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PAPER

CRIMINALISTICS

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An RNA Expression Method for Aging Forensic Hair Samples

ABSTRACT: A common limitation to most forensic trace evidence analysis is the ability to determine the time at which the evidence was deposited at the crime scene. This issue of timing is vitally important as it may not only reveal when the crime occurred, but could exclude potential suspects from the investigation. Using a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay, we monitored the relative expression ratio (RER) of two different RNA species (18S and β-actin) in hair samples that were aged naturally over a period of 3 months. No gender or age-of-donor biases were observed, and results were linear up to 60 days. After 60 days, the results were more variable and gave unreliable estimates of time since deposition. Overall, the results presented in this paper suggest that the age of hair samples containing follicular tags can be approximated using a second-order polynomial, although with limitations: Age = $3.31\text{RER}^2 - 2.85\text{RER} - 0.54$ ($R^2 = 0.98$).

KEYWORDS: forensic science, hair, trace evidence, RNA degradation, quantitative polymerase chain reaction, deposition age estimation

Determining the age of physical evidence or more specifically the time it was deposited at a crime scene is a common limitation of many different types of trace evidence. Linking a particular piece of evidence or person to a crime scene is vitally important in any criminal investigation. The time that this interaction occurs is significant and without it, the value of the evidence can be significantly decreased or in some cases completely lost.

Chemical evidence, such as paints, inks, and drugs (to list a few), tends to age in chemically predictable ways (1), and therefore, developing methods to measure these changes (and age these types of samples) have been relatively successful (2). Aging biological evidence is more difficult owing to the unpredictable degradation processes that are inherently involved with such material. The success rate in developing techniques to age the more common types of biological evidence recovered from crime scenes, such as blood (3,4), semen, hair, fingerprints (5), and bone (6,7), has varied considerably. The techniques themselves have been just as diverse ranging from simple visual examinations to more complicated techniques, such as GC-MS, X-ray diffraction, and DNA/RNA analysis. In the last few decades, there has been a focus on developing methods for determining the age of bloodstains due primarily to the prevalence of blood at major crime scenes. Spectroscopic (8), entomological (9), enzymatic (10), and chromatographic methods (11,12) have all been tried as well as electron paramagnetic resonance spectroscopy (13) and even specialized microscopy techniques (atomic force microscopy). Unfortunately, the results from these studies have been approximate at best and have failed to pinpoint the age accurately.

Perhaps the most promising work to date has involved RNA degradation studies. Anderson et al. (3) developed a reverse transcription quantitative polymerase chain reaction (RT-qPCR) assay to show that the ratio between two different types of RNA changed in a linear manner over the course of 150 days. There were a number of advantages to this approach. By examining a ratio of relative quantities, the analysis is independent of the initial sample volume, high sensitivity is achieved through the PCR amplification step, species-specific probes increase the assays specificity, and the method allows for the simultaneous extraction of DNA and RNA. Their results also suggest that there is the potential to apply this method to various other tissues as the targeted RNA species are universally expressed in all tissue types. Based on these assumptions, we have used a previously untested sample type (hair) to carry out a similar study.

Hair is one of the most common types of trace evidence found at crime scenes owing to the fact that each human loses on average 100 telogen phase hairs a day, it is easily removed in a struggle, and hair is generally very persistent, especially on rough fabrics and beneath fingernails (14). Hair can often provide corroborating evidence to link a person or object to a crime scene, provide a drug history of the donor, and in some cases it may even identify the source of the evidence via DNA profiling. However, to our knowledge, there are no available methods to reliably determine the age of hair samples. We have therefore applied the RT-qPCR technique to estimate the age of hair samples based on the degradation rates of two different RNA species, β-actin and 18S rRNA.

Methods

Hair Collection and Sampling

Hair samples were collected from a total of 10 individuals, all of who were white Caucasian of either English or Irish ethnic background; five individuals were aged between 20 and 25 years old (two women and three men), and five individuals were aged between 40 and 45 years old (three women and two men). The hairs were plucked from the head of each volunteer using tweezers and examined microscopically to ensure that a hair root (follicular

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tag) was attached. The last 1 cm of each hair (inclusive of the follicular tag) was cut and stored in sterile 1.5-mL Eppendorf tubes. This was repeated so that each tube contained 10 hair roots. One tube constituted one sample. A total of 20 hairs (two samples) were collected from each person for each time point (0 [control], 1, 5, 15, 30, 45, 60, 75, and 90 days), ensuring there were duplicate samples for each time frame. This created a total of 180 samples for all time points. The samples were then left to age at room temperature for the specified time periods to simulate natural aging.

RNA Extraction

RNA was isolated from each sample at the various time periods using an organic extraction method (TRI reagent BD; Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions with the exception of a few minor modifications. For each sample, 500 μL of TRI reagent was added to each of the Eppendorf tubes containing the 10 hair follicles along with 200 μL of PCR grade water. The tubes were then vortexed for 15 sec followed by 10-min incubation at 50°C. Instead of chloroform, 100 μL of 1-bromo-3-chloropropane was added to each tube and the samples were again vortexed for 15 sec. The samples were incubated for 5 min at room temperature and then centrifuged at 12,000 × *g* for 15 min at 4°C. The aqueous layer was transferred to a new sterile 1.5-mL Eppendorf tube, and 500 μL of cold (4°C) 2-propanol and 3 μL of a polyacrylamide carrier (Bioline, U.K.) were added. The samples were mixed gently, incubated at room temperature for 10 min, and centrifuged at 12,000 × *g* for 10 min at 4°C. The pellet was washed with 1 mL of 75% ethanol vortexed briefly and centrifuged at 12,000 × *g* for another 5 min at 4°C. The supernatant was removed and the wash step repeated. The RNA pellet was allowed to air dry for 5 min and resuspended in 20 μL of RNase-free ddH_2O. Two negative control samples (containing no hair) were carried out at each time point and processed in the same manner. All samples were treated with DNase I to avoid genomic DNA carryover. The RNA quantity was measured using Implen's NanoPhotometer (Implen GmbH, Munich, Germany).

Reverse Transcription

Reverse transcription was carried out using random priming and SuperScript II (Invitrogen, Paisley, U.K.) reverse transcriptase chemistry. Before the actual RT reaction, the random hexamers (Invitrogen), deoxynucleotriphosphate mixture (dNTP), and ddH_2O were mixed to final volume of 7 μL. Five microliters of RNA sample was then added and the mixture incubated for 5 min at 65°C. Samples were immediately chilled on ice and spun briefly before 5 × first stand buffer (Invitrogen) and DTT were added. The samples were briefly vortexed before incubation at room temperature for 2 min. Finally, 200 U of SuperScript II reverse transcriptase was added to each tube, giving final volume of 20 μL containing 500 μM dNTPs, 50 mM Tris–HCl, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 200 U of SuperScript II, and 0.1 μg random hexamers. The reaction components were gently mixed by inverting the tubes a couple of times before being incubated for 10 min at room temperature followed by 42°C for 50 min. The reaction was terminated by incubating the samples at 70°C for 15 min, and the samples were stored at −20°C prior to further use.

Quantitative Real-time PCR

A real-time PCR master mix was prepared and consisted of the following: Primers and Probes β-actin control reagent kit, Ribosomal RNA control kit, TaqMan Universal PCR Master Mix with UNG (all from Applied Biosystems, Foster City, CA), and nuclease-free ddH_2O. The final optimized concentrations used in the reactions were as follows: 200 nM β-actin probe (FAM dye) and 300 nM for both the forward and the reverse β-actin primers; and 200 nM 18S rRNA probe (VIC dye) and 100 nM for both the forward and the reverse 18S rRNA primers. Twenty-three microliters of the prepared PCR master mix was added to each well of the MicroAmp® Optical 96-well reaction plate (Applied Biosystems). Two microliters of each reverse transcribed RNA sample was added to the appropriate well to make a final reaction volume of 25 μl. Triplicate biological replicates were run for each RNA sample along with negative control and human genomic DNA as a positive control (Bioline, London, U.K.). The reaction plates were centrifuged briefly prior to qPCR analysis.

For qPCR analysis, an AB 7500 qPCR instrument was used according to manufacturer's instructions. The data were analyzed using SDS software (version 1.4.0; Applied Biosystems). The fluorescence threshold was set in the middle of the exponential (linear) phase of the PCR. A passive reference dye (ROX) was included in each sample as an evaporation and pipetting control, and an exogenous control (cDNA interplate calibrator) was used to adjust any variation between runs.

Statistical Analysis

Threshold cycle (Ct) values were exported into GenEx statistical software (version 4.3.7; BioEPS GmbH, Munich, Germany), which is designed specifically for qPCR data. Values were preprocessed which involves normalizing raw fluorescence Ct values against interplate calibrators, individual efficiency rates, qPCR repeats, and reference controls. This normalization process addresses the issues raised by Pfaffl (15), and the resulting Ct values were corrected according to his compensating formula, which involved replacing the base number of 2 with 1 + E (where E is the amplification efficiency rate).

Results were tested for normal distribution and upon conformation were subjected to regression analysis (geometric mean, SD, SEM, %CV and independent *t*-tests) using InStat 3 statistical software (version 3.06; GraphPad, La Jolla, CA). The relative expression ratios (RERs) were calculated from the normalized data. Owing to the differences observed in the target amplification efficiency rates, the formula proposed by Pfaffl (15), which compensates for any slight variation, was used to calculate the RERs:

$$\text{Relative Expression Ratio} = (1 + E_{\text{target}})^{\Delta Ct\ (\text{target})} / (1 + E_{\text{reference}})^{\Delta Ct\ (\text{reference})}$$

where E is the efficiency of the individual target species and ΔCt is the change in the Ct values.

To ensure there were no inhibitors present, the efficiency rates in a selected number of test samples (actual) were determined to ensure they were similar to those predicted from the standard curve (expected). For the purpose of this study, the values were considered similar enough to use the expected rates of 95.2% (18S) and 92.5% (β-actin) to quantify all test samples.

Primers and Probes Selection for qPCR

Human-specific primers and probes were obtained from commercially available kits (Applied Biosystems). 18S primers and probes were obtained from the TaqMan Ribosomal RNA Control Reagent Kit (Part Number: 4308329), while the β-actin primers and probes

originated from the TaqMan Human ACTB Endogenous Control Kit (Part Number: 4352935E). The primers and probes were selected based on their success by Anderson et al. (3).

Results and Discussion

Assay Optimization

Prior to any sample analysis, it was imperative to ensure that the qPCR assay was working efficiently and any limitations corrected for. This required full primer optimization (Figs 1 and 2) and complete efficiency testing of the optimized multiplex assay as well as on a selection of random test samples. The primer combinations that had the greatest efficiency (lowest Ct values) in the singleplex optimization studies were tested as a multiplex assay. Multiple combinations were tested, although only results from the two most efficient multiplex assays are presented here.

In assay 1, primer concentrations were the same as those stipulated by Anderson et al. (3) in their analysis of blood samples. Using a standard curve method, the efficiency rates for each species were calculated (β-actin: 83% and 18S: 88%). A second multiplex assay (assay 2) was tested using the optimal primer concentrations, as defined in the singleplex optimization study. The primer concentrations for β-actin were unchanged (300 nm); however, the concentration of both the forward and reverse 18S primers was increased to 100 nm. Efficiency rates of 92% (β-actin) and 95% (18S) were obtained. This particular combination of primers outperformed all other combinations tested (data not shown).

The efficiency rates from five hair samples, chosen at random, were determined and compared to the rates obtained from the multiplex assay 2. This was essential to ensure there were no inhibitors present in the test samples. The mean efficiency rates of the five random samples were 97.2% (18S) and 94.1% (β-actin), and this was comparable to 95.2% (18S) and 92.5% (β-actin), respectively, from assay 2. Detailed results can be found in Table 1. The differences observed were small at 2% and 1.6%, respectively; however, the variation in this data set was significant (data not shown).

Stability of 18S rRNA and β-actin mRNA

The results presented here suggest the two selected targets are suitable for this type of analysis, given the key criteria were to have two targets that degraded at consistent and yet different rates. 18S rRNA appears to be stable for approximately 45 days (excluding the noticeable effects during the first day) therefore may be a suitable candidate gene for this sample type over this particular period of time. After 45 days, the 18S species began to show signs of degradation and this was evident in the increase in Ct values but more accurately described by the decrease in the relative expression (Fig. 3). The relative expression of β-actin mRNA continually decreased with time, suggesting that the degradation process was relatively uniform. A sharp initial decrease in the expression of both species was observed between day 0 and day 1, indicating an accelerated rate of degradation. This rate was similar to those seen with samples older than 60 days.

RNA Quantification: Sensitivity

One of the prior concerns regarding the extraction of RNA was whether sufficient amounts could be obtained from very small starting quantities (10 follicular tags). Although various authors (3,14) have claimed to have recovered sufficient quantities of RNA from a single hair, the figures published regarding the exact quantities vary considerably (average RNA yield was 112.5 ng per hair follicle).

The quantity of total RNA in each sample was determined using a NanoPhotometer. The average yield of total RNA extracted per sample (10 hair roots) from all 180 samples was 88.25 ng. The mean quantities displayed a discernable trend with a continual decline in the amount of RNA extracted over the 90 days. Spectroscopic analysis of the RNA recovered from hair samples estimated

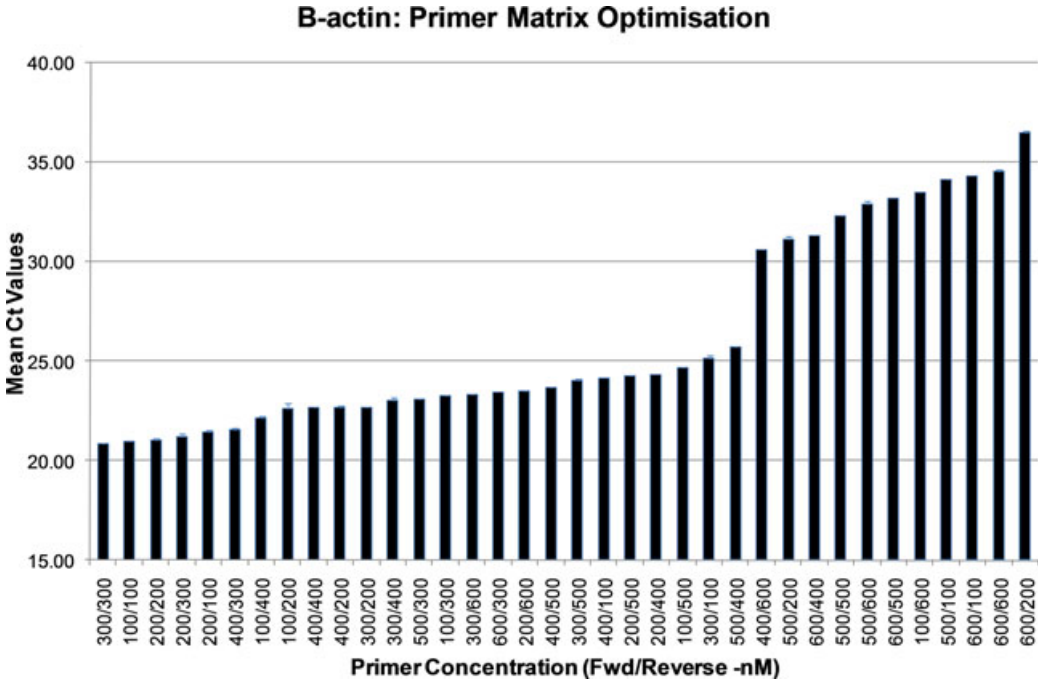

FIG. 1—β-*actin primer optimization. Primer concentrations of 300 nm for both forward and reverse primers were the most efficient as indicated by the lowest mean Ct value. A singleplex SYBR green detection system (Applied Biosystems) was used for the analysis. Error bars represent +1 SD.*

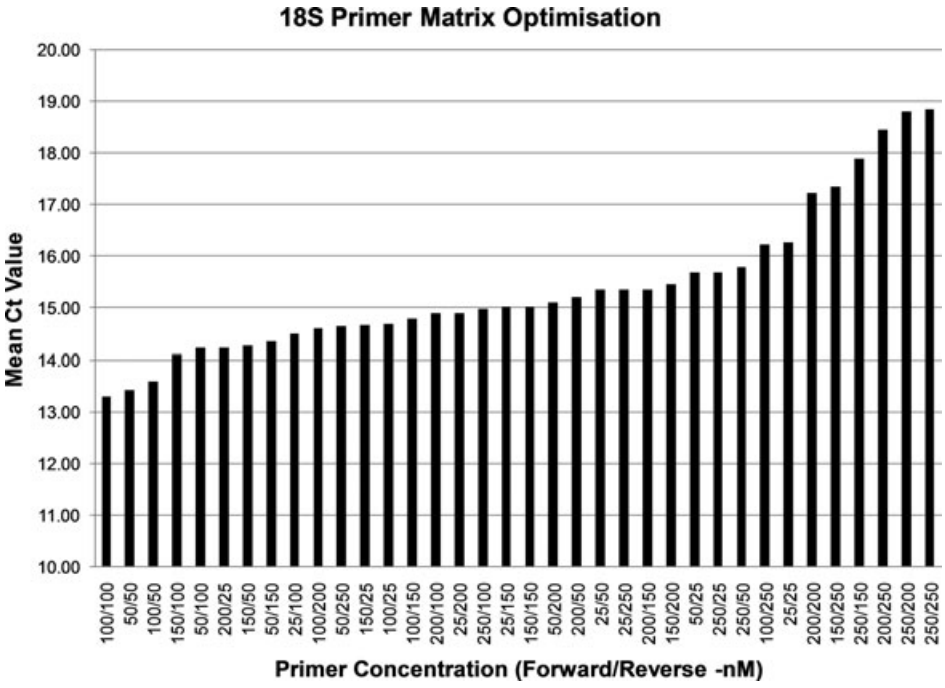

FIG. 2—*18S primer optimization. Primer concentrations of 100 nm for both forward and reverse primers produced the most efficient reaction rate. Error bars represent +1 SD.*

TABLE 1—*The 18S and β-actin efficiency rates of five random test samples (actual rates) were determined and compared to the efficiency rates obtained from the optimized multiplex assay (expected rates).*

Random Sample	Mean Ct Values		
18S	Neat	1/10	1/100
Sample 1	20.76	23.99	27.35
Sample 2	21.28	24.50	27.84
Sample 3	19.84	23.34	26.57
Sample 4	24.52	27.91	31.49
Sample 5	25.60	29.08	32.68
β-actin			
Sample 1	25.15	28.56	32.34
Sample 2	27.42	30.45	34.29
Sample 3	27.01	30.23	33.85
Sample 4	32.15	35.39	38.84
Sample 5	33.08	35.53	40.24

Efficiency Rate (%)	Optimized Multiplex Assay	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5
18S	95.2	100.9	101.7	98.2	93.6	91.6
β-actin	92.5	89.7	95.5	96.0	99.0	90.2

a 79% loss of total RNA from 0 to 90 days; however, at 90 days, there was still a sufficient amount of RNA recovered for subsequent processing using qPCR.

The level of sensitivity was tested by reducing the number of hair strands (follicular tags) used per sample. Real-time PCR signals from both the *β*-actin and 18S species were consistently detected in samples containing as little as three follicular tags. 18S was detected in samples containing a single hair strand; however, success was inconsistent (three of 10 samples) and the Ct values obtained were outside the dynamic range of the assay. This would have implications in an operational setting where only a single hair strand was recovered from a crime scene.

Inter- and Intra-donor Variation

Inter-donor variation was statistically calculated using the normalized qPCR data. A number of features were observed; though given the size of the population used in this study, further investigation is required to confirm these findings. The inter-donor variation of this data set was determined at each time period and ranged from 3.26% (5 days) to 8.22% (90 days) with a mean value of 5.98% across all aging times. The inter-donor variation showed a time-wise dependent increase, suggesting that external degradation factors are not consistent. The small inter-donor variation observed at the beginning of the study suggests low levels of natural biological variance *in vivo*. This is an important feature for this type of forensic analysis. Although the inter-donor variation increased with time, the values obtained were comparable to previously published data. Biological samples are often a source of significant variation especially dynamic tissue types, such as whole blood (3,4), where a single sample yields an average expression value from numerous different cell types. With hair samples, only a single cell type is analyzed which may explain why the variation was less than reported values from studies involving blood tissue.

Intra-donor variation varied from 0.39% (donor 6) to 1.05% (donor 10) with an overall average of 0.67%. The small intra-donor variation may be a direct reflection on the physical nature of hair in that it may be a more consistent entity than other tissue types. There was no evidence to suggest any gender or age bias associated with this variation (based on nonparametric tests).

Relative Expression Ratio

The common occurrence of hair samples found at crime scenes means they are a relevant sample type for aging studies. Our results demonstrate how the RER of two different species of RNA, 18S rRNA and *β*-actin mRNA, extracted from the follicular tag of hair samples, changed in a linear manner over time, thus

allowing an age estimation to occur. The RERs obtained from hair samples that had been aged for up to a period of 90 days under relatively uncontrolled conditions (room temperature, room humidity, and in the presence of daylight) are presented in Fig. 4. Samples were intentionally aged under these conditions to mimic forensic-type environments and to assess the robustness of the proposed method.

The RERs behaved in a linear and reproducible fashion with a relatively strong correlation with samples aged up to 45–60 days but as the time proceeded, the rate of increase tapered off and a

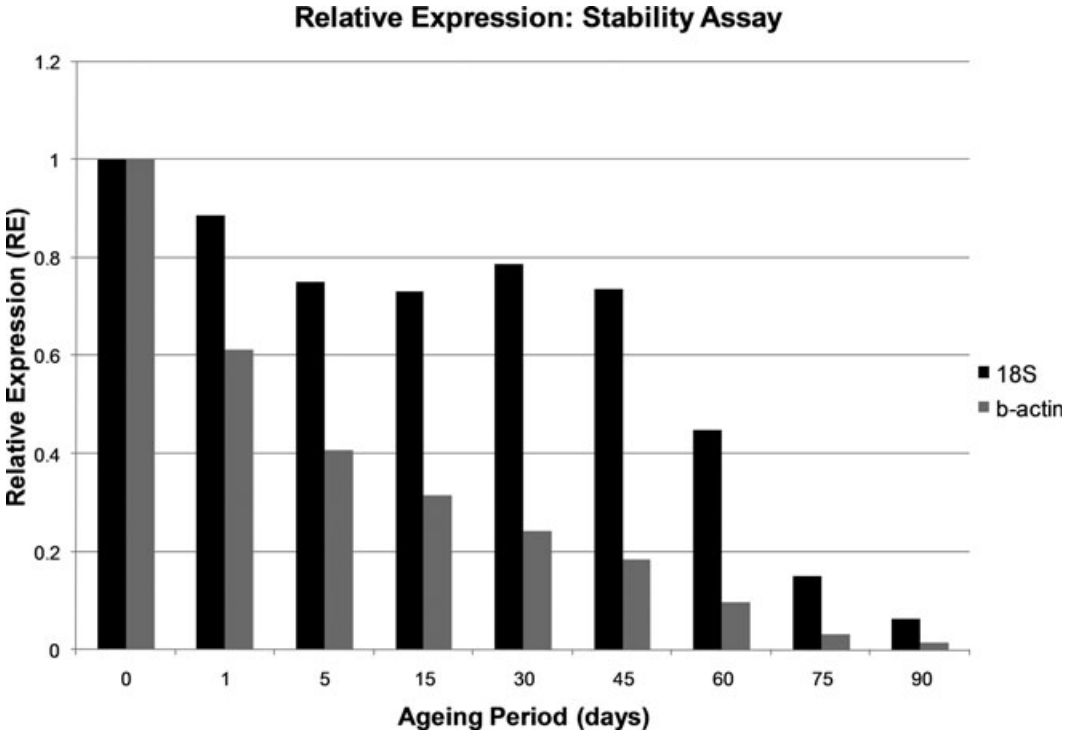

FIG. 3—*There is an initial decline in the expression of both species during the first day of aging.* β*-actin continues to degrade in a consistent manner over the time of the study, whereas the expression of the 18S species does not changed significantly between day 1 and day 45, highlighting its stability.*

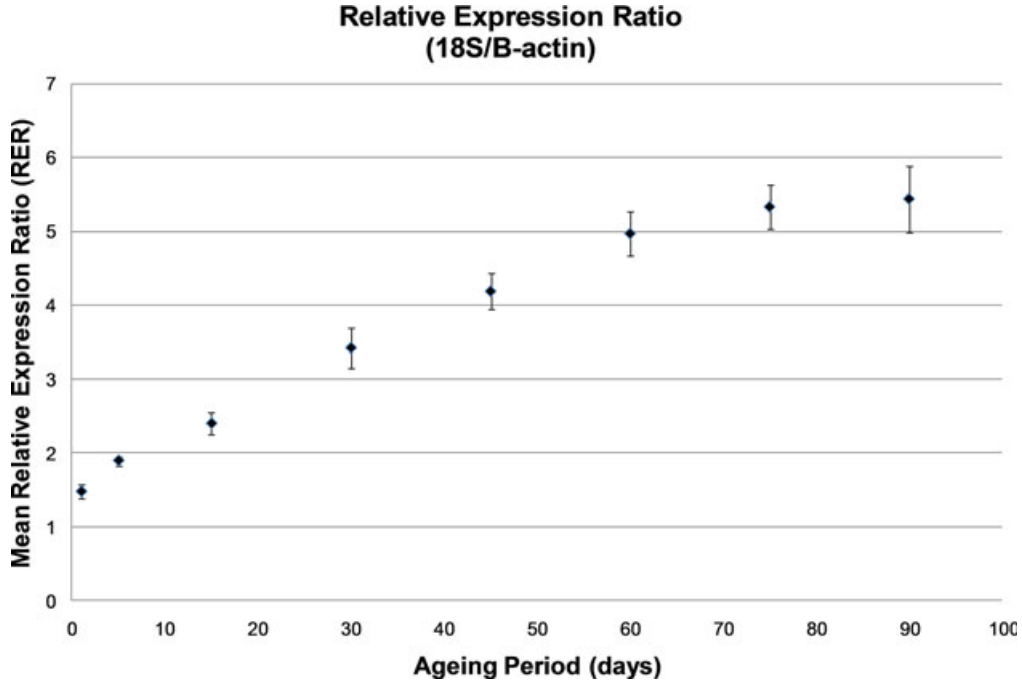

FIG. 4—*The mean RERs obtained from human hair samples aged over a 90-day period are presented above. The RERs increase in a linear manner in samples aged between 0 and 60 days. After 60 days, this trend appears to change and the plateau observed results in the loss of discrimination (age) using this method. Error bars represent ±1 SD.*

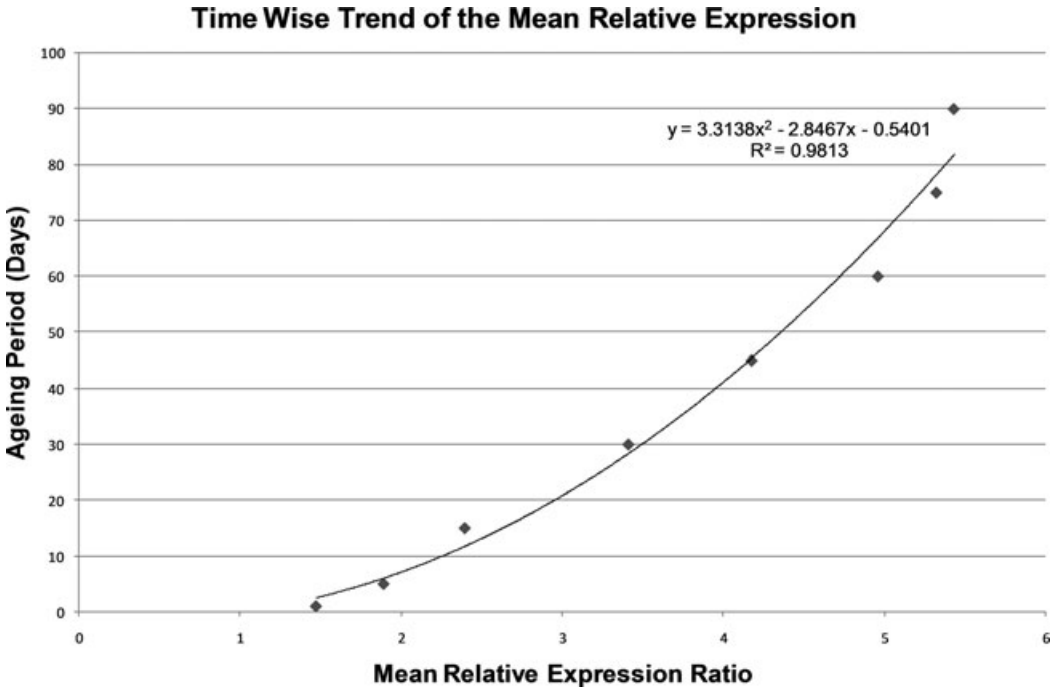

FIG. 5—*Time-wise trend in the RER. A second-order polynomial can be applied to the mean RERs over the 90-day aging period allowing for sample age estimations.*

plateau effect was observed. There was a noticeable decrease in the 18S relative expression in samples older than 60 days. This sudden increase in degradation rate was comparable to that experienced by β-actin mRNA which behaved consistently over the entire 90 days. It is uncertain as to why the 18S species began to degrade around this period of time but it is thought that it may be because of the breakdown of the ribosomal complex, which is thought to be primarily responsible for protecting the 18S rRNA (3). Breakdown of this complex would release the 18S species into the same environment as the β-actin mRNA and thus be exposed to the same degradation factors.

Another notable feature of this study involved the higher rate of degradation experienced by both species of RNA during the first day of aging. This could be a result of increased intra-cellular enzymatic activity, which is dependent on the natural aqueous environment common to all living cells. This increase rate was consistent across all 10 donors.

The error rate or confidence with any estimation varied over the 90-day period. Samples that had aged for <30 days could be distinguished within a 10- to 15-day age range. However, as the time frame increased, the ability to distinguish one sample from other decreased and the estimated age range became larger to the point where samples could not be distinguished at all after 60 days.

The RER values presented with a normal (Gaussian) distribution, and therefore, accurate interpretation could be made from the relevant statistical analysis although consideration must be made to the small number of samples tested. Independent paired *t*-tests and *F*-tests were performed on the mean RERs for this data set. The results suggested that the difference in the means between genders was not significant. A similar statistical analysis was performed to determine whether there was any evidence to suggest an age bias in the data. We could not detect any age-related effects on the mean RERs in this sample set. Given the small population size (10), any statistical findings should be viewed with caution but based on the results presented here, there is sufficient evidence to warrant further investigation.

Combined Data and Trend Analysis

Figure 5 illustrates the degradation kinetics, and based on these RER values, the aging process can be approximated using a second-order polynomial ($R^2 = 0.98$):

$$A = 3.31RER^2 - 2.85RER - 0.54$$

where A is the age of the hair in days and *RER* is the relative expression ratio.

To provide age estimations with a higher degree of confidence, it is suggested that a range rather than an exact figure be stipulated. Because the analysis is based on a single calibrator (slow-degrading housekeeping gene), multiple aging markers combined with geometric means could improve the estimation significantly.

Although a polynomial equation has been proposed to age hair samples using the resulting RERs, the authors acknowledge the limitations associated with this study. The population tested was small ($N = 10$), the lineal relationship between sample age and RER only exists for samples younger than 60 days (although could be improved with additional data), there is a limit to the amount of material required (minimum of three hairs), and hair sample type is specific (must contain a follicular tag).

Conclusion

This paper provides further support for RNA as a suitable candidate for forensic analysis owing to the highly sensitive processing techniques and its stability in forensic type samples. Both 18S and β-actin species have proven to be suitable targets for age estimations of hair samples, and this is predominately because of their patterns and rates of degradation, although this may be limited time-wise. The accuracy and precision observed suggests that a more reliable approach would be to provide an estimate range, rather than an exact value. In many criminal cases, especially those involving serious crime where the scene is generally processed

immediately, the range will be too large to be of any value. However, in certain situations, this level of detail may provide corroborating evidence or merely present as an investigative tool.

As well as using increased sample numbers, future research will focus on determining the cause of the increasing inter-donor variation and the effects of the various environmental factors. Different targets will be examined in an attempt to find suitable candidates for discriminating samples aged for longer than 60 days, and we are currently evaluating other suitable housekeeping genes for this type of analysis.

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