewed journal.