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Determination of time since deposition of blood stains
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Introduction

DNA analysis has revolutionized the field of Forensic Science by allowing for unambiguous identification of the person from whom a biological sample has been obtained. DNA provides a spatial link between a suspect and a crime scene or other location relative to a crime investigation. In many instances, this information is sufficient for conviction of the perpetrator of a crime. The limitation of traditional DNA, however, is that it provides no information on when the biological material was deposited. It only indicates that, some time in the past, the person visited the scene, it could have been months or years before the material was collected. Temporal linkage between the biological material and the commission of a crime is especially important in situations involving victims and suspects with close personal ties. In these instances, finding biological material from the suspect in the home or other pertinent location associated with the victim is not unexpected. The reverse can also be true, for instance finding the blood of the victim in the vehicle of the suspect. In the absence of knowledge about time of deposition, it is impossible to link the evidence to the crime. A temporal linkage could also be valuable for determining the location of suspects being tracked. For example, identification of a biological specimen could possibly link Osama Bin Laden to a specific cave during a specific time range. This could help to establish an approximate distance in which the suspect could have traveled in the given time based on the deposition of the biological sample.

Previous Techniques Developed to Determine the Age of a Blood Sample

Several approaches have been used in an attempt to determine the age of a bloodstain or other biological material. The majority of methods rely on the transformation of hemoglobin into its derivatives, and the changes in color and solubility which accompany them. Fiori (1962) summarized a method that correlated the age of the bloodstain with the progressive diffusion of Cl^- around the stain, which can be fixed as AgCl. Upon reduction, a black border will form around stains which are more than two months old. The size of the border will increase in increments up to nine months, indicating an approximate age of sample. Enzyme assays have also been used with limited success. In general, they allow for a statement that the sample is either less than or more than a certain age. Schwarz (1936) examined "peroxidase" in bloodstains as a method of determining age. The peroxidase was shown to be a direct indication of the amount of hemoglobin remaining in the stain. Guaiacum was used as a substrate for peroxidase, which produced a blue color in the presence of hemoglobin. These studies suggested that the intensity of the blue color varied with age. Another more recent approach (Rajamannarr, 1977) looked at the serum protein profile by immunoelectrophoresis in stains as a function of their age from 15 days to one year. A characteristic pattern of disappearance of various proteins at test points along the time line was constructed. All of the proteins were undetectable at 365 days. In contradiction, Sensabaugh, 1971, found albumin to be detectable by its immunological reaction in a dried blood sample eight years old. This contradiction has been explained by a change in electrophoretic mobility of albumin in aging bloodstains. The above approaches are incapable of discriminating blood samples from different species, provide too narrow or unreliable a window of time to be of much use, are limited to bloodstains and could have misleading results due to sample size.

Our Approach to Estimating Time of Deposition

One approach to determining the age of a biological sample is that of Carbon-14 (C14) dating. C14 is the radioactive form of Carbon and decays into Nitrogen14 (N14) at a constant rate. C14 is constantly being regenerated in the upper layers of the atmosphere by cosmic rays striking N14. Plants take up and incorporate both C14 and C12 into their cells. Animals that consume the plants likewise incorporate both types of Carbon into their cells. Once death occurs, however, no more C14 gets taken into the body and the C14 clock begins to tick. The ratio of radioactive C14 to non-radioactive C12 will change in a predictable fashion over time. Unfortunately, the half-life of C14 (5,730 years) is such that it is of little use to the forensic scientists. The concept of monitoring the changing ratio of two chemicals or compounds over time, however, has merit.

We have chosen to examine RNA stability as our method to determine the age of a biological sample. RNA has been proposed as a means to identify tissue type and one study looked at RNA levels relative to time of death but in general, the focus in the forensic science community has been on DNA. There are several reasons why we chose RNA over DNA. RNA is more abundant than DNA, existing in several 1000s of copies per cell as opposed to two copies for most DNA sequences. RNA is also relatively labile and is more susceptible than DNA to degradation due to factors such as high pH, RNases, certain chemicals, and possibly UV light. Most RNA species have a characterized *in vivo* half-life and a tightly regulated mechanism of decay. In contrast, as long as a cell is alive, its DNA remains intact. Many internal sequences and secondary structures regulate *in vivo* RNA decay. For example, *c-myc* contains a CRD (coding region instability determinant) that interacts with a 68-kDa CRD-BP (coding region instability determinant binding protein) which contains two RNA recognition motifs. When the CRD-BP is bound to c-*myc* mRNA, the CRD of the mRNA is shielded from endonucleolytic attack and the mRNA can only be degraded by standard degradation procedures. If the CRD-BP dissociates from the mRNA, the CRD becomes exposed to the endonuclease. The mRNA is then rapidly degraded by endonucleolytic cleavage within the CRD. This is just one example of the highly regulated *in vivo* decay of RNA. The *ex vivo* mechanism of RNA decay, however, has not been thoroughly investigated but our results indicate it is also a predictable process.

Many studies have indicated that DNA associated with nucleosomes (assemblages of histone proteins on which DNA is wound) is less susceptible to degradation by either endogenous or exogenous nucleases than is linker DNA located between the nucleosomes. This tight association with histone proteins provides protection to the DNA. We speculated that ribosomal RNA (rRNA, an integral component of ribosomes), due to tight association with ribosomal proteins, would also be relatively sheltered from degradation. In contrast, messenger RNA (mRNA) is not as tightly associated with proteins and may be more susceptible to degradation.

To estimate the relative amount of the two target RNAs recovered in a sample, we have utilized an approach called Real-time Reverse Transcriptase Polymerase Chain Reaction (Real-time RT-PCR). This approach first converts total RNA into complementary or copy DNA (cDNA) using the enzyme reverse transcriptase. The double stranded cDNA can then be amplified using appropriate primers in a traditional PCR reaction. Unlike traditional PCR, however, inclusion of a labeled probe complementary to the target RNA and internal to the primers allows the investigator to monitor the rate of PCR amplification of the targeted cDNAs. The rate of amplification is directly proportional to the amount of starting RNA. By labeling two different target RNA sequences with probes that emit light in different portions of the light spectra, you can monitor the relative rate of

amplification of two different target RNAs. This approach is perfectly internally controlled for experimental error such as pippetting errors or differences in the amount or efficiency of the enzyme(s) used in the assay.

Advantages

1. Both RNA and DNA can be isolated from the same sample allowing for both estimates of time of deposition and identification of the person from whom the sample was obtained
2. Analysis of ratios is independent of sample size
3. All experimental manipulations are identical and conducted simultaneously on both target RNAs
4. Probes can be made species-specific eliminating false data from other sources
5. Both 18s and beta-actin are expressed in all cells and thus can be used on different tissue types and not just blood
6. Both 18s & beta-actin are abundantly expressed RNAs thus only small sample sizes are required
7. The abundance of the RNAS also allows for estimates over a relatively broad range of time
8. Technically similar to current DNA PCR techniques thus requiring little additional training of personal

Results

Isolation of both RNA and DNA from the same bloodstain

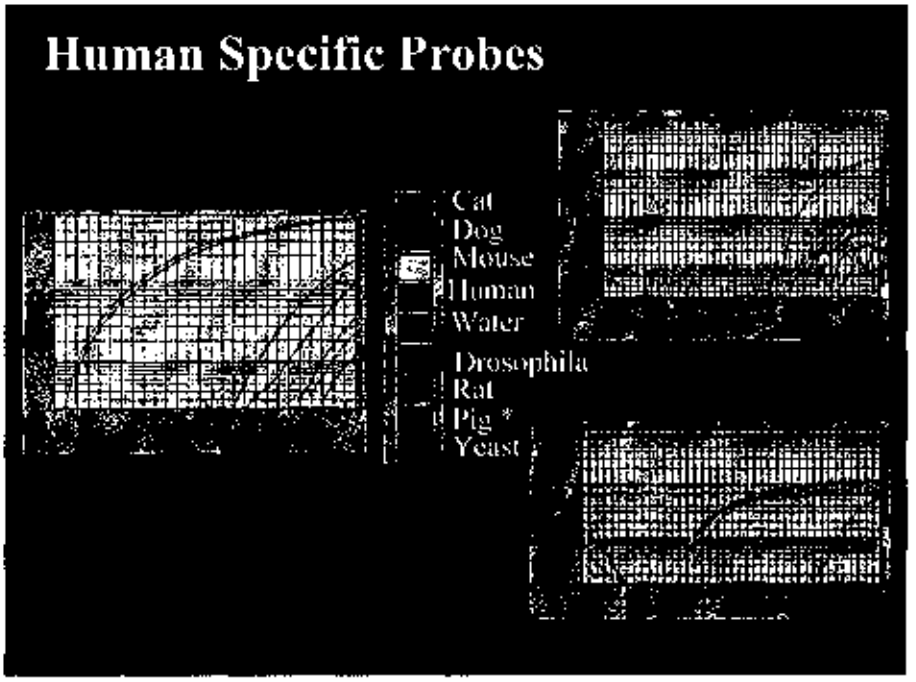
Since both RNA and DNA belong to a class of compounds known as nucleic acids, their chemical properties are similar. This allows simultaneous, but separate, isolation of RNA and DNA from the same bloodstain. We can thus perform both "temporal PCR" to estimate the time of deposition using the RNA component and STR identification on the DNA portion of the sample. Utilization of one set of techniques does not destroy the evidence for the other one. Using Real-time primers and probes that were either specific for RNA or DNA, we have examined the two isolates for contamination by the other nucleic acid and found no detectable RNA present in the DNA fraction and vice versa. The RNA specific primers and probes were constructed so that they spanned an exon-exon boundary so that only mature, processed RNA and not genomic DNA could be detected. For the DNA primers and probe, we chose non-transcribed portions of the genomic DNA.

Species-specific probes

Commercially available TaqMan probes from Applied Biosystems have been used to generate our preliminary data presented in Figure 2 and Table 3. These probes detect RNA from a very broad range of organisms. By utilizing polymorphisms between organisms, we were able to develop primer and probe combinations that are more species-specific (Figure 1). Values on the Y-axis represent a measure of the amount of fluorescence emitted by the TaqMan probes while the PCR amplification cycle is on the *X*-axis. The more RNA that is present in the starting material, the sooner exponential growth of the curve is obtained. Each cycle represents a 2-fold difference in starting RNA levels. In the case of beta-actin, our probes only detect human beta-actin RNA from among the species we have tested. Our best efforts on 18s, however, have produced primers and probes that are nearly human specific, only detecting pig 18s to any appreciable extent. While other species can be detected at high PCR cycle values, they would contribute a negligible amount to the overall signal. Contamination of the crime scene with pig blood, however, could alter the 18s values. We continue to try to improve our primers and probes to make them unique to humans and to optimize reaction conditions. If we are unable to develop one that does not recognize pig RNA, we can develop pig RNA specific primers and probes (to non-18s and thus less conserved RNA) to detect the presence of pig blood and to estimate how much it would contribute to the overall signal. The similarities of human and pig 18s could thus be overcome by the inclusion of an additional test

to identify the absence or presence of pig blood. In the case of a positive signal for pig blood, estimates of the amount of pig RNA present, using the pig specific primers and probes, can be subtracted from the 18s signal.

Figure 1. Detection of RNA from the indicated species with our species-specific probes Isolation of RNA from blood, hair follicles, and saliva



Estimates of RNA quantity

Table 1 indicates our success in recovering sufficient RNA to test for time of deposition from bloodstains, hair follicles, and saliva. All tissue types yielded sufficient RNA to conduct Real-time RT-PCR tests. The techniques and reagents we develop using blood should, therefore, be applicable to other tissue types as well. Confirmation of this with each tissue type will need to be performed but our initial results indicate that should present no problems.

Table 1. Recovery of RNA from different biological tissues based on Ribogreen.

1 µl fresh blood spotted onto cloth	40ng
Saliva swab	300ng
1 hair follicle	200ng

We estimated how much RNA is required for our tests based upon both the amount of biological material and the amount of RNA needed to obtain reliable Real-time RT-PCR data. Our Real-time system is more sensitive than what can be measured using Ribogreen (we could not detect samples smaller than 2 ng of RNA with Ribogreen). We have determined the amount of RNA present in our non-diluted samples using Ribogreen, performed a series of dilutions on the RNA to determine our ability to detect a signal with Real-time RT-PCR, and extrapolated the amount of RNA needed. With our smallest amplicons for 18s and beta-actin (60 base pairs), we can obtain useful data for RNA isolated from 1 µl of fresh blood (fresh blood spotted onto cotton cloth and RNA immediately

isolated) and then diluted 10 (~0.4 ng of total RNA) to 100 (~0.04 ng of total RNA) fold. In theory, we could obtain useful data from a fresh bloodstain as small as 0.01 µl. It should be noted that only 1/10 of each reverse transcriptase reaction is used in each Real-time assay therefore a total of 9-10 assays could be performed with this small amount of RNA. Clearly, our system is sensitive enough to detect RNA from any sample that might be identified as biological material. We have not tested our techniques with Luminal or other reagents used to identify bloodstains. These compatibility studies need to be conducted.

Recovery of RNA from different surfaces

We have successfully recovered RNA from three-day old bloodstains on various surfaces. These include the following cloths: cotton-polyester blends, wool, silk, (not shown), and cotton and polyester (Table 2). We have also recovered RNA from newspaper, bricks, and both varnished and unvarnished wood surfaces (Table 2). Simply using distilled/deionized water or a 25% ethanol-water solution, as is often used to recover DNA, did not provide satisfactory results. The newspaper and cloth RNA samples were isolated by hydrating the samples with Tri Reagent BD from Molecular Research Corp while for the hard surfaces, we were able to simply scrape up the sample using a razor blade. We suspect that hydration with water and ethanol-water reactivated RNase activity resulting in a low yield. We will, therefore, examine solutions containing various inhibitors of RNase activity for the recovery of samples not amenable to scraping.

Table 2. Total RNA recovered from different surfaces using Ribogreen and known quantities of RNA.

Cotton (10 µl of 3 day old blood)	100ng of total RNA
Polyester (10 µl of 3 day old blood)	100ng of total RNA
Brick (10 µl of 3 day old blood)	90ng of total RNA
Plastic (10 µl of 3 day old blood)	70ng of total RNA
Newspaper (10 µl of 3 day old blood)	60ng of total RNA
Unfinished Wood (10 µl of 3 day old blood)	100ng of total RNA
Finished Wood (10 µl of 3 day old blood)	70ng of total RNA

Age of Blood Trend

After preliminary results on bloodstains from a single person confirmed our original hypothesis that *ex vivo* 18s and beta-actin RNAs decay at different rates, we examined samples from four females and four males (variability between persons). Blood was drawn from each individual on three separate occasions (within person variability). From each date of blood draw for each person, RNA samples were isolated from three separate bloodstains (variability due to isolation). Each RNA sample was then assayed twice (variability due to assay). The means of ratio analysis of females and males are presented in Figure 2. The ratio is the value of 18s over that for beta-actin. Table 3 indicates the amount of variation we observed with these data. Both Standard Error and Standard Deviation are presented. The four female donors ranged in age from 22 to 36 years while the four males ranged from 21 to 55 years of age. The sample size within each age category was too small to make any meaningful comparisons.

Figure 2. Pooled population data (Bars indicate standard error).

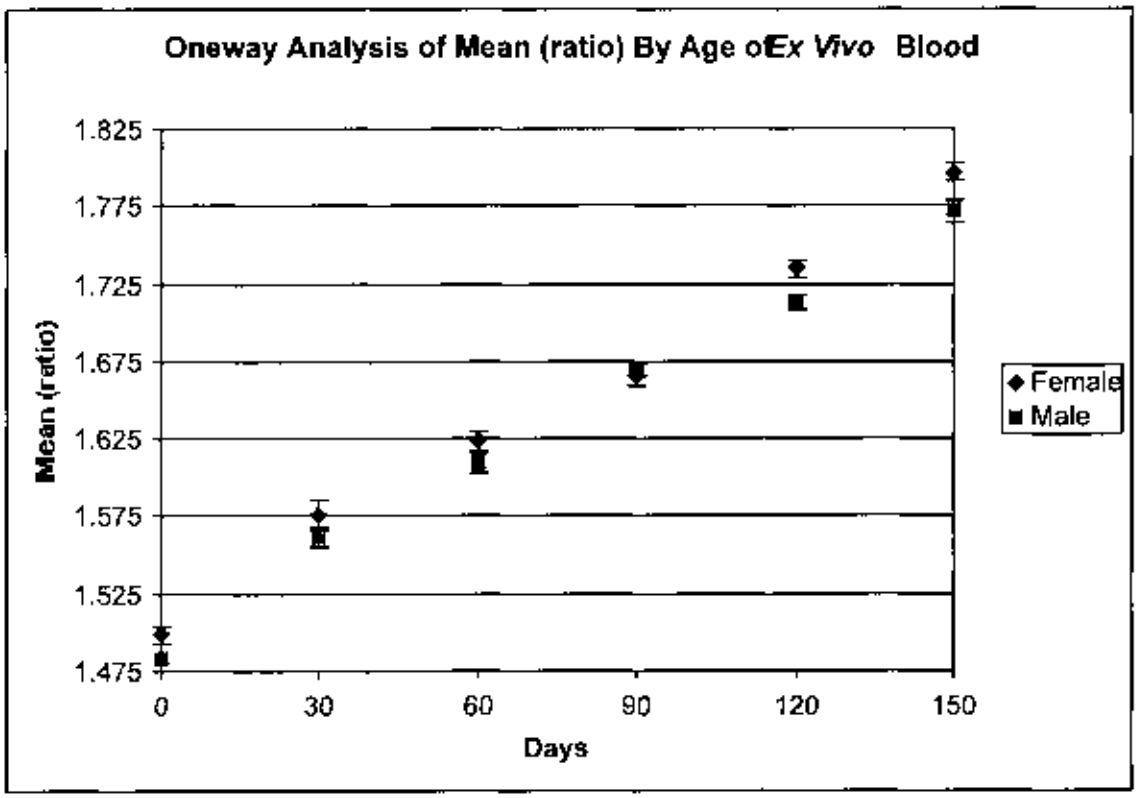


Table 3. Variation within data

Sex	Days	n	Mean of Ratio	Std Error	Std Deviation
F	0	72	1.49836801	0.00615666	0.05224095
F	30	72	1.57645711	0.00865694	0.07345655
F	60	72	1.62297068	0.00686272	0.05823207
F	90	72	1.66532709	0.00722973	0.06134627
F	120	72	1.73479106	0.0058206	0.04938946
F	150	72	1.79649773	0.00582809	0.04945299
M	0	72	1.48339593	0.00866593	0.07353289
M	30	72	1.56068628	0.00602969	0.05116365
M	60	72	1.61002923	0.00771633	0.06547522
M	90	72	1.66892991	0.00450743	0.03824681
M	120	72	1.71256782	0.00473909	0.04021251
M	150	72	1.77163733	0.00684049	0.05804345

n = (3 dates of blood draw) (3 bloodstains per date) (2 assays per stain) (4 people) = 72

The plot in Figure 2 shows a strong linear relationship between the mean ratios and day (age of blood) for both females and males. Although there is a suggestion of gender effect in Figure 2 and perhaps a gender-by-day interaction, it is not significant using the preliminary sample sizes. A model fitting the above sources of variability has a highly significant linear day effect ($P<0.0001$) and has an adjusted R^2 of 78.2% (male and female data were pooled together for these tests).

Estimates of Variability

The statistical analysis suggests that between person variability accounts for approximately 10.8% of the variability for the random effects. The random terms are: between persons, within persons, variability due to RNA isolation, and residual variation (which includes duplicate assays). Within person variability accounts for another 3.8% of the total while variability due to experimental error in the isolation of RNA accounts for an additional 3.4%. The remainder of the variability may be caused by factors, such as age and sex of donor, technical error in duplicate assays, and the interactions of these factors. Preliminary data suggest that there may be greater variability within a person (variability in blood draw dates) for females than males. This could be due to hormonal levels affecting the starting ratio of 18s to beta-actin RNAs. One way to eliminate this source of variability is by looking at different amplicon sizes (see below) detecting the same RNA "species". By looking at different regions of the same RNA species or type (for example 18s versus 18s), the amount of starting material at day zero would be identical for the two assays, i.e. present in equimolar amounts. Experimental error could also potentially be reduced by improved protocols. Between persons variability, however, may not be reducible unless age of donor, sex, or other factors are contributing to the equation. Larger population studies may allow identification of other sources of variability and allow for its reduction, permitting a better defined time line for when a sample was deposited.

The above data were generated using commercially available 18s and beta-actin primers and probes combinations. Neither, however, are human specific. Theses commercial primers and probes have been fully optimized for multiplexing and hence reduced variability. For our purposes, human specific primers and probes are required. The above graph provides a "proof of concept" that our original hypothesis is feasible. These results have been accepted for publication in the journal Forensic Science International and, although not yet published, the manuscript is available electronically. The electronic journal NewScientist, produced in England, wrote a brief news release of the results.

Having established the viability of our approach, we began a study of the impact of environmental factors on the relative rates of RNA decay with a series of different human specific primers and probes.

Amplicon size

Our results clearly indicate that 18s rRNA decays more rapidly than does beta-actin mRNA. The primers and probes used, and the distance, in terms of base pairs, that they span can also affect the ability and strength of the signal obtained. Amplicon size refers to the size of PCR fragment being expanded (from the forward primer to the reverse primer). The larger the amplicon, the more rapidly we detect a decrease in the RNA signal. This is not surprising. If degradation is a stochastic event, then the larger the fragment of RNA being examined, the greater the likelihood that some degradation has occurred and hence loss of signal.

We have generated amplicons that range in size from 60 base pairs up to 500 base pairs for both 18s and beta-actin. Loss of signal can be detected fairly early in the 500 base pair amplicons while little loss is seen in those for 60 base pairs even after 150 days. With our smallest amplicons, we can obtain useful data from bloodstains that are 15 months old, the oldest sample we have tested. This observation can be used to both eliminate issues related to the starting ratio of 18s to beta-actin and also to extend the time line for estimating time of deposition by using a broad range of amplicon sizes. By using different combinations of amplicons, we hope both extend the time over which reliable data can be collected and, by integrating the results from different sets, obtain a more accurate estimate of the age of a sample. We continue to develop new amplicon sets and seek ways to optimize the reactions.

Ongoing Research

Environmental Variables

We have generated preliminary results that indicate that both temperature and humidity have an effect upon the rates of the decay. We have also shown that full intensity natural light has no discernable effect on the decay rates. We are in the process of extending these studies to take into account these variables when estimating the age of a sample.

Population Variables

We have initiated a population study looking at variables such as ethnicity, age, and sex. Blood from African-Americans, Asian-Americans, and European-Americans of both sexes and young (21-25 years old) and older (40-50 years old) have been collected and are aging.

Shorter time-line

We have begun a study to look at changes in RNA levels that occur within the first few hours (zero hour versus four hours old) of blood deposition using DNA chip technology. Our initial results indicate that a large number of RNAs have significantly altered levels, some higher, some lower, in aqueous samples. We will extend these studies to dried samples and seek to develop assays that allow more precise estimates of younger samples. Examining larger amplicon sizes of 18s and beta-actin will also be used to estimate the age of younger samples. Once established for blood, a similar examination of semen will be conducted.

Summary

We have demonstrated the feasibility of using differences in decay rates of two different RNAs as a means of estimating the age of a biological sample. One manuscript has been accepted for publication in the Forensic Science International journal. This article has received favorable publicity. We anticipate additional publications deriving from this work. The funds provided by NIJ provided the necessary resources to enable a demonstration of the concept. Funding of this research has now been obtained from the Technical Support Working Group to enable the work to continue. While much remains to be done before this technique is ready for the crime laboratory and court system, we are well on our way to obtaining this goal thus providing a temporal linkage of biological material to the commission of a crime.

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A method for determining the age of a bloodstain

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Abstract

DNA allows for the unambiguous identification of the person from whom a biological sample was derived but provides little information about when the sample was deposited. This information only indicated that the biological material was deposited at the crime scene prior to the collection of evidence. The ability to determine the age of a biological sample would greatly benefit the forensic science community. If there were independent evidence that the biological sample was deposited at the time of the crime, then its age would reveal when the crime occurred. If the time of the crime were known through another means, then the age of the biological sample could potentially exclude the human source as a suspect. We have used real-time reverse transcriptase PCR to show that the ratio between different types of RNA (mRNA versus rRNA) changes over time in a linear fashion when dried human blood from eight individuals was examined over the course of 150 days. Although other approaches have been used in the past to estimate the age of a biological sample, our approach offers the following advantages: enhanced detectability of small samples, simultaneous isolation of DNA and RNA from the same sample, species-specific probes, and an increased window of usefulness.

Keywords: Forensics; Blood; RNA decay; Real-time PCR

1. Introduction

DNA analysis by polymerase chain reaction (PCR) has revolutionized the field of forensic science by allowing for identification of the person from whom a biological sample has been obtained. DNA analysis provides a spatial link between a suspect and a crime scene or other location relevent to a crime investigation. One limitation of traditional DNA analysis, however, is that it provides no information on when the biological material was deposited. It only indicates that the biological material was deposited at the crime scene prior to the collection of evidence [3,5]. However, that visit could have taken place months or even years before the actual collection of material. Temporal linkage between the biological material and the commission of a crime is especially important in situations involving victims and suspects with close personal ties. In these instances, finding biological material from the suspect in the home or other pertinent location associated with the victim is not unexpected. The reverse can also be true, for instance, finding the blood of the victim in the vehicle of the suspect. In the absence of knowledge about time of deposition, it is often impossible to link the biological samples to the commission of the crime.

Several approaches have been tested in an effort to determine the age of a bloodstain. The majority of methods rely on the transformations of hemoglobin into its derivatives, and the changes in color and solubility that accompany them. Fiori [2] summarized a method that correlated the age of the bloodstain with the progressive diffusion of chloride (Cl^-) around the stain, which can be fixed as AgCl. Upon reduction, a black border will form around stains which are more than two months old. The size of this border will increase in increments up to nine months, indicating an approximate age of the sample. Enzyme assays have also been used with limited success. In general, they allow for a

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statement that the sample is either less than or more than a certain age [4]. Schwarz (1936) investigated peroxidase in bloodstains as a method of determining age. Guaiacum is used as a substrate for peroxidase, which produces a blue color in the presence of hemoglobin. The strength of the signal is proportional to the amount of hemoglobin present and based on the results, the intensity of the blue color was suggested to decline with age [8]. Unfortunately, there are drawbacks to the aforementioned techniques. Both of the approaches mentioned above are incapable of discriminating between samples of different species, provide too narrow a window of time to be of much use, and can produce misleading or nonexistent results due to differences in sample sizes.

In order to establish a more reliable mechanism to determine the age of biological samples, RNA stability over time has been examined. RNA is a good candidate for such studies for several reasons. Many RNA species are highly abundant, RNA is relatively labile, and polymorphisms between species can be exploited to provide species-specific tests. Using RT-PCR, previous research has shown that GAPDH mRNA can be detected from dried blood samples that have aged up to 6 months [1]. These results indicated that RNA in dried blood samples might be stable enough for forensic science investigations. Recent research has shown different types of RNA decay at different rates. The inducible gene IL-3 mRNA was found to decay more rapidly than the mRNA of the GAPDH housekeeping gene in postmortem rat lung tissue [5]. RNA exists in a variety of different forms (mRNA, tRNA, rRNA), and it is hypothesized that the different types of RNA may also decay at different rates resulting in a change in ratios of RNA species as time progresses. This approach to estimating the age of a sample is analogous to that used for Carbon-14 dating. In the case of Carbon-14 dating, the ratio of radioactive Carbon-14 declines relative to non-radioactive Carbon-12 and it is this ratio that is used to estimate the age of a sample. In order to test the hypothesis that different types of RNAs decay, *ex vivo*, at different rates, we have compared the relative stability of a rRNA to a mRNA.

In this paper, it is suggested that the ratio of two types of RNA molecules (β-actin mRNA and 18 S rRNA) in experimental human bloodstains changed in a linear fashion during a 150-day study. This phenomenon is likely to form the basis for a more precise and accurate method than the previously mentioned techniques for determining the age of biological evidence.

2. Methods

2.1. Blood collection and sampling

Blood was drawn from eight individuals (four males and four females) of European ancestry. Males ranged in age from 21 to 55 and females ranged in age from 22 to 36. Ten milliliters of blood were collected in non-coated BD Vacutainers (Fisher Scientific, Pittsburgh, PA) on three separate occasions from each donor. Ten microliters aliquots were immediately spotted on the same piece of 100% cotton fabric and stored at 25 °C and 50% humidity in an EC22560 Environmental chamber (Lab-Line, Melrose Park, IL). A separate piece of fabric was used for each individual. Blood was drawn three times a week for two individuals randomly selected. The blood collection procedure was performed over a 4-week period. Samples were removed from the chamber and processed when they reached the desired ages of 30,60, 90, 120, and 150 days.

Our protocol was approved by the West Virginia University Institutional Review Board for the Protection of Human Research Subjects (IRB #15833).

2.2. RNA extraction

TRI Reagent BD (Molecular Research Center, Cincinnati, OH) was used to isolate RNA from the dried blood samples at the various ex vivo ages. For each time point (*ex vivo* age) and for each blood draw, RNA from three separate 10 μl bloodstains was isolated for each subject. For each subject, therefore, a total of nine RNA samples were isolated for each time point. Two hundred microliters of water and 3 μl of a polyacryl carrier (Molecular Research Center, Cincinnati, OH) were added to 750 μl of TRI Reagent BD. The dried bloodstains were cut from the fabric and added directly to this solution, which was then vortexed briefly and incubated at 50 °C for 10 minutes (min). One hundred microliters of 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, OH) was added to each sample, then vortexed for 15 seconds followed by a room temperature incubation (23 °C) for 3 min. Samples were then centrifuged for 15 min at 4 ° C. All centrifugations were performed at 12,000 × *g* at 4 °C. The upper aqueous layer (containing the RNA) was transferred to a new tube (~500 μl) and 500 μl of cold isoproponal was added. The interphase contained the high molecular weight DNA that was extracted for other experiments (see below). The samples were inverted 2 times and incubated at room temperature for 7 min. The samples were then centrifuged for 8 min. The liquid supernatant was discarded and 1 ml of 75% ethanol (EtOH) was added to wash the RNA pellet. The samples were briefly vortexed and centrifuged for 5 min. The liquid supernatant was removed and the RNA pellets were allowed to air-dry for 5 min at room temperature. To resuspend the RNA pellet, 40 μl of RNase free water (Fisher Scientific, Pittsburgh, PA) was added and incubated at 55 °C for 10 min. A sham RNA isolation of cotton containing no bloodstain was performed with every assay as a negative control.

2.3. DNA extraction

DNA was extracted from the interphase and organic (lower phase) layer after the phase separation during the RNA isolation of the same bloodstain. The blood-stained

fabric was removed from the organic layer and washed in 500 μl of DNAzol (Molecular Research Center, Cincinnati, OH). The fabric containing the DNA was then placed in 1 ml of DNAzol, 10 μl of 10% sodium dodecyl sulfate (SDS) and 20 μl of 20 mg/ml of proteinase K. The samples were incubated at room temperature for 2 days with occasional vortexing. The samples were centrifuged for 10 min at 10,000 × *g* and the supernatant was removed. Three microliters of polyacryl carrier and 500 μl of 95% EtOH were added to each sample, tubes were inverted several times, and incubated at room temperature for 3 min. The samples were then centrifuged at 5000 × *g* for 5 min and washed with 1 ml of 95% EtOH, and centrifuged twice at 2000 × *g* for 2 min followed by washes. The supernatant was discarded and the DNA pellet was air dried for 5 min and then resuspended in 20 μl of nuclease free water.

2.4. Reverse transcription

Applied Biosystem's TaqMan Gold RT-PCR kit was used for the reverse transcription reaction. A reverse transcription master mix (final concentration: 1× TaqMan buffer A, 5.5 mM magnesium chloride, 500 μM each dATP, dCTP, dGTP and dUTP, 2.5 μM random hexamers) was made, aliquoted into individual PCR tubes and stored at −20 °C until time of use. Forty microliters of the RNA suspension, 2.0 μl of RNase inhibitor (0.8 U) and 2.5 μl of multiscribe reverse transcriptase (3.25 U) was added to each reaction. Samples were pulse centrifuged and placed in a Techne Touchgene Gradient thermocycler (Burlington, NJ) under the following conditions (25 °C for 10 min, 48 °C for 30 min and 95 °C for 5 min). A no-enzyme control was run with every assay.

2.5. Real-time PCR

A real-time PCR master mix composed of: Applied Biosystem's β-actin (401846) control reagents, Applied Biosystem's 18 S rRNA (4308329) control reagents, and Applied Biosystem's TaqMan Universal PCR Master Mix was generated to be used for RNA detection. The following list represents the final concentrations of each component of the reaction: 50 nM 18 S rRNA forward primer, reverse primer and 200 nM probe (VIC dye layer) 300 μM β-actin forward primer, reverse primer and 200 μM probe (FAM dye layer), and 1 × TaqMan Universal PCR Master Mix. 34.75 μl of the real-time PCR master mix was added to real-time optical tubes (Applied Biosystems, Foster City, CA). Fifteen and a quarter microliters of each cDNA sample was added to the tubes for a total volume of 50 μl. The samples were briefly centrifuged before real-time analysis. Duplicate samples were run for each RNA sample. Samples were placed in Applied Biosystem's 7700 Sequence Detection System and run on default conditions. Positive (control cDNA) and negative (water) controls for real-time analysis were included. The sham RNA isolations (cotton cloth lacking a bloodstain) were analyzed at this point for detection of false positives. Analysis of data was preformed using Applied Biosystem's Sequence Detection Software Version 1.7. In real-time PCR, the relative mRNA level is indicated as the Ct value, which is the PCR cycle number first showing settled fluorescence intensity of amplified RT-PCR products of the target RNA (exponential amplification). The Ct value is inversely proportional to the amount of target mRNA sample [9–11]. Our Ct values for each subject for each time point represent means of eighteen measurements. Three RNA samples were isolated from each subject on three different occasions and replicates of each RNA isolate were analyzed for real-time analysis.

2.6. Relative RNA quantification

Results were analyzed and threshold values adjusted as described by the manufacturer. In brief, this was achieved by increasing the threshold bar to ensure it was above any background noise of the real-time PCR run. A set threshold was identified for both β-actin and 18 S rRNA and kept constant for all assays. Control cDNA was used to adjust for experimental variation caused by the 7700 machine. All runs were normalized to the same control cDNA before statistical analysis. Results were exported into Microsoft Excel to generate the values needed for further analysis.

2.7. RiboGreen quantification of RNA

RiboGreen (Molecular Probes, Eugene, OR) was used to determine the concentration of the RNA samples. Fifteen microliters of a RiboGreen/TE dilution was added to 15 μl of total RNA isolated from various blood samples. The samples were analyzed and the fluorescence determined using Applied Biosystem's 7700 real-time PCR machine. Concentrations were determined by comparison to a series of known RNA dilutions.

2.8. Species-specificity and primer design

Primers and probes were designed to be human-specific using the sequence comparison program SEQUED (Applied Biosystems, Foster City, CA). mRNA Genbank sequences from human and other species were aligned and the polymorphic regions identified. Primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA). Both primers/probes were evaluated using RNA obtained from several non-human organisms: cat, dog, rat, mouse, pig, and yeast. RNA was obtained from cat and dog blood donated by a local veterinarian. Rat, mouse and pig RNA was purchased from Pharmingen (San Diego, CA). Yeast RNA was isolated using the standard protocol for the Roche High Pure RNA Isolation Kit (Indianapolis, IN). No amplification ensured that the primers and probe were human-specific. A probe specific for DNA was also designed to test for DNA contamination of the RNA

sample and to determine the efficiency of DNA recovery when isolated from the same blood sample. DNA-specific primers/probe were designed based on a non-transcribed region of the human GAPDH gene (GAPNT). The DNA-specific probe (GAPNT) was multiplexed with an RNA-specific probe from Applied Biosystems (hsBA) to test for both RNA and DNA in the same sample. Optimal conditions are as follows: GAPNT—250 nM probe, 500 nM forward and reverse primers, hsBA—50 nM probe and forward and reverse primers.

2.9. Statistical analysis

Analysis was performed using a nested analysis of variance using the method of restricted maximum likelihood [7]. Date of draw is nested within subject. Time (age of blood) was nested within date. Samples (RNA isolates) were nested within times and duplicate (real-time) assays were nested within samples. All factors except subject and times were taken to be random. The response variable is the ratio of Ct value for the 18 S gene over that for the β-actin gene (relative ratio).

3. Results

Results from the dried blood of eight subjects processed over 150 days are presented in Fig. 1. The ratio of the Ct value of 18 S over that for β-actin is a linear function of age of blood for both females and males. Although there is a suggestion of gender effect in Fig. 1 and perhaps a gender-by-day interaction, it is not statistically significant using this sample size. A model fitting the above sources of variability has a highly significant linear day effect ($P < 0.0001$) and has an adjusted R^2 of 78.2% (male and female data were pooled together for these tests). The data from Fig. 1 is also presented in tabular form in Table 1, indicating the amount of variation observed.

3.1. Estimates of variability

The statistical analysis suggests that inter-person variability accounts for approximately 10.8% of the variability for the random effects. The random terms are: intra-person, inter-person, variability due to RNA isolation, and residual variation (which includes duplicate assays). Intra-person variability accounts for another 3.8% of the total with the majority being seen in females. Variability associated with experimental error in the isolation of RNA accounts for an additional 3.4%. The remainder of the variability may be caused by various factors, such as age and sex of donor, technical error in duplicate assays, and the interactions of these factors. Preliminary data suggest that there may be greater variability within a person (variability in blood draw dates) for females than males. The greater variability seen in females may be due to altered hormonal levels encountered during the monthly estrus cycle affecting the starting ratio of 18 S to β-actin RNAs. This will need to be further pursued with a larger population size.

3.2. Levels of detectability

Using RiboGreen, the total amount of RNA present in 10 μl fresh blood samples was determined to be about 100–150 ng. A series of dilutions were performed on the RNA to determine the ability to detect a signal with real-time

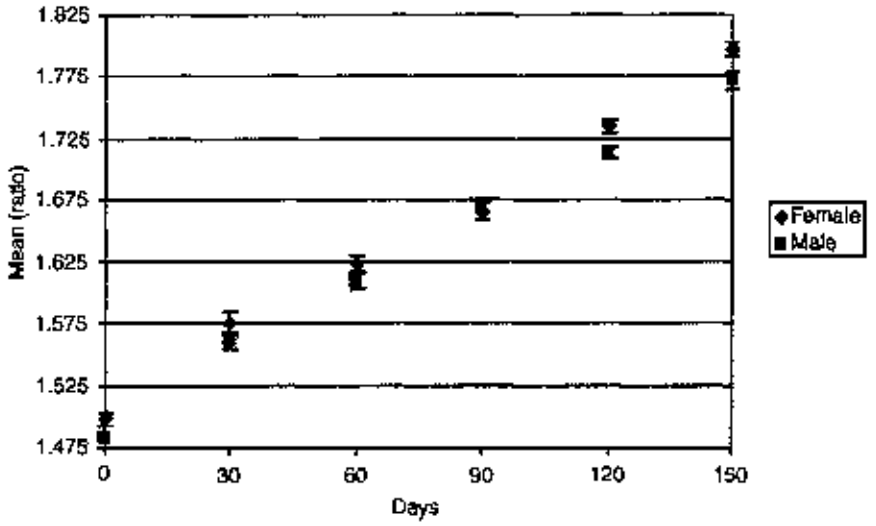

Fig. 1. One way analysis of mean (ratio) by age of ex vivo blood. It shows the change in RNA levels as a function of bloodstain age. Data represent the ratio of 18 S rRNA to β-actin mRNA as determined by real-time reverse transcriptase PCR. A model fitting the above sources of variability has a highly significant linear day effect ($P < 0.0001$) and has an adjusted R^2 of 78.2%.

Table 1
The variability associated with the results presented in Fig. 1

Sex	Days	n	Mean of ratio	Standard error	Standard deviation
F	0	72	1.49836801	0.00615666	0.05224095
F	30	72	1.57615711	0.00865694	0.07345655
F	60	72	1.62297068	0.00686272	0.05823207
F	90	72	1.66532709	0.00722973	0.06134627
F	120	72	1.73479106	0.0058206	0.04938946
F	150	72	1.79649773	0.00582809	0.04945299
M	0	72	1.48339593	0.00866593	0.07353289
M	30	72	1.56068628	0.00602969	0.05116365
M	60	72	1.61002923	0.00771633	0.06547522
M	90	72	1.66892991	0.00450743	0.03824681
M	120	72	1.73479106	0.00689064	0.04986556
M	150	72	1.79649773	0.00258789	0.02587559

N = (3 dates of blood draws) (3 bloodstains per date) (2 assays per stain) (4 people) = 72.

RT-PCR. We have successfully amplified both β-actin and 18 S RNA from 1 μl of fresh blood (fresh blood spotted onto cotton cloth and RNA immediately isolated) and then diluted 0.5-fold (~7 ng of total RNA) to 0.25-fold (~3 ng of total RNA). In theory, under the environmental conditions examined, we could amplify both β-actin and 18 S RNA using real-time PCR at levels above background from a fresh bloodstain as small as 0.25 μl. RiboGreen analysis of the RNA isolated from 0- to 90-day-old 10 μl spots of blood dried on cotton, estimated a 19% loss of total RNA from 0 to 1 day, 29% loss from 0 to 30 days and a 56% loss from 0 to 90 days (data not shown). The described real-time RT-PCR method examines specific regions on specific genes and is not intended to analyze total RNA loss, making this technique even more sensitive. Because we are examining a ratio of two RNAs, different volumes of blood should give the same ratio even if differences are observed in the actual amount of starting material. Table 2 shows the recovery of RNA from 1, 5, and 10 μl dried blood stains. As the volume of the blood decreases, the Ct values for β-actin and 18 S increase, as expected, but the difference between the two remain the same. We would expect this trend to remain the same over time but this concept still needs to be further investigated. It should be noted that only 1/6 of each reverse transcriptase reaction is used in each real-time assay, therefore, a total of five to six assays could be performed with this small amount of RNA. Clearly, this system is sensitive enough to detect RNA from nearly any sample that might be identified as biological material.

Table 2
The Ct values associated with the indicated volumes of blood.

β-Actin	18 S	ΔCt	Amount of blood (μl)
27.87	19.56	8.31	1
27.67	19.1	8.57	–
25.21	16.39	8.82	–
25.29	16.57	8.72	–
22.87	14.43	8.44	5
22.83	14.2	8.63	–
23.1	14.24	8.86	–
23.01	14.1	8.91	–
21.48	13.27	8.21	10
21.34	12.83	8.51	–
21.58	13.05	8.53	–
21.17	12.5	8.67	–

3.3. RNA- and DNA-specificity

Because the 18 S and β-actin probes are not RNA-specific, the RNA samples were for DNA contamination by targeting two different nucleic acid regions. We generated a DNA-specific (GAPNT) probe that recognizes a 5′ non-transcribed region of the human GAPDH gene and has been confirmed to recognize control genomic DNA (Fig. 2B). This was multiplexed with a commercially available RNA-specific probe from Applied Biosystems (hsBA) which spans an exon–exon boundary in the β-actin gene. This probe was also determined to be RNA-specific (Fig. 2A). GAPNT yielded no signal for the RNA sample, the sample lacking reverse transcriptase (RT) and the negative (water) control (Fig. 2A). The amplification of hsBA confirmed the presence of RNA in our samples. Fig. 2A shows that hsBA only recognizes the RNA (cDNA) isolated from the dried sample. There was no signal from either the minus RT control or for water. DNA was also isolated from the same dried blood sample and Fig. 2B shows that it is free of detectable RNA contamination and is only recognized by GAPNT. The RNA probe (hsBA) and water did not give a signal.

3.4. Species-specificity

Although not used to generate the data in Fig. 1, we have demonstrated the feasibility of developing a species-specific set of primers/probes for 18 S and β-actin. Primers and

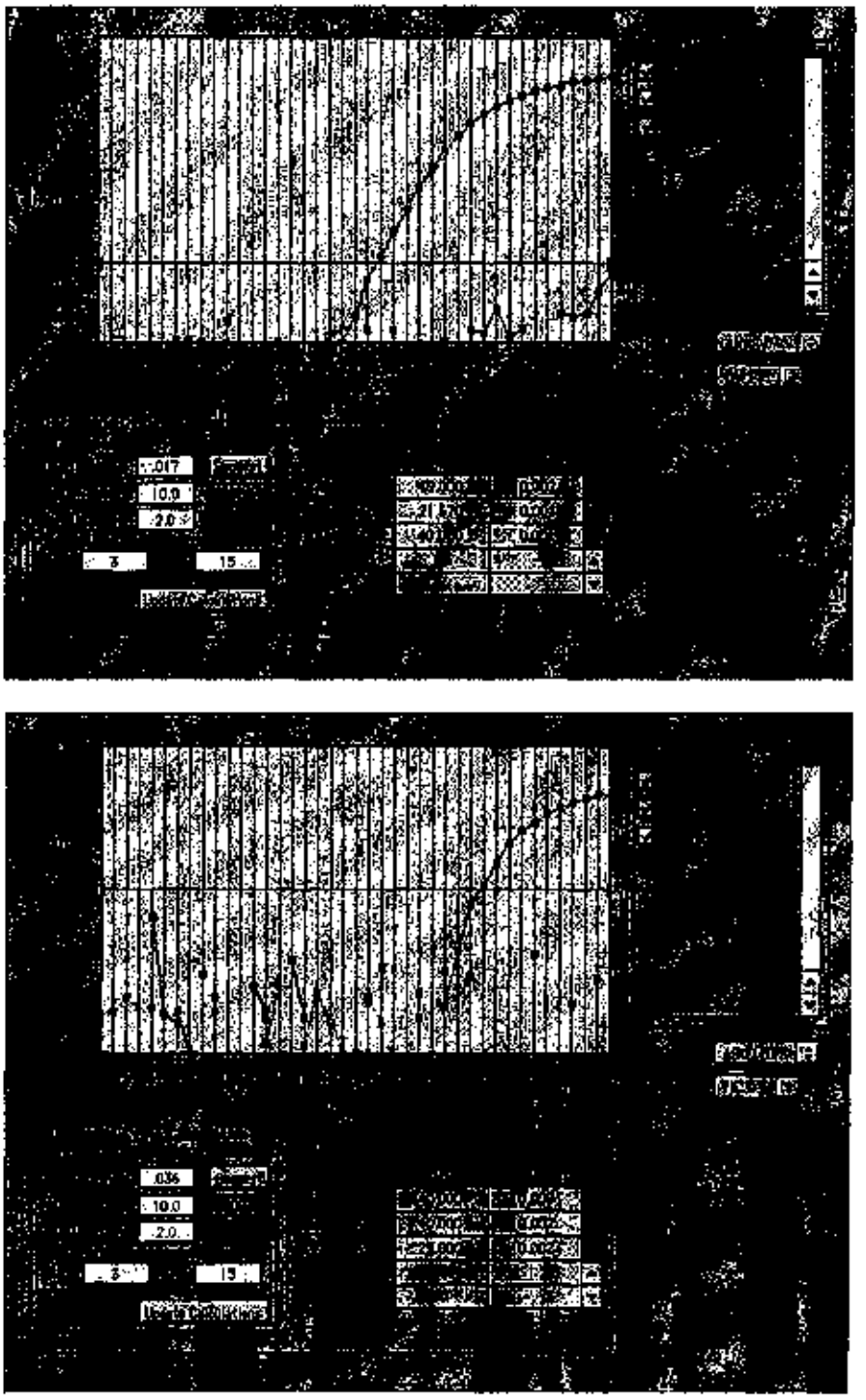

Fig. 2. Simultaneous RNA and DNA isolation from the same blood sample. Real-time amplification plots of RNA (red), cDNA (green), and DNA (blue) recovered from the same 10 μl blood sample. Part (A) shows the RNA-specific (FAM layer) primer/probe only amplifies the cDNA from the blood sample. Part (B) shows that the DNA-specific (VIC layer) primer/probe only amplifies the DNA from the blood sample.

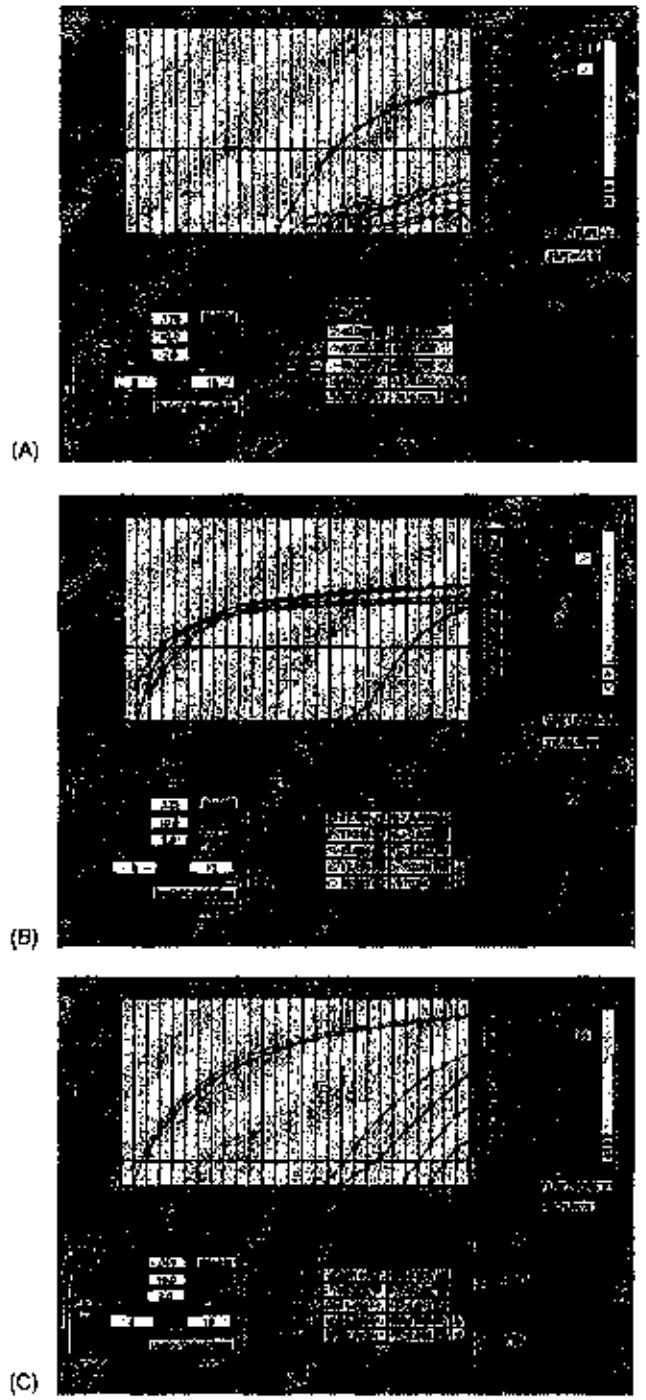

Fig. 3. Amplification of RNAs with human-specific primers and probes. Amplification of RNA from different non-human sources using human-specific primers/probe for β-actin and 18 S. RNA was amplified from human (dark blue), pig (light blue), mouse (yellow), rat (light green), dog (dark green), cat (pink), and yeast (medium blue). Part (A) shows the amplification of these cDNAs using a human-specific primer/probe set for β-actin (BA500 FAM dye layer). Water (red) was used as a negative control for the Real-time reaction. Part (B) shows the amplification of the same cDNAs from (A) with a Universal 18 S primer/probe set (VIC layer). Part (C) shows the amplification of the above mentioned cDNAs with primers/probe for the human-specific detection of 18 S (18 S4 VIC layer).

probes generated for 18 S and β-actin were designed to be species-specific based on polymorphic regions of the coding region of the genes. They were tested on RNA from cat, dog, mouse, rat, yeast, human, and pig. Fig. 3A shows that the probe for β-actin (BA500) only recognized RNA from human. Fig. 3B shows a multiplex of BA500 and Universal 18 S. Because 18 S is used for universal detection it recognizes cDNA from all species and was used as a positive control to test the cDNAs used. At this point, the18 S probe (18 S4) gives a significant signal for both human and pig (Fig. 3C). If this cannot be resolved, the RNA sample can be examined with pig-specific probes to ensure that the human sample has not been contaminated with pig RNA or the PCR product can be sequenced. Each Ct value represents a two-fold difference in starting material. Because 18 S is so prevalent, it reaches exponential amplification at cycle 14 when human RNA is amplified. A signal given at cycle 33 (mouse) represents a 2^{19}-fold difference contributing 0.00019%, which is considered to be at background levels. If our human samples were contaminated with mouse RNA, the mouse RNA would not significantly alter the results obtained with the human RNA due to the limitations of detectability of mouse RNA. Our primers and probes were also shown to yield no signal from sham-RNA isolations of various fabrics (data not shown).

4. Discussion

The results presented in this paper demonstrate that the determination of the relative ratios of two different RNAs, 18 S and β-actin, can be used to estimate the age of a bloodstain. Fig. 1 shows the rate of decay of two RNA species in dried blood over a 150-day period under controlled conditions. The Ct value of 18 S did not appreciably change over the course of 150 days but the Ct values for β-actin became significantly reduced as a function of time thus the relative ratio of 18 S rRNA to β-actin mRNA increased over time.

Both 18 S and β-actin are considered "housekeeping genes" which are expressed in all cell types at relatively high levels, thus their RNA products are likely to be recovered from crime scene evidence. The universal expression of these genes means that our analysis could potentially be applied to tissue types other than blood. Comparisons of different blood draws from the same individual and between different individuals suggest relatively constant steady-state levels of the housekeeping genes we have chosen.

18 S rRNA is a very abundant species with several thousands of copies per cell and it exists almost exclusively in a complex of ribosomal proteins comprising the small subunit of the ribosome that, we hypothesized, would provide a protective environment for the rRNA. This association may help prevent nuclease attack by shielding the rRNA from enzymatic or chemical agents which in turn makes the RNA more stable, acting in a fashion similar to histone protection of DNA. In contrast, even when loaded with polyribosomes, electron micrographs show significant gaps where the mRNA is accessible between the ribosomes. After examining several housekeeping mRNAs (data not shown), β-actin was chosen, which, although less abundant than the ribosomal species, is still a fairly abundant mRNA. Like most mRNAs, it is not permanently incorporated into a protective protein complex and thus, our data indicate, degrades more rapidly then 18 S.

The age estimate of a bloodstain may provide temporal linkage between the deposition of the blood and the time a crime was committed. Conversely, these results may be helpful in excluding samples that do not correspond to the time when a crime was committed. For example, if a bloodstain is determined to be over two months old and the crime was committed only two weeks ago, that particular bloodstain can be excluded from the crime scene evidence. In the absence of other knowledge, this technique may also be of use to approximate when the crime was committed based on the criteria mentioned above. If a bloodstain is known to have been deposited at the time a crime was committed, then the ratio of 18 S rRNA to β-actin mRNA could potentially be used to estimate the time the crime occurred.

We are in the process of examining the effects of environmental factors (microorganisms, temperature, humidity, and full spectrum light) on RNA decay. We are also expanding the study population to look at the specific effects of sex, age, and ethnicity and to apply the technique to other biological samples such as saliva and hair. We have successfully isolated RNA from an undetermined amount of saliva and a single hair follicle (data not shown).

The results that we have presented in this paper provide support for the hypothesis that there are differences in *ex vivo* RNA decay rates. It is suggested that β-actin mRNA is less stable and decays more rapidly than 18 S rRNA in *ex vivo* bloodstains. Using real-time reverse transcriptase polymerase chain reaction with the simultaneous detection of the 18 S rRNA and β-actin mRNA we have shown that relative RNA ratios can be used to estimate the time of blood deposition. Our proof of concept results show that both RNAs decay in a consistent and predictable manner. This has the potential to become a more precise and accurate means of dating bloodstains and potentially other biological samples in comparison to the previously existing techniques. The method proposed is advantageous over the previous techniques of dating dried bloodstains for the following reasons. By examining RNA ratio, the analysis is independent of sample size. Only relatively small samples are required for the test, with samples as small as 1 μl of blood being successfully processed. There is also potential for application to tissues other than blood with tissue-specific mRNA profiles to determine the tissue from which the sample is derived [6]. Species-specificity of the primers and probes help to exclude false signals due to contamination. With many methods, investigators must decide which test to apply to a given sample since many procedures preclude other analysis on the same sample. This method

allows DNA to be co-isolated with RNA, providing simultaneous analysis for the identification of the person from whom the biological sample was derived and estimating when it was deposited. Additionally, all of the experimental manipulations occur simultaneously to both target RNAs in the same tube thus eliminating potential issues such as pipeting errors or differences in enzyme efficiency. Finally, in the dried blood samples, the time period over which useful estimates can be made is at least on the order of 150 days.

The opinions, findings, and conclusions or recommendations expressed in this publication are those of the authors and do not necessarily reflect the views of the Department of Justice.

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