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RNA analysis as a method to determine the age of a biological sample

Stacey E. Anderson
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RNA Analysis as a Method to Determine the Age of a Biological Sample

Stacey E. Anderson

**Dissertation submitted to The Eberly College of Arts and Sciences at West Virginia
University in partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy
In
Cellular and Molecular Biology**

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Keywords: RNA decay, Real-time PCR, Forensic Analysis

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ABSTRACT

RNA Analysis as a Method to Determine the Age of a Biological Sample

Stacey E. Anderson

DNA analysis by Polymerase Chain Reaction (PCR) allows for the unambiguous identification of the person from whom a biological sample was derived but provides no information about when the sample was deposited. The ability to determine the age of blood or a biological sample would be of significant benefit to the forensic science community. This would provide a temporal linkage between the blood of the perpetrator and the commission of a crime or it could be used to exclude evidence that does not correspond to the time a crime was committed. It was hypothesized that *ex vivo* RNA decay was a predictable process and real-time PCR indicated that different types of RNA and different sized regions of RNA influenced our capabilities of RNA detection in dried blood stains. The ratio/difference in Ct values between different types and regions of RNA (rRNA vs. mRNA), was identified to change over time in blood stains. Under the conditions examined in the present study, the ratio between the identified RNA species changed in a consistent and reliable way. Environmental influences on RNA detection in dried blood were explored; temperature and humidity both influenced RNA detection but full spectrum light did not. In order to establish a shorter time frame of detection, DNA microarrays were used to identify RNAs that may undergo changes in RNA levels *ex vivo* due to a stress response. Preliminary results identified several candidate RNA that had up-regulated levels from 4-96 fold in the first 1-4 hours after deposition. Although other approaches have been used in the past to estimate the age of a biological sample, this approach offers the following advantages: DNA and RNA can be co-isolated from the same sample; probes can be made species specific; relatively small samples can be assayed; and, the window of time estimates for dried blood is at least 0 to 150 days. This work provides a starting point for a technique that may prove useful in estimating the age of a biological sample. With the integration of the above mentioned techniques, this method has the potential to solve a problem the forensic community has been investigating for over 100 years.

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TABLE OF CONTENTS

Title Page	i
Abstract	ii
Acknowledgments	iii
Table of Contents	iv-v
List of Tables	vi
List of Figures	vii-ix
Introduction	1-11
Chapter One-A Method to Determine the Age of a Biological Samples	
Introduction	12
Methods	12-28
Results	28-38
Discussion	38-41
Chapter Two-The Effect of Amplicon Size on RNA Detection in Dried Blood Stains	
Introduction	42
Methods	43-55
Results	55-79
Discussion	80-84
Chapter Three- Environmental Effects on RNA stability in Blood	
Introduction	85
Methods	85-87
Results	88-102
Discussion	103-106
Chapter Four- DNA Microarray analysis of <i>ex vivo</i> Blood stains	
Introduction	107
Methods	107-112
Results	113-127
Discussion	127-134

Chapter Five-A Method to Determine the Age of a Blood Stain-An Extended

Review

Introduction	135
Methods	135-139
Results	139-151
Discussion	152-154

Final Discussion	155-165
-------------------------	----------------

References	166-170
-------------------	----------------

Appendix	171-172
-----------------	----------------

Curriculum Vitae	173-174
-------------------------	----------------

LIST OF TABLES

Table 1-1. Threshold and Baseline Values for All Primer/Probe Sets.	23
Table 1-2. Controls.	23
Table 1-3. Subject Information for Dried and Aqueous Trials.	25
Table 1-4. Mean RNA recovery.	29
Table 1-5. Recovery of RNA from ACD and non-ACD treated blood.	32
Table 1-6. Confidence Intervals Associated With a Given RNA Ratio.	38
Table 2-1. Sequences of Amplicons.	44
Table 2-2. Identification of RNAs used for Analysis.	48
Table 2-3. Mean RNA Recovered from Blood Deposited on Different Surfaces.	74
Table 2-4 A-B. Real-time Analysis of RNA Isolated from Blood Deposited on a Different Surfaces	76
Table 2-5. Sensitivity of Experiment using Real-time Analysis.	78
Table 2-6. Sensitivity of Experiment using RiboGreen Analysis	79
Table 4-1. Primer and probes sequences required for PCR amplification of each of the identified mRNAs.	110
Table 4-2. Optimal primer and probes concentrations required for PCR amplification of each of the identified mRNAs.	112
Table 5-1. Subjects Involved in Final Population Study	134
Table 5-2. Statistical Analysis Associated with Final Population Study	149
Table 5-3. Confidence Intervals Associated with 18S (180 bp)/BA (180 bp)	145
Table 5-4. Confidence Intervals Associated with 18S (180 bp)/18S (500 bp)	151
Table 5-5. Confidence Intervals Associated with BA (60 bp)/BA (300 bp)	151

LIST OF FIGURES

Figure 1-1. Flow Chart for Aqueous Blood Experiment.	27
Figure 1-2. Housekeeping RNA Prevalence in Dried Blood.	30
Figure 1-3A-B. Simultaneous RNA and DNA Isolation from the Same Blood Sample.	33
Figure 1-4. RNA Decay in Aqueous Blood Over Time.	35
Figure 1-5. One Way Analysis of Mean (ratio) by Age of <i>Ex Vivo</i> Blood Under Constant Conditions.	37
Figure 2-1. Locations of Amplicons in RNA	47
Figure 2-2. Analysis of RNA Isolated from Dried Blood Using 18S (60 bp)/BA (60 bp).	56
Figure 2-3. Real-time Analysis of RNA Isolated from Dried Blood Samples.	58
Figure 2-4. Species-Specificity of Primer/Probe Sets.	60
Figure 2-5. RNA Detection of Various Fabrics	62
Figure 2-6. Variation in Isolation Technique.	64
Figure 2-7. RNA Stability in TRI Reagent BD	65
Figure 2-8. The Effect of -20 °C Storage on RNA Detection.	67
Figure 2-9. RNA Detection of Different Blood Volumes.	69
Figure 2-10. STR Analysis of DNA Isolated From Dried Blood Stains.	71-72
Figure 3-1. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp)	89
Figure 3-2. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp).	90
Figure 3-3. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using BA (60 bp)/BA(300bp)	91
Figure 3-4. The Effects of Temperature on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp).	94
Figure 3-5. The Effects of Temperature on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp).	95

Figure 3-6. The Effects of Temperature on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp).	96
Figure 3-7. The Effects of Humidity on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp).	97
Figure 3-8. The Effects of Humidity on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp).	98
Figure 3-9. The Effects of Humidity on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp).	99
Figure 3-10. The Effects of Re-hydration on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp).	100
Figure 3-11. The Effects of Re-hydration on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp).	101
Figure 3-12. The Effects of Re-hydration on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp).	102
Figure 4-1. Scatter plot analysis identifying the results from the first DNA microarray.	115
Figure 4-2. Messenger RNA levels in 200 µl of aqueous blood.	116
Figure 4-3. Messenger RNA levels in 30 µl of dried blood.	118
Figure 4-4. Messenger RNA levels in Blood Deposited on Different Surfaces.	119
Figure 4-5. Messenger RNA levels in 1 ml of dried blood	120
Figure 4-6. EGR1 mRNA levels in Blood Collected with Different Additives.	122
Figure 4-7. GOS1 mRNA levels in Blood Collected with Different Additives	123
Figure 4-8. RGPS mRNA levels in Blood Collected with Different Additives.	124
Figure 4-9. Scatter plot analysis identifying the results from the second DNA microarray.	126
Figure 4-10. Messenger RNA levels in 200 µl blood stains.	128
Figure 4-11. Messenger RNA levels in 100 µl blood stains.	129
Figure 4-12. Messenger RNA levels in 100 µl blood stains.	130
Figure 4-13. Messenger RNA levels in 200 µl blood stains.	131
Figure 5-1. Preliminary Analysis using 18S (60 bp)/BA (60 bp).	140

Figure 5-2. Preliminary Analysis using 18S (180 bp)/18S(500bp).	141
Figure 5-3. Preliminary Analysis using BA (60 bp)/BA (300 bp).	142
Figure 5-4. Preliminary Analysis using 18S(180 bp)/BA (180 bp).	143
Figure 5-5. One Way Analysis of Mean (Difference) by Age of <i>Ex Vivo</i> Blood Under Constant Conditions using BA (60 bp)/BA (300 bp).	145
Figure 5-6. One Way Analysis of Mean (Difference) by Age of <i>Ex Vivo</i> Blood Under Constant Conditions using 18S (180 bp)/18S (500 bp).	146
Figure 5-7. One Way Analysis of Mean (Difference) by Age of <i>Ex Vivo</i> Blood Under Constant Conditions using 18S (180 bp)/BA (180 bp).	147

Introduction

Problem

DNA analysis has revolutionized the field of forensic science by allowing for the identification of the person from whom a biological sample has been obtained [1]. DNA analysis provides a spatial link between a suspect and a crime scene or other location relevant to a crime investigation. In many instances, this information is sufficient for conviction or exoneration of the suspected perpetrator of a crime. DNA analysis does not, however, indicate when a sample might have been deposited at the crime scene [2]. A sample's deposition could have taken place months or even years before a specific crime was committed. Samples could also have been deposited after the commission of a crime. 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 a suspect in the home, vehicle, or other pertinent location associated with a victim is not unexpected. DNA analysis can thus verify from whom the blood or other biological evidence came from but provides no information on when the material was deposited. For example, if a suspect's blood were found in the vehicle of the victim, the accused could argue that the sample had been deposited before the crime was actually committed, and at this point in time there is no scientific way to show otherwise. Evidence of this type can be very pertinent to the outcome of trials of this type, if the age of the sample can be determined.

Establishing the temporal linkage of biological evidence could also help to determine locations that have been visited by a particular individual. Establishing the age of an

identified sample could assist in the tracking of wanted suspects. This information could estimate an approximate distance in which the individual could have traveled based on the predicted age of the biological sample.

Previous Techniques Developed to Determine the Age of a Blood Sample

Several approaches have been used to assess the age of a blood stain or other biological material. The majority of the methods have relied on the transformations of hemoglobin into derivatives, and the changes in color and solubility which accompany these transformations [3]. In 1962, Fiori summarized a method that correlated the age of a blood stain with the progressive diffusion of chloride ions around the stain, which can be fixed *in situ* as AgCl [4]. Upon reduction, a black border forms around stains that are more than 2 months old. The size of the border will increase in increments up to 9 months due to the progressive diffusion of Cl⁻ around the stain, indicating an approximate age of sample. This method does not take into account the size of the stain and does not differentiate between human and other blood. Enzyme assays have also been used to date specimens, although with limited success. In general, they indicate either the presence or absence of enzyme activity. Studies have shown that activity persisting for a given amount of time suggest that a sample is either less than or more than a certain age [reviewed in 3]. In 1936 Schwarz measured catalase and peroxidase activity in blood stains as a method to determine specimen age [5]. The peroxidase activity was determined using guaiacum which, in the presence of hemoglobin, would catalyze the guaiacum bluing reaction with hydrogen peroxide. The results of the experiment suggested that the intensity of the blue color varied with age. Another more recent approach developed by Rajamannarr in 1977 examined the serum

protein profile by immunoelectrophoresis of stains as a function of their age of deposition from 15 days to 1 year [6]. A characteristic pattern of disappearance of various globulin proteins and albumin at test points along the time line was constructed. All of the proteins assayed were undetectable at 365 days. In contrast, Sensabaugh, in 1971, found albumin to be detectable by its immunological reaction in a dried blood sample 8 years old [7]. This contradiction has been explained by a change in electrophoretic mobility of albumin in aging blood stains [7]. High performance liquid chromatography (HPLC) analysis of unknown degradation products of hemoglobin present in dried blood stains has also been examined as a method to attempt to determine the age of a blood stain [8]. The results suggested that the ratio of peak areas, represented by the amounts of the unknown compounds examined decreased in size over time. This method was never established as a routine technique in forensic casework. The majority of the previously mentioned approaches are incapable of differentiating blood samples from different species, are limited to blood tissue, and could have misleading results due to the inability to control for varying amounts of blood.

Effective Means for Estimating Age

Archeologists have developed an effective means for estimating the age of a sample using carbon-14 dating. This approach is based on the ratio of radioactive carbon-14 relative to non-radioactive carbon-12. Carbon-14 has a constant rate of decay while levels of carbon-12 do not change over time. This results in a changing ratio that can be used to estimate the age of an organic sample [9]. A problem with carbon-14 dating is that the time line of detection is thousands of years and, therefore, can not be used to estimate time of blood sample deposition. However, developed criteria for carbon-14 dating can provide guidance in the development of a shorter time line of detection. By analogy to Carbon-14 dating,

biological molecules suitable for analysis should be both abundant and labile. One biological molecule that fits this description is RNA.

Why RNA?

DNA is the nucleic acid that is typically utilized in crime scene analysis, but there are several reasons why RNA was selected over DNA for estimating age of a biological sample. RNA is more abundant than the corresponding DNA template, existing in several 1000s of copies per cell as opposed to the 2 copies typical of most DNA sequences [1]. RNA is chemically and biologically more labile than DNA, particularly at elevated temperatures ($>65^{\circ}\text{C}$) and in the presence of alkaline conditions. These sensitivities are further compounded by the endonucleolytic and exonucleolytic activities of a variety of persistent ribonucleases that exist almost everywhere [10]. On the other hand, DNA has been shown to be more stable because it has been detected in certain tissue for tens of thousands of years [11]. Additionally, forensic analyses of DNA samples that are decades old are today routine and the DNA remains stable in dried blood stains as long as no bacterial overgrowth occurs [12]. All of these reasons support the use of RNA as a means of determining the age of a biological sample. In order to establish a more reliable mechanism to determine the age of biological samples, this study examines the relative stability of different RNA species as a function of time.

Most RNA species have a characterized *in vivo* half life and a highly regulated mechanism of decay [13]. The mechanisms of decay for mRNAs can be very diverse with components such as the 5' cap, secondary structure in the 5' untranslated region, premature

termination codons, specific sequences in the open reading frame, 3' untranslated region sequences including the iron response element of the transferrin mRNA and the AU rich target degradation sequence present in many unstable mRNAs, and the poly(A) tail influence decay [14]. Many internal sequences and secondary structures also influence *in vivo* RNA decay [15]. 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 [16]. When the CRD-BP is bound to *c-myc* mRNA, the CRD of the mRNA is shielded from endonucleolytic attack. If the CRD-BP dissociates from the mRNA, the CRD becomes exposed to endonucleases. The mRNA is then rapidly degraded by endonucleolytic cleavage within the CRD. Two other examples of regulated *in vivo* decay of RNA are the iron response element (IRE) on the transferrin mRNA and the AU rich element (ARE) contained in many unstable mRNAs [17, 18]. Five stem-loop structures located within the 3' untranslated region are capable of binding the IRE – binding protein (IRE-BP). The affinity of this protein for these sequences is regulated by cellular iron, and changes in affinity occur through dissociation and reassociation of an iron-sulfur cluster. When intracellular iron is abundant, the IRE-BP does not bind the IRE and the transferrin receptor mRNA is relatively unstable. Under low iron condition, the IRE-BP binds to the IRE and the transferrin receptor mRNA is stable. It is thought that the IRE-BP prevents the binding of destabilizing proteins. The ARE sequence is an AU-rich region contained within the 3' untranslated region of an mRNA. Similar to the mechanism behind the IRE related decay, an AU rich element binding-protein (ARE-BP) will bind the sequence and stabilize the mRNA. If the ARE-BP does not bind the ARE, the mRNA is targeted for decay. The exact mechanism behind binding of the ARE-BP has not yet been established.

At this point in time, the *ex vivo* mechanisms of RNA decay have not been thoroughly investigated.

A paper from 1992 reported that in addition to DNA, RNA could be extracted from dried blood specimens on filters that were used for screening newborns for genetic defects. Although these filters are normally discarded after DNA analysis, these researchers have diagnosed carriers for sickle cell disease and β -thalassemia using the RNA isolated from these blood stains [19]. The RNAs were stable enough for cDNA synthesis by reverse transcriptase and amplification by PCR in samples that had aged for up to 4 years stored at 4⁰ C or room temperature.

In addition, others have shown that reverse transcription PCR (RT-PCR), could detect GAPDH (glyceraldehyde 3-phosphate dehydrogenase) mRNA in dried blood stains present on cotton fabric that had aged up to 6 months at room temperature [20]. These results indicate that RNA in dried samples might be stable enough for forensic science investigations. Other research looking at the degradation profile of mRNA in dead rats has shown that interleukin-1B, induced by intravenous injection of lipopolysaccharide 6 hours prior to sacrifice, decays more rapidly than does the mRNA for the GAPDH housekeeping gene in post mortem lung tissue of rats examined at various intervals over a 3 day period [21]. This suggests that in the lung tissue of a decaying corpse different RNA species may degrade at different rates.

RNA Quantification/Theory of Operation

A number of methods are available for the measurement of mRNA levels including northern blot, RNase protection assays, *in situ* hybridization, and real-time PCR. Of these,

real-time PCR is the only method that provides sufficient sensitivity and specificity to measure low level amounts of mRNA (<10 copies/reaction to $>10^7$ copies/reaction) in small amounts of tissue because it exponentially amplifies small amounts of nucleic acid [22]. This sensitivity enables the detection of rare mRNAs, mRNAs from small amounts of tissue, and mRNAs that are expressed in mixed cell populations [23]. Other methods such as northern analysis require large amounts of RNA (10 ng) that are not usually provided by forensic samples.

Development of equipment used for real-time monitoring of fluorescence within PCR tubes and the development of the chemistry for fluorescent detection of PCR products has led to major improvements in the ease and reproducibility of quantitative RT-PCR. Methods for fluorescence monitoring of the PCR process employ fluorogenic probes based on fluorescent resonance energy transfer systems such as Taqman and Molecular Beacons [24]. Real-time monitoring of the PCR reaction using these technologies allows faster and more accurate measurement of RNA levels than conventional PCR quantification [25]. In-tube fluorescence monitoring of the appearance of PCR products eliminates post-PCR analysis of product, such as ethidium bromide gels, thus removing the errors and time associated with these steps [26, 27]. Because of the sensitivity of the technique and many of the other above mentioned reasons, real-time PCR has been chosen to assess the RNA levels in the specimens studied.

During real-time PCR, forward and reverse primers hybridize to specific sequences of a target cDNA with the TaqMan probe hybridizing to a target sequence internal to these primers [28]. The DNA Polymerase enzyme cleaves the TaqMan probe with its 5'-3' polymerization associated nuclease activity during extension phases of PCR. A Taqman

probe consists of an oligonucleotide with a 5' reporter dye and a downstream 3' quencher dye. A fluorescent reporter dye, such as FAM (6-carboxy-fluorescein), is covalently linked to the 5' end of the oligonucleotide. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence, primarily by Forster-type or fluorescence resonance energy transfer [24]. Fluorescence resonance energy transfer measures the interaction between 2 molecules based on the distance between the reporter and quencher. Each fluorophore has a 2-peak spectrum in which the first peak is the excitation peak, and the second is the emission peak. For the combined fluorescence resonance energy transfer effect, the emission peak of the reporter has overlap with the excitation peak of the quencher. The net result is that the reporter emits less energy than normal because some of the energy it would radiate as light gets transferred to the quencher. The reporter dye and quencher dye are separated upon cleavage, resulting in increased fluorescence of the reporter. At any given cycle within the exponential phase of PCR, the amount of fluorescence is thus a function of the initial number of template copies. Differences in amplification rates are indicative of differences in the amount of starting material [28]. The cycle number at which exponential amplification is detected is termed the threshold cycle or Ct value. The 3' end of the TaqMan probe is blocked to prevent extension of the probe during PCR. The fluorescence signal is generated only if the target sequence for the probe is amplified during PCR. Because of these requirements, non-specific amplification is not detected [29].

A benefit of using real-time PCR is the ability to simultaneously detect more than 1 species or sequence of RNA, in a single tube, in a process called multiplexing. The probes for each of the RNAs are labeled with different dyes which absorb different wavelengths and

can thus be analyzed simultaneously. Both the VIC and FAM reporter dyes emit fluorescence in a range that is shared between the two but each one absorbs different extreme wavelengths of the spectra (500 nm-660 nm) so they can be multiplexed. The real-time PCR machine controls for overlapping wavelengths of the 2 dyes by filtering out the overlapping regions and thus, limiting the specific range of each individual dye. This allows for simultaneous assays of both RNAs examined [30]. Since all manipulations are performed on both RNAs simultaneously, a ratio of RNA levels is generated which is internally controlled. The ratio should be the same regardless of sample size, pipetting errors, or enzymatic efficiency.

Hypothesis

RNA, transcribed from a DNA template, can serve different functions within a cell. Messenger RNAs (mRNA), for instance, contain the information specifying the amino acid sequence of polypeptides. Messenger RNAs form an association with ribosomes in order to synthesize the polypeptides. This association is temporary and transient, with the large and small subunits of the ribosomes assembling on the mRNA to initiate translation and then disassembling once synthesis of the polypeptide is complete. Further, the ribosomes do not remain in a single location on the mRNA but slide along the mRNA as the polypeptide is synthesized leaving relatively large areas of the mRNA not covered by the ribosomes [31]. Electron micrographs demonstrate that large areas of the mRNA are exposed to the cellular environment [32]. In contrast, ribosomal RNA (rRNA) forms a tight and stable association with the ribosomal proteins [33]. This tight association with the ribosomal proteins may protect and shield rRNA from degradation, analogous to protection afforded to DNA by its association with histones in the formation of nucleosomes [34]. The absence of such a tight

association between mRNA and proteins could render them more susceptible to degradation. Although not directly tested, it is believed that one type of RNA (rRNA) will be more stable than a second type (mRNA) due to, differential association with proteins. Ribosomal RNA would degrade at a much slower rate than mRNA and the ratio of these two will change in a predictable fashion that is time dependent, acting analogously to the disappearance of carbon-14 over time.

The central hypothesis of this dissertation is that RNAs decay in *ex vivo* dried blood samples at different rates and that these differences can be used to estimate the age of *ex vivo* blood samples. In order to test this hypothesis, however, other experiments must be performed. First, robust protocols for the isolation of RNA from dried blood stains must be developed, with only small amounts of variability contributed by different investigators using the same method. Second, mRNA and rRNA species will need to be selected for real-time analysis of the RNA isolated from the dried blood stains, and procedures for comparisons of the relative amounts of the different RNA species will need to be generated and optimized. Third, procedures ensuring that false-positive signals from DNA do not confound the interpretation of the data must be developed. These control experiments will allow for rigorous evaluation of the hypothesis being tested. After preliminary technical work, additional factors that may be critical in real-world analysis must also be considered. For example, what quantity of blood, fluid or dried, or other material, is needed to conduct these tests? Is the technique restricted to blood or can other biological samples also be used? Can DNA identification and estimates of sample age using RNA be performed with the same sample? Varied environmental agents and conditions, such as temperature, humidity, and light must also be examined to determine if they affect the rates of specific RNA species

degradation. PCR primers and probes that allow for only human-specific analysis need to be developed in order to eliminate any RNA signal that might be due to sample contamination with blood or other material from other species. A sample collected at a crime scene may be stored for a period of time before it can be examined, and so a technique for sample storage must be produced. Because blood stains can be found on a variety of surfaces, the recovery and efficiency of RNA from the various media using this method need to be investigated. A time frame of detection of RNA species in blood will need to be determined and tested on a population of sufficient size to permit rigorous statistical analysis. The variability within and between persons and people will need to be examined. *Bauer et al* detected RNA from blood stains that had aged out to 6 months, because of this, a time line of at least 6 months can be expected [20]. In order to help establish a shorter time line, altered specific RNA levels as a result of an *ex vivo* environment will be pursued, identified, and examined. The development of a technique that addresses all of these factors has the potential to be a more accurate and precise method of dating blood stains than any method currently employed.

Chapter One-A Method to Determine the Age of a Biological Sample

Introduction

The focus of the studies done in this chapter are aimed at identifying the most efficacious of the available methods to isolate RNA from dried blood, the selection of candidate RNAs, the generation and optimization of real-time analysis, and technique application for sample analysis. The results presented in this chapter suggest that the Ct value of 18S rRNA does not change over the course of 0-150 days but the Ct values for β -actin mRNA become significantly reduced as a function of time. It is the intent of this study to examine if these changes are predictable and can be used for estimating the age of a biological sample.

Methods

Blood Collection

Human blood was collected in Becton Dickenson (BD) vacutainers (Fisher Scientific, Pittsburgh, PA). Thirty aliquots of blood were deposited onto cotton fabric (10 μ l) or into 1.5-ml tubes (50 μ l) and both were stored at 25 $^{\circ}$ C and 50% humidity inside an environmental chamber.

RNA Extraction

Roche High Pure RNA Isolation Kit

RNA was isolated from dried blood stains using a spin column method that selectively binds RNA to a glass fiber fleece. RNA was isolated using the protocol accompanying the High Pure RNA isolation Kit (Roche, Indianapolis, IN) except for the modifications described below. Three hundred μl of phosphate buffered saline (PBS) was added directly to the 50 μl “aqueous” blood sample and then the samples were homogenized with RNase free disposable pestles blood for 10 strokes to break up clotted blood. “Aqueous” blood is defined as blood stored in 1.5-ml tubes and not dried on a selected medium. Four hundred μl of the provided lysis buffer was then added to the samples and the tube was inverted 6 times. The lysed homogenate was then transferred to a spin column provided by the kit and the protocol provided by the kit was followed from this point. The Roche kit was also used to isolate RNA from 10 μl dried blood stains. The same protocol as described above was followed with the addition of an initial 10 minute incubation at 50 $^{\circ}\text{C}$ in 400 μl of lysis Buffer and 300 μl of PBS. The samples were also vortexed briefly prior to incubation instead of homogenizing with a pestle. The remainder of the protocol was followed exactly.

TRI Reagent BD

RNA was isolated from 10 μl dried blood stains using a mono-phase solution containing both phenol and guanidine thiocyanate (TRI Reagent BD, Molecular Research Center, Cincinnati, OH). The TRI Reagent BD protocol for RNA isolation was followed including the modifications described below. The dried blood sample was directly added to a

solution containing 200 µl of water, 3 µl of a polyarcyl carrier (Molecular Research Center, Cincinnati, OH), and, 750 µl of TRI Reagent BD. To isolate RNA from the 50 µl “aqueous” blood samples, the 200 µl of water was replaced with 20 µl of 5 M acetic acid in the initial TRI Reagent solution. The remainder of the procedure was identical for both dried and aqueous samples. The RNA pellet recovered from the initial blood sample was resuspended in 40 µl of RNase free water (Fisher Scientific, Pittsburgh, PA) and incubated at 55 °C for 10 minutes. A sham RNA isolation of cotton or TRI Reagent BD containing no blood stain was performed with every assay to serve as a negative control.

Qiagen

The QIAamp RNA Blood Mini Kit (Qiagen, Santa Clarita, CA), was used to isolate RNA from 10 µl dried blood samples using the provided protocol for total RNA isolation from tissues. Silica-gel based spin columns with selective binding properties were used for the RNA isolation procedure. The protocol provided with the QIAamp RNA Blood Mini Kit was followed exactly except for the addition of an initial 10 minute incubation at 55 °C.

Zymo

RNA was isolated from 10 µl dried blood stains using the ZR Total RNA Isolation Kit (Zymo, Orange, CA). The protocol provided with the Zymo kit was followed exactly except for the addition of an initial 10 minute incubation at 55 °C. The RNA was eluted from the provided spin column into an RNase-free tube using 16 µl of RNase-free water added directly to the column matrix (provided by kit) with a brief centrifugation. Samples were brought up to a 100 µl volume by adding 84 µl of RNase-free water.

Reverse Transcription

Applied Biosystem's Taqman Gold RT-PCR kit was used for all reverse transcription reactions mentioned in this dissertation. A reverse transcription master mix (final concentration: 1X TaqMan buffer A; 5.5 mM magnesium chloride; 500 μ M each dATP, dCTP, dGTP and dUTP; and 2.5 μ M random hexamers) was made, aliquoted into individual PCR tubes and stored at -20°C until time of use. Forty μ l of the RNA sample, 2.0 μ l of RNase inhibitor (0.8 U), and 2.5 μ l of Multiscribe reverse transcriptase (3.25 U) was added to each reaction. Samples were centrifuged briefly (1-2 seconds) and placed in a Techne Touchgene Gradient thermocycler (Burlington, NJ) under the following conditions (25°C for 10 minutes, 48°C for 30 minutes and 95°C for 5 minutes). A no-enzyme control was run with every assay.

RiboGreen Quantification of RNA

RiboGreen (Molecular Probes, Eugene, OR) was used to estimate RNA recovery from dried blood stains. RiboGreen analysis was performed according to the manufacturer's guidelines. All RNAs analyzed were compared to RNA standards provided with the kit. The fluorescence of each sample was measured using Applied Biosystem's 7700 real-time PCR machine with Sequence Detection v1.6.3 software. A specific protocol for this procedure is provided in the Appendix (A-1). Prior to reading the fluorescence of the experimental samples, a background reading was performed to serve as a control. Background fluorescence was subtracted from the mean fluorescence to generate the final value.

Kit Comparison

RNA was isolated from dried blood stains which had aged for 1 to 2 weeks on cotton. A variety of methods were used to isolate the RNA including RNA isolation kits manufactured by Zymo, Qiagen, Roche and MRC. RNA was isolated from 2 dried blood samples using each kit according to the protocols described above. The efficiency and yield of each extraction procedure was determined based on RiboGreen analysis. All RNA samples were brought up to 200 µl with water to standardize the final RNA volumes for RiboGreen analysis. This experiment was executed by 3 different investigators to assess which kit/procedure demonstrated the least amount of preparation variability between RNA samples using the above mentioned RNA isolation procedures. Results are based on the average of 4 measurements (2 RNA isolates and 2 real-time assays).

Human Endogenous Control Plate (HEPC)

Applied Biosystem's HECP consisted of Taqman primers and probes for 11 housekeeping genes [18S rRNA (18S), acidic ribosomal protein (huPO), β -actin (huBA), cyclophilin (huCYC), glyceraldehyde-3-phosphate dehydrogenase (huGAP), phosphoglycerokinase (huPGK), β_2 -Microglobulin huB2M), β -glucuronidase (huGUS), hypoxanthine ribosyl transferase (huHPRT), transcription factor IID, TATA binding protein (huTBP) and transferrin receptor (huTfR)] and an internal positive control. RNA was isolated (as previously described) from triplicate 10-µl dried blood stains at 0, 2, 12 and 24 months after deposition using TRI Reagent BD. The RNA was converted to cDNA as previously described. All cDNA samples for each time point were combined into the same tube. For each RNA examined with the plate, replicate cDNA samples from each time point

were used for analysis. A master mix containing 25 µl of Taqman Universal PCR master mix, 10 µl of cDNA and 15 µl of water was generated in a quantity based on 24 reactions. Four mixes were made to represent each of the 4 different aged blood stains. Fifty µl of the reaction mix was added to each well of the plate. The plate was briefly centrifuged and then incubated on ice at room temperature for 30 minutes. Real-time PCR and analysis was performed as described below. This entire procedure was repeated. Results are based on the averaged value of 2 real-time reactions and 2 separate plate readings for a total of 4 measurements.

RNA Recovery

Whole blood was collected from a single human subject on 3 different occasions in plain and acid citrate dextrose (ACD) solution B coated BD vacutainers by standard venipuncture. RNA was isolated from triplicate 10-µl samples from blood collected in plain and ACD treated vacutainers and analyzed at 0, 7, 14, and 21 days after deposition. The blood collected in plain vacutainers was aliquoted into in a 1.5-ml tube (10 µl), capped, and stored at 25 °C inside an environmental chamber, prior to coagulation. The blood containing ACD was stored at 4 °C in the same 7-ml vacutainer used for blood collection. Replicate RiboGreen assays were performed on each RNA isolate. Results are based on the averaged value for 3 blood draws, 3 RNA isolates, and 2 real-time assays for a total of 18 measurements.

Paired Student's t-test

A paired Student's t-test was performed on the values obtained from RiboGreen analysis of the different aged RNA samples isolated from blood collected in plain or ACD coated vacutainers [35]. Comparisons were made between 0 and 7 days, 0 and 14 days and 0 and 21 days for the RNA isolated from the blood collected in each vacutainer type. An N value of 3 was used because 3 blood draws were taken from the same individual. Significance was demonstrated based on a P-value less than 0.01.

DNA Extraction

Both DNA and RNA were isolated from the same blood stain using TRI Reagent BD and DNazol. After the RNA in the aqueous phase was processed, DNA was extracted from the blood stained cloth using a modified version of the DNazol protocol for genomic DNA isolation from mouse tail clips [36]. 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% SDS (sodium dodecyl sulfate) and 20 µl of 20 mg/ml proteinase K. The samples were incubated at room temperature for 2 days with occasional vortexing. The remainder of the DNazol protocol was followed exactly. To isolate DNA from dried blood samples deposited on surfaces other than fabric, such as plastic, the remaining aqueous phase overlying the interphase was removed at the phase separation step of the RNA isolation protocol using TRI Reagent BD.

An additional 3 µl of polyaracyl carrier was added, and DNA was precipitated from the interphase and organic phase with 0.4 ml of 100% ethanol. The protocol for DNA isolation using TRI Reagent BD was followed from this point. The DNA pellet recovered from the initial blood samples was air dried at room temperature for 5 minutes and then dissolved in 20 µl of nuclease free water and rehydrated at 55 °C for 10 minutes.

DNA Detection

A Taqman probe specific for DNA was designed to test for DNA contamination of RNA isolated from dried blood stains using TRI Reagent BD. This probe was also used to determine the efficiency of DNA recovery when isolated from a RNA-depleted blood sample. DNA-specific primers/probes were designed based on a 5' non-transcribed region of the human GAPDH gene (GAP201NT). The specific primer and probe sequences for GAP201NT are presented in Figure 2-1. The DNA-specific probe (GAP201NT) was multiplexed with an RNA-specific probe that amplifies an exon-exon boundary of β -actin to test for both RNA and DNA in the same sample. The RNA-specific probe was purchased from Applied Biosystems (4333762F) [hsBA aka BA (180 bp)]. Optimal conditions were as follows: [GAP201NT- 250 nM probe (VIC), 500 nM forward and reverse primers], [BA (180 bp)- 50 nM probe (FAM) and forward and reverse primers]. A master mix that contained the primers and probes for β -actin and GAP201NT was generated as described in the primer optimization section below.

Real Time PCR

Primer Optimization

All custom designed primers and probes were synthesized by and ordered through Applied Biosystems. All primers and probes were used at a final concentration of 100 μ M. The primary stock was diluted 10-fold to generate a secondary concentration of 10 μ M. Optimal primer and probe concentrations for use in real-time PCR range from 25-900 nM. An optimization matrix was performed on each primer/probe combination (singleplex) used in the experiments described in this dissertation to determine which primer concentrations produced maximum PCR product. This was defined by setting up 16 independent assays where the amounts of forward (FP) and reverse primer (RP) varied between 50 and 900 nM concentrations while the probe was at a final concentration of 250 nM. An example singleplex matrix is demonstrated below.

50 nM FP, 50 nM RP	250 nM FP, 50 nM RP	500 nM FP, 50 nM RP	900 nM FP, 50 nM RP
50 nM FP, 250 nM RP	250 nM FP, 250 nM RP	500 nM FP, 250 nM RP	900 nM FP, 250 nM RP
50 nM FP, 500 nM RP	250 nM FP, 500 nM RP	500 nM FP, 500 nM RP	900 nM FP, 500 nM RP
50 nM FP, 900 nM RP	250 nM FP, 900 nM RP	500 nM FP, 900 nM RP	900 nM FP, 900 nM RP

(FP=forward primer, RP=reverse primer)

An initial mix containing 10 μ l of control cDNA, 25 μ l of TaqMan Universal PCR Master Mix and 250 nM probe (1.25 μ l) was generated for 20 reactions and added to each of

the wells. The primers and probes at concentrations indicated above were added to each well and water was used to bring the total reaction volume to 50 μ l. Real-time PCR analysis was performed using Applied Biosystem's 7700 and amplification curves of each reaction were analyzed. Optimal primer concentrations were identified by the reaction having the lowest Ct value and the highest Δ RN (amount of normalized fluorescence measured on the y-axis). The same procedure was used to identify optimal primer concentrations for a multiplex reaction except the primers for the most abundant/stable amplicon were kept constant (singleplex optimal concentrations) and the primers for the less abundant/stable amplicon were varied as described above. Both curves were examined after real-time analysis. Optimal concentrations were identified based on the lowest Ct value and the highest Δ RN for the less abundant/stable and the lowest Δ RN for the more abundant/stable identified based on all reactions in the matrix. Primer concentrations are limited by selecting concentrations which yield a lower Δ RN for the more abundant/stable amplicons. This prevents these primers from utilizing the majority of the PCR master mix and thus reducing the amplification of the less abundant RNA species.

RNA Detection

A real-time PCR master mix composed of Applied Biosystem's TaqMan Universal PCR Master Mix , primers and probes for Applied Biosystem's β -actin (part number 401846) control reagents (BA) and, Applied Biosystem's 18S rRNA (part number 4308329) control reagents (18S) was used for analysis of aqueous blood population study and first trial dried blood population study (described below). The following list represents the final concentrations of each component of the reaction: [50 nM 18S rRNA forward primers and

reverse primers, 200 nM probe (VIC dye layer), 300 μ M β -actin forward primers, reverse primers, 200 μ M probe (FAM dye layer), and 1X TaqMan Universal PCR master mix].

Thirty-five μ l of the real-time PCR Master Mix was added to real-time optical tubes (Applied Biosystems, Foster City, CA) and 15 μ l of each cDNA sample was then added to the tubes for a total volume of 50 μ l.

Relative RNA quantification

All samples were briefly centrifuged before real-time analysis, placed in Applied Biosystem's 7700 Sequence Detection System, and run on default conditions for 40 PCR cycles. Results were analyzed and threshold values adjusted as described by Applied Biosystems [37]. In brief, this was achieved by increasing the threshold bar to ensure it was above any background noise (non-exponential amplification) of the real-time PCR run and at a point where there is little to no variation between duplicate assays. A set threshold was identified for both components of the reaction (β -actin mRNA and/or 18S rRNA) and kept constant between all assays (Table 1-1). The baseline is identified as a defined range of cycles before amplification of the PCR product occurs. This was set to be specific for each RNA and to ensure that the initial amplification of each RNA begins at a cycle that is greater than the maximum value of the baseline. 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. Controls that were included in all parts of the technique described in this dissertation are provided in Table 1-2.

Table 1-1. **Threshold and Baseline Values for All Primer/Probe Sets.** Primer/probes sets other than 18S/BA are further described in Chapters 2-5. #1 represents the first component of the primer/probe set and #2 represents the second component of the primer/probe set.

Primer/Probe Set	Baseline #1 (Ct value)	Threshold #1	Baseline #2 (Ct value)	Threshold #2
18S (60 bp)/BA (60 bp)	3-13	0.017	3-15	0.021
18S (180 bp)/18S (500 bp)	3-8	0.237	3-15	0.03
BA (60 bp)/BA (300 bp)	3-15	0.017	3-15	0.048
18S (180 bp)/BA (180 bp)	3-13	0.179	3-15	0.083
18S/BA-Commerically available	3-15	0.019	3-15	0.056

Table 1-2. **Controls.**

RNA ISOLATION	Sham isolation			
REVERSE TRANSCRIPTION	- RT			
REAL-TIME PCR	Control cDNA	Negative control- Water	DNA /RNA specific probes	Analysis of all above mentioned controls

Blood Collection and Sampling

Aqueous Blood

Ten ml of blood was drawn from 11 (5 males and 6 females) subjects of European ancestry on 3 separate occasions for the first trials utilizing “aqueous” blood. “Aqueous” blood is defined as 50 µl of blood stored in capped 1.5 ml tubes. Males ranged in age from 21-55 (median age=45 years) and females ranged in age from 22-36 (median age=27.5 years). Thirty aliquots (50 µl) were immediately placed into capped 1.5-ml tubes for the first trial. Samples were stored at 25 °C in an EC22560 Environmental chamber (Lab-Line, Melrose Park, IL) until ready for RNA isolation. Blood was drawn 3 times a week from 2 individuals. Blood samples were collected over a 4-week period for the subjects involved in this study. Samples were removed from the chamber and processed at 0, 1, 2, 3, 6, and 10 days after deposition. Age and sex for all subjects tested are provided in Table 1-3. Our protocol was approved by the West Virginia University Institutional Review Board for the Protection of Human Research Subjects (IRB #15833).

Dried Blood

Ten ml of blood was drawn from 8 (4 males and 4 females) subjects of European ancestry on 3 separate occasions for the first trials utilizing dried blood. Males ranged in age from 21-55 (median age=38 years) and females ranged in age from 22-36 (median age=29 years). The 8 subjects who participated in the dried blood study also participated in the previously mentioned “aqueous” blood study. Thirty aliquots (10 µl) for each individual

were immediately spotted on the same piece of 100% cotton fabric. All samples were stored at 25 °C and 50% humidity in an EC22560 Environmental chamber (Lab-Line, Melrose Park, IL) until they were ready for RNA isolation. A separate piece of fabric was used for each blood draw and individual. Blood was drawn 3 times a week for 2 individuals. Blood samples were collected over a 4-week period for the subjects involved in this study. Samples for dried blood trial 1 were removed from the chamber and processed at 0, 30, 60, 90, 120, and 150 days after deposition. Age and sex for all subjects tested for trial one are provided in Table 1-3

Table 1-3. **Subject Information for Dried and Aqueous Trials.** Information about the subjects involved in both the dried (D) and aqueous (A) population experiments. Gender, age and experiment participation are indicated.

SUBJECT	SEX	AGE	DRIED (D)/AQUEOUS (A)
1	FEMALE	24	A
2	FEMALE	25	D/A
3	FEMALE	36	D/A
4	MALE	45	D/A
5	MALE	55	D/A
6	FEMALE	36	A
7	FEMALE	30	D/A
8	MALE	32	D/A
9	FEMALE	22	D/A
10	MALE	21	D/A
11	MALE	46	A

Real-time Analysis of Blood for First Trials

Real-time PCR was performed on cDNA for all time points. Duplicates of each cDNA from each time point for each individual and blood draw were assayed. This totaled 18 measurements for each time point per individual (3 draws X 3 samples/draw/time point X 2 assays/sample X 11 = 198 measurements) for the aqueous blood trial (Figure 1-1) and (3 draws X 3 samples/draw/time point X 2 assays/sample X 8 = 144 measurements) for the dried blood. BA and 18S primer sets were multiplexed and used to analyze the cDNAs. Complementary DNAs from the same individual were analyzed in the same real-time run. Because ABI's 7700 can run 96 samples at once, 2 real-time runs were performed to analyze all time points for each individual (6 time points X 18 measurements = 108 reactions). A control cDNA (same cDNA aliquot reverse transcribed from the same RNA sample and stored at -20 °C until time for use) was included in every run and controlled for machine/primer and probe and master mix variability. All results were adjusted based on this value. Thresholds were set for both BA and 18S (Table 1-1) before the Ct values were exported into Excel. Ct values for both BA and 18S were analyzed by examining the ratio of BA to 18S. Sham RNA isolations and no reverse transcriptase controls were analyzed using real-time PCR at this point for detection of false positives. Real-time analysis of data was performed using Applied Biosystem's Sequence Detection Software Version 1.7.

Figure 1-1. Flow chart for aqueous and dried trials. Time points indicated below represent the time points for the aqueous trial, time points for the dried trials are 0, 30, 60, 90, 120, and 150 days.

Statistical Analysis

For the first dried blood trial, analysis was performed with a nested analysis of variance using the method of restricted maximum likelihood [38]. 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 sex and age of blood were taken to be random. These include: intra-person (different blood draws), inter-person (different subjects), variability due to RNA isolation, variability due to duplicate real-time assays and residual variation (unknown sources of variability not accounted for by model). The response variable is the ratio of Ct value for the 18S gene over that for the β -actin gene (relative ratio). In addition non-linear regression models were fit to ratio and age of blood for the dried blood-first trial calibration confidence intervals were determined from those results [39].

Results

RNA isolation technique comparison

To determine the best method for isolating RNA from small volumes of dried blood, several commercially available kits and reagents were examined. Because these procedures were not specifically designed for isolation of RNA from dried blood, each one was critically evaluated and the one yielding the greatest amounts of RNA with the least amount of variability among samples and investigators was selected for future analysis of the method

described in this dissertation. Results are presented in Table 1-4. Extraction with TRI Reagent appeared to yield the largest amount of RNA, as estimated by RiboGreen analysis, while application of the Zymo kit yielded the least. Investigators A and C recovered RNA estimated to be of concentrations about 70-80 ng per sample using TRI Reagent BD, while investigator B recovered about 40 ng of estimated RNA concentration. Extraction of RNA using the Qiagen kit recovered the second greatest amount of RNA, with investigators A and C recovering about 50 ng of estimated RNA. Application of Roche and Zymo kits usually resulted in recovery of 7 ng or less of estimated RNA from the samples, with measurements at or near background levels. Real-time analysis on RNA isolated from dried blood stains using the different kits supported the RiboGreen results concerning the RNA yields (data not shown).

Table 1-4. **Mean RNA recovery.** Values shown for each investigator and kit represent the mean RNA quantity as estimated by RiboGreen. Standard error is included in table.

Investigator	Qiagen (ng)	Roche (ng)	TRI Reagent (ng)	Zymo (ng)
A	50±5	7±3	80±10	*N/D
B	40±2	5±3	40±7	N/D
C	50±6	7±2	70±5	7±4

*, N/D means not detected.

Housekeeping gene selection

The rRNA species selected for analysis was 18S. This selection was due to a greater success in generating human-specific primers and probes for 18S than for 5S and 28S. β -actin was selected as our mRNA species after analyzing the results from a housekeeping gene expression panel. Applied Biosystem's Human Endogenous Control Plate was used to determine relative levels of several housekeeping genes in dried blood specimens. The 11 housekeeping genes examined are as follows:

Figure 1-2. Housekeeping RNA Prevalence in Dried Blood. A Ct value of 35-40 is considered near background levels with Ct values at 40 representing no detection. RNAs examined include, 18S rRNA (18S), acidic ribosomal protein (huPO), β -actin (huBA), cyclophilin (huCYC), glyceraldehyde-3-phosphate dehydrogenase (huGAP), phosphoglycerokinase (huPGK), β_2 -microglobulin (huB2M), β -glucuronidase (huGUS), hypoxanthine ribosyl transferase (huHPRT), transcription factor IID TATA binding protein (huTBP) and transferrin receptor (huTfR). Standard error bars are included.

18S rRNA (18S), acidic ribosomal protein (huPO), β -actin (huBA), cyclophilin (huCYC), glyceraldehyde-3-phosphate dehydrogenase (huGAP), phosphoglycerokinase (huPGK), β_2 -microglobulin (huB2M), β -glucuronidase (huGUS), hypoxanthine ribosyl transferase (huHPRT), transcription factor IID TATA binding protein (huTBP), and transferrin receptor (huTfR). An Internal Positive Control (IPC) was included to help interpret negative results that could have been caused by PCR inhibitors. In the absence of inhibitors, IPC co-amplifies with target DNA and gives a specific signal. The IPC sequence is artificial so that PCR primers do not amplify anything in the test samples. β -actin was determined to be one of the most abundant mRNA species detected in dried blood samples. Figure 1-2 shows the Ct values for each of these cDNAs at the indicated time points. All cDNAs show the expected trend of increased Ct values over time. The lowest Ct values for all time points were associated with 18S, β -actin and β_2 -microglobulin. Out of the 2 mRNA species, β -actin was selected for initial analysis because of its commercially available pre-developed primers and probes. The amplicons range in size from 74-153 bp for the various mRNAs tested. Amplification using primers for 18S rRNA generated a 186 bp amplicon.

Recovery of RNA

Because of the modifications to the original protocol for RNA isolation using TRI Reagent BD (see methods), the recovery of RNA from paired specimens was examined. RNA recovery from blood with and without anticoagulants was compared. The estimated RNA concentrations, based on RiboGreen analysis, for the different types of blood are presented in Table 1-5. Eighty ng of estimated RNA was recovered from the blood containing ACD compared to 100 ng of RNA from the coagulated blood at 0 days. After the

blood samples had aged for 1 week, the estimated recovery of RNA from coagulated blood dropped to about 25 ng (75% less than day-0 samples) and 12.5 ng after 3 weeks (88% less than day-0 samples). Fifty ng of RNA was recovered from the blood containing ACD for time points 1, 2, and 3 weeks (37.5 % less than day-0 samples). It appears that RNA can be recovered from blood stored either way. No significant loss in RNA recovery is observed from the blood collected with ACD. A significant loss in RNA recovery is observed from the blood collected in plain vacutainer from 0 to 7 days and 0 to 14 and 0 to 21 days.

Table 1-5. **Recovery of RNA from ACD and non-ACD treated blood.** Values shown represent the mean RNA quantity as estimated by RiboGreen absorbance. Standard error is included in the table. N=3.

Vacutainers	0 days (ng)	7 days (ng)	14 days (ng)	21 days (ng)
ACD	80 $\pm$ 10	50 $\pm$ 9	50 $\pm$ 10	50 $\pm$ 8
PLAIN	100 $\pm$ 10	25 $\pm$ 3*	12.5 $\pm$ 6*	10 $\pm$ 5*

*, statistically significant differences determined using a paired Student's t-test comparing all time points to 0 days.

RNA- and DNA-Specificity

GAPNT201 was identified to recognize control genomic DNA (Figure 1-3B). BA (180 bp) was determined to be RNA-specific (Figure 1-3A). GAPNT201 yielded no signal for the RNA sample, the sample lacking reverse transcriptase (-RT) or the negative (water) control (Figure 1-3A). The amplification of the BA (180 bp) amplicon strongly suggests the presence of RNA in our samples. Figure 1-3A illustrates that the

Figure 1-3A-B. **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- μ l blood sample. Figure 1-3A shows the RNA-specific primer/probe only amplifies the cDNA from the blood sample. Figure 1-3B shows that the DNA-specific primer/probe only amplifies the DNA from the blood sample. There was no amplification obtained using water as a template (data not shown).

A

Ct value for cDNA (green) is 21.6 and Ct values for RNA (red) and DNA (blue) are > 40.

B

Ct value for DNA (blue) is 29.8 and Ct values for RNA (red) and cDNA (green) are > 40.

BA (180 bp) amplicon only recognizes the RNA (cDNA) isolated from the dried sample. There was no signal from either the -RT control or for water. DNA was isolated from the same dried blood sample and Figure 1-3B shows that it is free of detectable RNA contamination and is only recognized by GAPNT201. The RNA probe [BA (180 bp)] and water did not give a signal.

Aqueous Blood-Trial One

Figure 1-4 shows the Ct value ratio between β -actin and 18S isolated from blood stains collected from 11 individuals aged over a 10-day period. The graph represents pooled data from all 11 individuals representing 198 measurements for each time point. It appears from the graph that there is a decreased amount of β -actin mRNA relative to that of 18S resulting in an increased RNA ratio over time.

Dried Blood-Trial One

RNA analysis of the dried blood of 8 subjects, processed at various time points over 150 days are presented in Figure 1-5. The ratio of the Ct value of β -actin over that for 18S is a linear function of age of blood for both females and males within the limitations of this experiment. Although there is a suggestion of gender effect and perhaps a gender-by-day interaction, these were not statistically significant using this sample size. A model fitting the sources of variability described in the methods 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 this test).

Information about all subjects who participated in both studies is included in Table 1-3

Figure 1-4. RNA Decay in Aqueous Blood Over Time. Figure 1-4 shows the change in RNA levels determined by ratio of Ct values as a function of bloodstain age. Data represent the ratio of β -actin mRNA to 18S rRNA as determined by real-time reverse transcriptase PCR. Standard error bars for each time point are included. n=11.

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 is a result of various factors that can not be accounted for by this model. Preliminary data suggest that there may be greater variability within a person (variability in blood draw dates) for females than males. This has also been investigated with a larger population size in Chapter Five.

Confidence Intervals

Confidence intervals were established to identify a range of age associated with a given RNA ratio established for a dried blood stain. Table 1-6 shows the 95% confidence intervals associated with the results from dried blood trial 1. Results were calculated based on relative ratios for 1 individual and all 8 individuals who participated in the study. The range of the confidence intervals is reduced by using the values for a single individual.

Figure 1-5. **One Way Analysis of Mean (ratio) by Age of *Ex Vivo* Blood Under Constant Conditions.** Data represent the ratio of β -actin mRNA to 18S rRNA 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%. Standard error bars for each time point are indicated on graph. $N=8$.

Table 1-6. Confidence Intervals Associated With a Given RNA Ratio. Confidence intervals are identified for a single individual and all individuals who participated in the study.

RNA Ratio	N=1	N=8
1.52	0-53 days	0-68 days
1.54	0-64 days	0-79 days
1.56	0-75 days	0-90 days
1.58	0-87 days	0-101 days
1.60	4-100 days	0-112 days
1.62	11-113 days	4-123 days
1.64	19-128 days	14-134 days
1.66	28-144 days	24-146 days
1.68	37-162 days	34-157 days
1.70	46-181 days	44-169 days

DISCUSSION

The results presented in this chapter suggest that the relative ratios of 2 different RNAs, 18S rRNA and β -actin mRNA, may be used to estimate the age of a bloodstain. Figure 1-5 shows the rate of decay of the 2 RNA species in dried blood over a given time period. The Ct value of 18S did not change over the time frame examined but the Ct values for β -actin became significantly increased as a function of time, thus, causing the relative ratio of β -actin mRNA to 18S rRNA to increase over time.

A standard method of RNA isolation has been adjusted to successfully extract RNA from dried blood samples. TRI Reagent BD protocol was modified and found to be the preferred method of RNA extraction compared to the other kits examined (Table 1-4). The TRI Reagent BD RNA isolation protocol recommends the use of blood freshly collected in vacutainers containing anticoagulants or isolated from blood stored in these vacutainers at 4 °C. The blood used for the experiments examined in this dissertation was not collected or stored in this manner. The blood used is representative of what could be found at a crime scene, therefore, this blood does not contain anticoagulants and is not stored using TRI Reagents BD's recommended conditions. Because of the differences in collection and storage of the blood, the RNA recovery of blood collected in ACD coated vacutainers and plain vacutainers was examined. Based on the results in Table 1-5, it appears that RNA can be recovered from both types of blood. Further experimentation will need to be performed to address more specific issues such as percentage of RNA loss and comparisons of RNA recovery isolated from the different types of blood. A significant loss of RNA recovery over time was identified for the blood collected in plain vacutainers. No significant loss in RNA recovery over time was identified for the blood collected with ACD solution B. The RNA recovered from blood in the presence and absence of an anticoagulant was found to be suitable for analysis with real-time PCR (data not shown).

From comparisons between Figures 1-4 and 1-5 it appears that the RNA stored in dried blood stains is more stable than RNA stored in aqueous blood. The time line for detection of RNA recovered from aqueous blood could only be extended to 10 days because the Ct values for β -actin were at or reaching background levels. The RNA examined in the dried blood stains was detected at Ct values much less than background for up to 150 days

but will need to be examined at points beyond 150 days to establish a longer time line of detection. Because the aqueous blood appears to remain in a more hydrated form than the blood deposited on cotton, it would be reasonable to assume that enzymatic activity could still occur in aqueous samples resulting in a shorter time line caused by increased RNA degradation. The idea that RNA is more stable in a dried form, prior to isolation, is consistent with previous work done on both RNA and DNA [1, 8, and, 19].

“Housekeeping genes” are expressed in all cell types and are critical for cellular survival [40]. RNA levels for these genes are thought to be relatively high with little variation. Because of this, housekeeping genes have been selected as our candidate RNAs. The housekeeping gene expression panel indicated to us that β -actin is one of the most abundant mRNAs present in dried blood stains. The ribosomal RNA species selected was 18S rRNA and it appeared from the expression panel high levels of 18S rRNA were also present in dried blood stains. The identification of polymorphic regions within 18S, which can later be exploited to generate human-specific primers and probes, further supported the selection of this RNA.

Statistical analysis of the results shown in Figure 1-5 suggests that females contribute more intra-person variability than males. The greater variability seen in females may be due to altered hormonal levels encountered during the monthly estrous cycle, or time of day in which the blood was drawn. These factors could affect the starting ratio of 18S to β -actin RNAs. Other work has shown that a single physiological dose of estradiol up-regulates estrogen receptor-alpha (ER), progesterone receptor (PR), GAPDH, c-fos, cyclophilin, and actin mRNAs in the endometrium of ovariectomized ewes [41]. This suggests that hormonal regulation may cause variability in steady state levels of certain housekeeping

gene. Chapter Five addresses this point by identifying the sources of variability associated with RNA detection in aged, *ex vivo* dried blood stains in a larger population

The confidence intervals established based on the results in Figure 1-5 suggest that analysis of RNA from a single individual may yield narrower intervals. The same trend was observed when confidence intervals were established for all 8 subjects individually (data not shown). If the person, whose blood stain was in question, could be identified using PCR based techniques, then there is an option of establishing a standard curve of relative ratios of RNA over time specific for the individual in question. Blood from the individual could be freshly collected, processed, and then the ratio of RNA in the unknown sample could be compared to the known results to estimate time of deposition. Narrower confidence intervals give less room for error with regards to age estimation.

The results in this chapter show that both types of RNA examined decay in a consistent and predictable manner. Although the confidence intervals generated for these experiments are large and probably would not be useful as a predictive model, Chapter Five focuses on reducing confidence intervals associated with a blood stain. The technique described in this dissertation has the potential to become a more precise and accurate means of dating aged blood stains than the previously mentioned techniques. The selected method of RNA isolation yields DNA-free RNA and provides for the simultaneous isolation of both RNA and DNA. The next avenue pursued was to further validate and refine the established method.

Chapter Two-The Effect of Amplicon Size on RNA Detection in Dried Blood Stains

Introduction

There are many issues regarding the method described in Chapter One that still need to be examined, that is the focus of this chapter. Various factors that may influence RNA detection in blood stains, such as the human-specificity of the primers and probes used for real-time PCR are investigated. Although, the initial results, shown in Chapter One, are based on analysis using commercially available primers and probes that are not human-specific, human-specific primers and probes were developed and used to further investigate RNA stability as a measure of blood stain age. The size of the projected PCR amplicons has been predicted to influence detection of the target RNA. For example, the larger the region of cDNA intended to be amplified; fewer available template molecules are expected due to the greater likelihood of fragmentation by agents contributing to RNA breakdown. This concept has the potential to enhance the sensitivity of this assay

Along with the design of human-specific primers and probes, in this chapter many factors that may affect the validity and sensitivity of the RNA detection method including the storage of sample, variability between investigators, analysis of RNA from different blood volumes, DNA analysis of dried blood stains, deposition of blood on different surfaces and technique sensitivity will all be addressed. The results presented in this chapter suggest that different types of RNA and different sized regions on the same RNA are degraded at different rates.

Methods

Primer Sets for Amplification of Different Sized Amplicons

Four primer sets that amplify different sized regions from β -actin and 18S are shown below. All mixes contain primers and probes for each region amplified and universal PCR master mix. Ten μ l of cDNA (used as a template for the real-time PCR reaction) was added to each reaction and the total volume was brought up to 50 μ l with water. Also provided below is the size of each region amplified, dye label and optimal concentration of the primers and probe.

Primer optimization was performed as described in Chapter One.

1. 18S (60 bp) – (VIC) 250 nM FP, 900 nM RP, 250 nM probe
BA (60 bp) - (FAM) 250 nM FP, 900 nM RP, 250 nM probe
2. 18S (180 bp) (VIC) 75 nM FP, 50 nM RP, 250 nM probe
18S (500 bp) (FAM) 900 nM FP, 900 nM RP, 250 nM probe
3. BA (60 bp) (VIC) 100 nM FP, 25 nM RP, 250 nM probe
BA (300 bp) (FAM) 500 nM FP, 500 nM RP, 250 nM probe
4. 18S (180 bp) (VIC) 900 nM FP, 250 nM RP, 250 nM probe
BA (180 bp) (FAM) 2.5 μ l (commercially available from Applied Biosystems)

Table 2-1. Sequence of Amplicons. Sequence information of the primers and probes used in all experiment (Chapter 2-5) of this dissertation. All sequences are written in a 5'-3' orientation.

NAME	Forward Primer	Reverse Primer	PROBE
BA (60 bp)	TCATTCCAAATATGAGATGCATTGT	GGACTGGGCCATTCTCCTTAG	AAGTCCCTTGCCATCCTAAAAGCCACC
18S (60 bp)	TGGATAACTGTGGTAATTCTAGAGCTAATAC	ATCCCCCCCCGCGAAG	TGCCGACGGGCGCTGACC
BA	TCACCCACACTGTGCCCATCTACGA	CAGCGGAACCGCTCATTGCCAATGG	ATGCCCCCCCCATGCCATCCTGCG
18S	*NA	NA	NA
BA (180 bp)	NA	NA	NA
18S (180 bp)	CGGAGAGGGAGCCTGAGAA	CTCCAATGGATCCTCGTTAAAGG	CGGCTACCACATCCAAGGAAGGCA
BA (300bp)	CTTCAACACCCCAGCCATGT	CTCTTGCTCGAAGTCCAGGG	CTGTGCTATCCCTGTACGCCTCTGGC
18S (500 bp)	TTCGGAAGTGAAGCCATGAT	CATGCCAGAGTCTCGTTCGTT	CATTCGTATTGCGCCGCTAGAGGTG
GAP201NT	TGTTTCATCCAAGCGTGTAAG	CCCTACTTTCTCCCCGCTTT	GTCCTGGGAACCAGCACCGATCAC

*NA, commercially produced primers and probes whose sequence information is not available.

Sequences of all primers used for amplification are provided in Table 2-1. Figure 2-1 and Table 2-2 provide other pertinent information about each primer/probe set including amplicon location within the RNA molecule.

Amplicon Size and Decay

Blood was collected from the same individual on 3 different occasions. Twenty aliquots (10 µl) were deposited on cotton and RNA was isolated from triplicate blood stains at 0, 7, 14, 21, and 50 days after deposition. The 4 newly designed primer/probe sets described above (Table 2-1) were used to analyze the RNA isolated from the blood over the period examined. Duplicate cDNAs were assayed giving a total of 18 measurements per time point (3 blood draws X 3 RNA isolates X 2 cDNA assays). Five real-time runs were performed to assay all cDNA samples using the 4 primer/probe sets. Thresholds were adjusted (Table 1-1) and the results were exported into Microsoft Excel to determine standard error and difference in Ct value.

Species-Specificity and Primer Design

The primers and probes for all of the non-commercially available primer/probe sets were designed to be human-specific using the sequence comparison program SEQUED (Applied Biosystems, Foster City, CA). Messenger RNA 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) to anneal to the identified polymorphic regions. The primers/probes were evaluated in real-time PCR reactions containing template RNA obtained from several non-human organisms (cat, dog, mouse, rat, pig, drosophila, and yeast). Little to no amplification compared to that

observed with the human RNA template, suggested that the primers and probe were human-specific. The location of each primer/probe range within the mRNA molecule is described in Table 2-2 and Figure 2-1. Each primer/probe set was used to amplify cDNA from each of the above mentioned organisms. Purified RNA from rat, mouse, and pig was purchased from Pharmingen (San Diego, CA). Yeast and drosophila RNA were isolated using the Roche High Pure RNA isolation kit according to the protocol provided with the kit (Chapter One). Blood from cat and dog was obtained though a local veterinarian and the RNA was isolated using TRI Reagent BD (Chapter One). All RNAs were converted to cDNA using the standard reverse transcription procedure described in Chapter One. The concentrations of all cDNAs were normalized to approximately 1 ng/ μ l. As a positive control, every cDNA was amplified using universally recognized, commercially available primers and probes for 18S rRNA purchased from Applied Biosystems. Amplification from every primer set with water was included as a negative control. This experiment was repeated.

Figure 2-1. Location of Amplicons in RNA. The nucleotide location within the RNA of the commercially available 18S (18S) was not provided.

Table 2-2. Identification of RNAs used for Analysis. Table 2-2 provides information related to each amplicons described in this dissertation.

RNA	NAME	AMPLICON SIZE	GENBANK ACCESSION NUMBER	LOCATION BASED ON GENBANK SEQUENCE	HUMAN SPECIFIC
β -actin	BA (60 bp)	84 bp	NM_001101	1448-1508	Yes
β -actin	BA (300 bp)	300 bp	NM_001101	421-751	Yes
β -actin	BA	300 bp	NM_001101	549-879	No
β -actin	BA (180 bp)	180 bp	NM_001101	9-189	Yes
18S	18S	187 bp	M10098	NA	No
18S	18S (60 bp)	64 bp	M10098	245-305	Yes
18S	18S (500 bp)	500 bp	M10098	991-1475	Yes
18S	18S (180 bp)	180 bp	M10098	531-711	Yes

RNA Detection from Various Fabrics

RNA was isolated from 1 centimeter by 1 centimeter triplicate samples of various fabrics including: cotton, polyester, silk, linen, and wool according to the previously described TRI Reagent BD protocol. All 5 previously mentioned primer/probe sets were used to assay duplicate cDNAs prepared from all the fabrics mentioned. Control cDNA was used as a template for all primer/probe sets to ensure the real-time mix worked and water as a template was included as a negative control. Results are based on the averaged value of triplicate isolations, duplicate real-time assays, and repeated experiments for a total of 12 measurements.

Additional TRI Reagent BD Experiments

Human blood was collected by standard venipuncture from a single individual in 10-ml plain vacutainers for all of the experiments mentioned below.

Variability Associated with TRI Reagent BD as a RNA Extraction

Method

Twenty aliquots (10 μ l) of blood were distributed onto cotton fabric and left to dry for several hours at room temperature. Three different investigators isolated RNA from triplicate samples at the same time, in the same lab using TRI Reagent BD. The RNA was reverse transcribed according to standard procedure and then duplicate cDNA assays were analyzed using the primer/probe sets, BA (60 bp)/BA (300 bp) and 18S (180 bp)/18S (500 bp). Results are based on the averaged value for triplicate samples and duplicate assays for a total of 6 measurements.

Stability in TRI Reagent BD

Ten μ l blood aliquots were placed directly into 12 1.5-ml tubes containing TRI Reagent BD solution (Chapter One). The samples were stored at room temperature for up to a 7-day period. RNA was isolated and reverse transcribed from triplicate samples at 0, 1, 4,

and 7 days after deposition. cDNAs were examined in a real-time reaction using BA (60 bp)/BA (300 bp) and 18S (180 bp)/18S (500 bp). Duplicate assays were performed for each cDNA and this entire experiment was repeated. Results are the averaged value of triplicate RNA isolates, duplicate real-time assays, and repeated experiments for a total of 12 measurements. The N value was determined to be 2 based on 2 separate blood draws from the same individual.

The Effects of Freezing on RNA Storage

The RNA isolation procedure (TRI Reagent BD) was initiated for 8, 10- μ l dried blood stains that had aged on cotton for 5 days at room temperature. After the step in the protocol when 75% ethanol is added, half of the samples were stored at -20 $^{\circ}$ C for a 3 day period and the RNA isolation procedure was completed on the remaining 4 samples. Two of these samples were reverse transcribed according to normal procedure and the other 2 were stored at -20 $^{\circ}$ C for subsequent RiboGreen analysis to determine RNA concentration. Three days later, the RNA isolation procedure was completed on the samples stored at -20 $^{\circ}$ C. Half of the samples at each condition were reverse transcribed and the other half were left as RNA and the concentration was analyzed using RiboGreen. Duplicate real-time assays were performed on the 4 (2 stored at -20 $^{\circ}$ C and 2 not stored at -20 $^{\circ}$ C) of the cDNAs using BA (60 bp)/BA (300 bp), 18S (60 bp)/BA (60 bp), and 18S (180 bp)/18S (500 bp). Measurements plotted are based on the averaged value of 2 RNA isolates, duplicate real-time assays and repeated experiments (2 X 2 X 2=8). The N value was determined to be 2 based on two separate blood draws.

Different Blood Volumes

RNA was isolated using TRI Reagent BD (Chapter One) from 1, 5, and 10 µl samples of 5 day old blood that had been stored on cotton fabric at room temperature and 50% humidity. RNA was isolated from duplicate samples of each blood volume and duplicate real-time assays of each cDNA were performed using BA (60 bp)/BA (300 bp), 18S (60 bp)/BA (60 bp), and 18S (180 bp)/18S (500 bp). Results are based on the averaged value of duplicate samples, duplicate real-time assays and repeated experiments for a total of 8 measurements.

Paired Student's t-test

A paired Student's t-test was performed on the measurements taken between the samples exposed to various conditions examined throughout this chapter [35]. Three total measurements were compared for each time point between experimental and control groups representing 3 separate blood draws for the same subject. All measurements (triplicate RNA isolates and duplicate assays) were averaged to generate a single value for each blood draw. Significance was demonstrated based on a P-value less than 0.01.

Short Tandem Repeat (STR) analysis of DNA

DNA was isolated (after prior removal and isolation of RNA) from 2-week old 10-µl dried human blood samples deposited on either plastic or cotton according to the procedures described in Chapter One. DNA was also isolated from hair and saliva after prior removal of the RNA (Chapter One). The DNA pellets were dried for 15 minutes by centrifugation at

12,000 X g in a speed vacufuge. The samples were sent to Affiliated Genetic (Salt Lake City, Utah) for STR analysis. The procedure was performed as described in the Appendix (A-2).

Sample Collection for Surface Experiments

Blood was collected from a single individual in 10-ml non-coated BD vacutainers on two separate occasions. Twenty (10 μ l) aliquots of blood (100 ng $\pm$ 13) from the same blood draw were placed on a variety of different surfaces including newspaper, plastic (150 X 15 mm, standard, sterile, polystyrene Petri dishes), unfinished wood, finished wood, cotton, polyester, brick and cinderblock. All surfaces, excluding plastic, were bleached and autoclaved prior to the deposition of blood. RNA was isolated from duplicate blood stains deposited on the same surface at 0, 1, 30, 60 and 90 days after deposition. Samples were incubated at room temperature (23 $^{\circ}$ C) and 30-50% humidity throughout the duration of the experiment. Sixteen hairs were also plucked from a single individual on 2 separate occasions and stored in uncapped 1.5-ml tubes at room temperature and humidity (see above). RNA was isolated at 0, 30, 60, and 90 days after deposition. An Omni swab (Whatman #WB10-0004, Clifton, NJ) was used to collect buccal cells from the cheek of a human subject. Sixteen saliva/buccal cell samples were collected from the same individual on 2 separate occasions, placed in 1.5-ml tubes and stored at room temperature and humidity (see above). RNA was isolated at 0, 30, 60, and 90 days after deposition. Four RNA samples were isolated from blood deposited on each surface and from hair/saliva. Two of the samples were reverse transcribed according to the previously mentioned procedure and stored at -80 $^{\circ}$ C

until the completion of the experiment. The other 2 RNA samples were resuspended in 200 µl of water and frozen at -80 °C until all samples were ready for RiboGreen analysis.

RNA Isolation for Surface Experiment

Dried Blood

To isolate RNA from blood stains deposited on fabric, the blood stains were cut out of the cotton and polyester fabrics and then placed into the TRI Reagent BD solution (Chapter One). To isolate RNA from blood stains deposited on solid surfaces, the blood stains were removed from the surfaces, including unfinished wood, finished wood, and brick, by scraping with a razor blade. The dried blood was then placed into the TRI Reagent BD solution using a pair of tweezers. The tip of the tweezers was dipped into the TRI Reagent BD prior to the collection of the blood stains, which increased the tweezers affinity for the dried blood scrapings. The blood stains were removed from plastic and newspaper using the moistened tweezers as mentioned above. TRI Reagent BD protocol was then followed to isolate RNA from dried blood deposited on each of the above mentioned surfaces.

Hair

A single hair was plucked from a human subject, trimmed to retain the root follicle, and placed in 1 ml of TRI Reagent (Molecular Research Center, Cincinnati, OH) and 3 µl of polyarcyl carrier. The remainder of the protocol was identical to the method of RNA isolation for dried blood using TRI Reagent BD.

Saliva/Buccal Cells

To isolate RNA from saliva/buccal cells, the tip of the applicator containing the sample was ejected into 1 ml of TRI Reagent and 3 μ l of polyarcyl carrier. The sample was then heated for 10 minutes at 55 $^{\circ}$ C. The Omni swab was then removed from the solution, placed into a spin basket contained in a 1.5-ml tube (Promega #V1221 Madison, WI) and centrifuged for 1 minute at 13,000 X g. The liquid recovered from centrifuging was then combined with the original solution. The remainder of the protocol was followed according to the TRI Reagent BD protocol for isolation of RNA from dried blood.

Reverse Transcription and Real-time PCR

All reverse transcription and real-time PCR was performed as described in Chapter One.

Analysis of Surface Experiment

The following primer/probe sets: 18S (60 bp)/BA (60 bp) and BA (60 bp)/BA (300 bp) were used to analyze all cDNAs during real-time analysis. Duplicate assays were performed on each cDNA, so each analysis generated 8 measurements (4 for each blood draw/collection). RiboGreen analysis was performed on all RNA samples according to standard procedure (Chapter One). Each 200 μ l RNA sample was divided in half to generate duplicate RiboGreen assays; therefore, each surface yielded 8 measurements per time point (4 measurements for each blood draw/collection).

Sensitivity of Technique/Limitations of Detection Method

RNA was isolated from duplicate blood stains of volumes, 10 µl, 5 µl, and 1 µl that were freshly deposited on cotton and left to air dry at room temperature and 50% humidity for 30 minutes. RNA was also collected from a freshly plucked hair follicle. The RNA pellets generated after the 75% ethanol precipitation step of the isolation procedure were resuspended in 100 µl of water. Of the 100 µl sample, 20 µl was used for conversion to cDNA, 20 µl was used for concentration analysis using RiboGreen, 20 µl was used for 3 serial 2-fold dilutions and 20 µl was used for 3 serial 10-fold dilutions. cDNAs from each dilution were analyzed with all previously described primer/probe sets and compared against known RNA standards using RiboGreen. Reverse transcription, RiboGreen and real-time analyses were performed as previously described (Chapter One). The results are based on the averaged value for duplicate RNA isolates, duplicate assays, and 2 blood draws for a total of 8 measurements.

Results

Analysis of Dried Blood-Trial One Results using Species-Specific

Primers/Probe

Samples from the dried blood-trial 1 (Chapter One) were analyzed using human-specific primers and probes designed for β -actin and 18S [18S (60 bp)/BA (60 bp)] and the

Figure 2-2. Analysis of RNA Isolated from Dried Blood Using 18S (60 bp)/BA (60 bp). Results presented are based on real-time using 18S (60 bp)/BA (60 bp). Standard error for each time point is included on graph. N=1.

results are presented in Figure 2-2. It appears from the data that there is no change in the difference in Ct value over time. Relative ratio was the response variable in the first dried blood trial, and difference in Ct value was examined when the cDNAs were analyzed using the human-specific primers and probes. If a change in RNA levels was occurring, it would result in a change in both response variables over time. These data raise the possibility that the difference in Ct value between both RNAs examined does not change over time. This concept is further investigated using a larger population size in Chapter Five. The results shown are based on the averaged values for three blood draws, 3 isolates per time point and duplicate assays for a total of 18 measurements.

Amplicon Size and Decay

Based on the results presented in Figure 2-3, analysis using the primer/probe set that amplifies 60 bp regions on 18S and β -actin [18S (60 bp)/BA (60 bp)] does not show a change in the difference in Ct value over the 50-day period examined. When the amplicon size is increased to 180 bp [18S (180 bp)/BA (180 bp)] a change in the difference in Ct value is observed. Because the same sized region is amplified for 18S and β -actin, amplicon size can not be the factor influencing the change in difference in Ct values over time. It appears that there is decreased detection for β -actin compared to 18S when the same sized regions are amplified. This raises the possibility that protein protection may be influencing RNA detection, causing 18S to be the more stable RNA species. No change was observed over time when the 60 bp regions were amplified on 18S and β -actin. It is possible that this is due to the small size of the region amplified. Analysis using BA (60 bp)/BA (300 bp) generates a large change in the difference in Ct value from 0-50 days. The same trend occurs when the

Figure 2-3. Real-time analysis of RNA Isolated from Dried Blood Stains. Difference in Ct value is specific for the primer/probe sets used for analysis (2^{nd} - 1^{st}). Mean difference in Ct value is plotted and standard error bars are included. N=1.

cDNAs were analyzed with 18S (180 bp)/18S (500 bp). These results prompted further investigations using various primer/probe sets to determine the age of a biological sample by identifying difference in RNA decay.

Species–Specificity

Figure 2-4 shows that cDNAs from all species examined are amplified using primers and probes for the commercially available 18S. Based on the results, the primers and probes for the commercially available β -actin (BA), BA (60 bp), and BA (180 bp) only amplify cDNA from humans. Primers and probes for BA (300 bp) amplify cDNA from human with background levels of detection of cDNA from cat and dog. Primers and probes for 18S (60 bp) amplify cDNA of human and pig while primers and probes for 18S (180 bp) amplify cDNA from the majority of the species with the greatest detection of cDNA from human, rat, cat and dog. Primers and probes for 18S (500 bp) detects human cDNA with background levels of detection of pig, cat, and dog cDNA. If these primers and probes cannot be made completely human-specific, the RNA sample in question can be examined using primers and probes specific for the other species identified that could have contaminated the human sample.

Figure 2-4. Species-Specificity of Primer/Probe Sets. Ct values are plotted on the x-axis and values at or above 35 are considered background and therefore are designated “0”. The mean Ct value is plotted for each amplicon and standard error bars are included. N=2.

Each Ct value represents a 2-fold difference in starting material. Because 18S is so prevalent in the cell, it reaches exponential amplification at low Ct values when human RNA is amplified. For example, 18S (60 bp) yields a Ct value of 13 for the amplification of human cDNA and a Ct value of 33 for the detection of rat cDNA. This represents a 2^{19} -fold difference contributing 0.00019% to the results obtained. This is considered to be background level. Therefore, if these human samples were contaminated with rat RNA, the rat RNA would not significantly alter the results obtained with the human RNA based on detection using primers and probes for 18S (60 bp). Values at 35 and higher are considered at background levels.

Real-time Detection of Background RNA Present in Different Fabrics

Results presented in Figure 2-5 suggest that the majority of the newly designed primer/probe sets did not detect RNA isolated from wool, cotton, polyester, linen, and silk. 18S (60 bp)/BA (60 bp) only detects RNA from the positive control at levels above background. This primer/probe set amplifies the smallest regions examined but still gives a very specific reading. The primers that amplify 18S (180 bp) appear to detect RNA from several of the fabrics tested, including silk and wool but the levels of detection were close to background (Ct value > 35). The primers that amplify 18S (500 bp) do not detect RNA at levels above background from any of the fabrics. Potentially misleading results caused by increased levels of 18S (180 bp) due to recognition of RNA present in natural fibers, could prevent accurate analysis of RNA recovered from blood stains that have been deposited on fabric.

Figure 2-5. RNA Detection of Background RNA Present in Various Fabrics. Results shown are based on sham RNA isolations of various natural fabrics. Mean Ct value is plotted and standard error bars are included. N=2.

All β -actin primers only detect RNA from the positive control. For the commercially available primer/probe set 18S/BA, 18S detects RNA from all of the fabrics with the lowest Ct value appearing to come from RNA detection in silk. These results show that 2 of the primer sets for 18S detect RNA from the fabrics examined. The commercially available 18S is universal and the primers and probes are designed to recognize RNA from an abundance of species, so this is not surprising. The Ct values plotted in the Figure 2-5 are based on the individual primer sets, although they were multiplexed with their respective partner for the real-time analysis.

Further Analysis of RNA Isolation Procedure Using TRI Reagent

BD

Many experiments were performed to identify potential advantages or limitations of using TRI Reagent BD to isolate RNA from dried blood stains. These experiments examined the variability contributed by the isolation procedure, the effects of storage of blood stains in the TRI Reagent on RNA stability, the effects of different volumes of blood on the difference in Ct value, and the analysis of DNA isolated subsequent to the RNA.

Variability Contributed by TRI Reagent BD

Further analysis was performed to determine if different investigators could recover similar amounts of RNA from blood stains of the same age using TRI Reagent BD. Based on the results, it does not appear that there are any differences with respect to the difference in

Figure 2-6. Variation in Isolation Technique due to Investigator. Investigators are designated A, B, or C. Mean difference in Ct value is plotted and standard errors bars are shown. Difference in Ct value is specific for the primer/probe sets used for analysis (2^{nd} - 1^{st}). N=3.

Figure 2-7. RNA Stability in TRI Reagent BD. Mean difference in Ct value is plotted and standard errors bars are shown. Difference in Ct value is specific for the primer/probe sets used for analysis (2^{nd} - 1^{st}). N=3.

Ct value obtained by all investigators using all primer/probes sets for analysis (Figure 2-6). This gives us no reason to believe that large amounts of variability are contributed by the investigator during the processing of samples.

Stability of RNA When Stored in TRI Reagent BD

To examine the effects of sample storage on RNA stability in blood samples, blood stains were placed into TRI Reagent BD solution and stored for various intervals over a 7-day period. Statistical analysis using the paired Student's t-test did not identify a significant difference between the difference in Ct values for 0 day compared to 1, 4, and 7 days for analysis using either primer/probe set. The results indicate that RNA regions examined in this experiment are stable for at least 7 days when stored in the TRI Reagent BD (Figure 2-7).

The Effects of -20 °C Storage on RNA Recovery

To further establish the flexibility in the TRI Reagent BD protocol, the effects of an additional -20 °C storage step during the RNA isolation procedure on RNA stability was examined. Based on analysis using a paired Student's t-test, there were no significant differences observed between the samples subjected to the -20 °C storage step versus the samples that were not for any of the primer/probe sets examined (Figure 2-8). RNA concentration was also estimated using RiboGreen, and based on analysis using a paired Student's t-test, no differences in concentration were identified between -20 °C (50±2) storage of the RNA in ethanol at a concentration of 75% versus no -20 °C (47±5) storage of the RNA over ethanol. These results indicate to us that storage of RNA in ethanol at a

Figure 2-8. The Effect of -20 °C Storage on RNA Detection. Mean difference in Ct value is plotted and standard errors bars are shown. Difference in Ct value is specific for the primer/probe sets used for analysis (2nd - 1st). N=3.

concentration of 75%, at -20⁰ C does not affect RNA detection of the regions examined.

Another benefit of using the TRI Reagent BD RNA isolation procedure is that samples can be stored half-way through the procedure, if time restraints should arise, without affecting RNA detection.

Different Blood Volumes

Real-time analysis using BA (60 bp)/BA (300 bp), 18S (60 bp)/BA (60 bp), and 18S (180 bp)/18S (500 bp) showed no difference in the difference in Ct value for all volumes of blood (Figure 2-9). Based on these results, it appears that the difference in Ct values is independent of sample volume. Individual Ct values are increased for the smaller volumes but the difference in Ct value remains the same. These results suggest that the technique described in this dissertation would yield the same results independent of sample size or volume.

STR analysis of DNA

Based on Affiliated Genetics' analysis, DNA isolated from 10-µl spots of blood deposited on cotton and plastic could be successfully genotyped. DNA isolated from hair and saliva/buccal could not be genotyped. Analysis using 3 and 10 µl of the 75 µl rehydration displayed a genotype for some of the markers using DNA from individuals 1, 2, 5, and 6 (see below). Analysis using 1 µl of the 75 µl rehydration resulted in no amplification of any of the DNAs tested. Figure 2-10 shows the results of the STR analysis

Figure 2-9. RNA Detection in Different Volumes of Blood. Mean difference in Ct value is plotted and standard errors bars are shown. Difference in Ct value is specific for the primer/probe sets used for analysis (2^{nd} - 1^{st}). N=2.

using the blood from fabric and cotton (10 μ l of 75 μ l) for all markers described in the methods section. Make note that differences between males and females, based on the sex determination marker, were observed. The inability to amplify the DNA isolated from hair and saliva may be due to the large rehydration volume.

Classification of DNA used for Genotyping Analysis

1. blood on cotton/female
2. blood on cotton/female
3. hair/female
4. hair/female
5. blood on plastic/male
6. blood on plastic/male
7. saliva/female
8. saliva/female

Figure 2-10A-B. STR Analysis of DNA Isolated From Dried Blood Stains. Ten μ l of the original sample was used to generate the results for A and B. Allelic Ladders, which represent a standard to which the lower panel is to be compared, are identified in the upper panels of both A and B. The lower panel in A represents DNA isolated from blood deposited on cotton. The lower two panels in B represent DNA isolated from blood deposited on cotton. The second panel in B identifies DNA from a male and the third panel in B identifies DNA from a female.

B

Detection of RNA over time from different surfaces

RNA recovered over a 90-day period from dried blood deposited on different surfaces, and isolated from hair and saliva/buccal cells, was examined using real-time PCR and RiboGreen. Fifty ng of RNA was recovered from a single freshly plucked hair follicle (Table 2-3). RiboGreen analysis predicted RNA concentrations above the estimated values of the standard curve for the RNA recovered from saliva/buccal cells. The samples were not diluted because the RiboGreen results contradicted the real-time results (see below). The amount of RNA recovered from blood after 1 day was very similar for all surfaces examined except brick and polyester. RiboGreen analysis estimated recovery of 80-100 ng of RNA from blood deposited on cotton, plastic, newspaper, unfinished wood, and finished wood. RNA isolated from blood deposited on polyester yielded over 100 ng of RNA and RNA isolated from blood deposited on brick yielded about 50 ng of total RNA. The amount of RNA recovered decreased over time for blood stains deposited on all surfaces and the RNA isolated from hair and saliva/buccal cells. After 90 days, RNA recovery from most surfaces yielded 40-60 ng of RNA.

Real-time analysis using 18S (60 bp)/BA (60 bp), showed no change in the difference in Ct value between BA and 18S isolated from dried blood stains deposited on all surfaces over the 90-day period examined (Table 2-4B). This is consistent with previous results (Figure 2-2). The difference in Ct value was very similar for RNA isolated from blood deposited on all surfaces examined; RNA isolated from hair was the only exception. This could be due to the high Ct value obtained for β -actin when RNA recovered from hair was used as a template for real-time analysis.

Table 2-3. Mean RNA Recovered From Blood Deposited on Different Surfaces. Absorbance was converted to ng of RNA by comparison to a standard curve. Standard error is included in table. The predicted RNA concentration for fresh blood was based on values obtained from freshly extracted blood prior to deposition.

Source of Sample	FRESH (ng) Prior to Deposition	1 DAY (ng)	30 DAY (ng)	60 DAY (ng)	90 DAY (ng)
Polyester	*100±13	110±20	85±25	90±24	90±16
Newspaper	100±13	80±10	60±7	50±6	40±12
Cotton	100±13	85±12	80±8	85±6	52±6
Plastic	100±13	87±12	80±8	64±6	60±8
Unfinished wood	100±13	95±10	50±8	33±5	35±6
Finished Wood	100±13	95±5	50±6	33±4	35±7
Brick	100±13	60±15	40±4	30±6	30±8
Hair	50±6	N/A	45±6	35±7	30±5
Saliva	Undetermined	N/A	Undetermined	Undetermined	Undetermined
Sham	2 ±0.5	3±0.5	3±1	2±0.5	1±0.5

* Approximately 100 ng of blood was deposited onto polyester, newspaper, cotton, plastic, unfinished wood, finished wood, and brick at time point 0 day. RNA was isolated from blood deposited on each of these surfaces at 1, 30, 60, and 90 days after deposition.

Real-time analysis using BA (60 bp)/BA (300 bp) showed an expected change in the difference in Ct value over the 90-day period (Table 2-4A). The differences in Ct values over time appeared to be similar for RNA recovered from blood deposited on unfinished wood, plastic, cotton, finished wood and RNA isolated from hair and saliva. RNA recovered from blood deposited on newspaper yielded a larger difference in Ct value for time point 1 day but was only detectable until 60 days. Many individual Ct values for the early time

points were near background levels suggesting that real-time analysis of RNA isolated from dried blood stains deposited on newspaper may be unreliable. Consistent with other results, real-time analysis of RNA recovered from blood deposited on polyester showed increased differences in Ct values over time (Table 2-4B). These values are inconsistent with the differences in Ct values estimated for the RNA isolated from blood deposited on the other surfaces examined. The difference in Ct values for the RNA isolated from blood deposited on polyester were lower than those established for the RNA recovered from the blood deposited on other surfaces and did not change much over the time frame examined. These results indicate to us that specific time lines of decay for blood deposited on different surfaces need to be developed to ensure accurate results.

Relative amounts of the region amplified predicted by Ct value corresponds to the relative concentrations predicted by RiboGreen analysis. For example, RNA isolated from blood deposited polyester yields the most RNA over the time frame examined when compared to the other isolated RNAs based on RiboGreen analysis (Table 2-3). RNA isolated from blood deposited on polyester also yielded the lowest Ct values based on real-time analysis (Table 2-4). RNA isolated from hair and saliva yielded the highest Ct values for all time points examined. The sham control yielded Ct values of 35-40 for samples analyzed from all time points.

Table 2-4A-B. Real-time Analysis of RNA Isolated from Blood Deposited on a Variety of Different Surfaces. Values shown represent the difference in Ct values (Δ CT) from RNA isolated from blood deposited on different surfaces and isolated from hair and saliva that had aged up to 90 days. Standard error is included in the table.

A

18S (60 bp)/ BA (60 bp)	1 DAY (Δ CT)	30 DAYS (Δ CT)	60 DAYS (Δ CT)	90 DAYS (Δ CT)
Brick	6.15 $\pm$ 0.65	6.91 $\pm$ 0.45	6.31 $\pm$ 0.63	6.432 $\pm$ 0.38
Cotton	7.12 $\pm$ 0.68	7.1 $\pm$ 0.63	6.6 $\pm$ 0.59	6.98 $\pm$ 0.39
Finished Wood	6.43 $\pm$ 0.61	7.18 $\pm$ 0.52	6.68 $\pm$ 0.36	7.0 $\pm$ 0.54
Newspaper	6.6 $\pm$ 0.73	6.35 $\pm$ 0.35	7.0 $\pm$ 0.61	6.48 $\pm$ 0.41
Plastic	6.9 $\pm$ 0.32	7.6 $\pm$ 0.34	7.3 $\pm$ 0.65	6.9 $\pm$ 0.36
Polyester	5.96 $\pm$ 0.26	6.96 $\pm$ 0.34	6.4 $\pm$ 0.59	6.4 $\pm$ 0.14
Unfinished Wood	6.30 $\pm$ 0.41	7.27 $\pm$ 0.16	6.22 $\pm$ 0.62	6.5 $\pm$ 0.69
Hair	13.53 $\pm$ 1.43	12.92 $\pm$ 1.75	11.7 $\pm$ 1.19	11.45 $\pm$ 1.05
Saliva	7.05 $\pm$ 0.86	7.0 $\pm$ 0.31	4.5 $\pm$ 0.85	7.0 $\pm$ 0.47

B

BA (60 bp)/ BA (300 bp)	1 DAY (Δ CT)	30 DAYS (Δ CT)	60 DAYS (Δ CT)	90 DAYS (Δ CT)
Saliva	1.66 $\pm$ 0.54	4.14 $\pm$ 1.07	5.95 $\pm$ 1.58	8.89 $\pm$ 0.97
Hair	2.10 $\pm$ 0.46	3.05 $\pm$ 0.78	3.85 $\pm$ 0.30	7.12 $\pm$ 1.27
Brick	3.68 $\pm$ 0.68	4.1 $\pm$ 0.53	5.2 $\pm$ 0.23	6.25 $\pm$ 0.25
Cotton	2.65 $\pm$ 0.20	3.63 $\pm$ 0.39	4.82 $\pm$ 0.32	7.42 $\pm$ 0.51
Finished Wood	2.89 $\pm$ 0.52	3.96 $\pm$ 0.42	5.77 $\pm$ 0.59	7.04 $\pm$ 0.44
Newspaper	3.34 $\pm$ 0.79	5.32 $\pm$ 0.16	*ND	ND
Plastic	2.66 $\pm$ 0.31	3.87 $\pm$ 0.27	6.27 $\pm$ 1.07	7.01 $\pm$ 0.30
Polyester	1.88 $\pm$ 0.39	3.67 $\pm$ 0.47	4.48 $\pm$ 0.32	4.49 $\pm$ 0.24
Unfinished Wood	2.60 $\pm$ 0.65	4.2 $\pm$ 0.29	5.41 $\pm$ 0.89	7.70 $\pm$ 0.31

*ND, detection of one of the regions amplified at a PCR cycle higher than 35.

Levels of Detectability

A series of dilutions was performed on RNA isolated from dried blood stains to determine the sensitivity of the method described in this dissertation. The values shown in Table 2-5 represent the Ct values obtained for cDNA amplified using each primer/probe set with individual Ct value listed for each region amplified. For the amplicon 18S (60 bp)/BA (60 bp), 24/28 would mean that 24 was the Ct value for 18S and 28 was the Ct value for β -actin. RNA isolated from 1 μ l blood samples, diluted 100-fold, was amplified using 18S (60 bp)/BA (60 bp) suggesting the sensitivity for the technique described in this dissertation (Table 2-5). Successful amplification was achieved using all primer/probe sets when RNA isolated from 1 μ l blood stains, diluted 6-fold, was used as a template. In theory, assuming all environmental and laboratory conditions are constant, both β -actin and 18S RNA could be amplified, using real-time PCR from a fresh bloodstain as small as 0.01 μ l.

RiboGreen analysis estimated the total amount of RNA recovered from 10 μ l fresh blood samples to be 80-90 ng and that of 1 μ l was estimated to be 15 ng. Seventy ng of RNA was recovered from a single hair follicle. All other estimated concentration of all dilutions are provided in Table 2-6.

Table 2-5. Sensitivity of Experiment using Real-time Analysis. The first measurement represents the Ct value for the first amplicon identified in the primer/probe set and the second measurement represents the Ct value for the second amplicon in the multiplex reaction. Six dilutions were performed on each of the 1, 5, and 10 µl samples. ND=Not Detected.

Dilution	18S (180 bp)/	18S/BA-	BA (60 bp)/	18S (180 bp)/	18S (60 bp)/
	BA (180 bp)	Commercially available	BA (300 bp)	18S (500 bp)	BA (60 bp)
	Ct Values	Ct Values	Ct Values	Ct Values	Ct Values
1 µl	16/25	17/29	25/27	16/27	17/24
2-fold	18/27	20/30	28/30	18/29	18/29
4-fold	20/29	21/31	30/31	19/30	19/30
6-fold	21/31	21/32	31/32	21/32	21/32
10-fold	22/33	ND	ND	22/33	22/33
100-fold	ND	ND	ND	ND	28/36
1000-fold	ND	ND	ND	ND	ND
5 µl	14/22	14/25	22/24	14/25	14/21
2-fold	16/25	17/29	24/26	16/28	15/23
4-fold	18/26	18/30	27/28	17/29	16/25
6-fold	19/28	19/30	28/29	18/31	18/27
10-fold	20/30	20/31	29/30	19/33	19/28
100-fold	22/32	ND	30/33	ND	20/30
1000-fold	ND	ND	ND	ND	26/32
10 µl	14/20	14/23	21/24	14/23	14/20
2-fold	16/22	14/27	22/26	15/25	15/22
4-fold	17/24	16/29	25/27	16/28	16/24
6-fold	18/25	17/28	26/28	18/29	17/25
10-fold	19/27	18/29	27/28	19/27	18/26
100-fold	20/28	ND	29/31	20/31	19/27
1000-fold	22/31	ND	ND	21/33	23/30

The method developed utilizes specific regions on specific RNAs and is not intended to analyze total RNA loss; this increases the sensitivity of technique described in this dissertation. It should be noted that only 1/10 of each reverse transcriptase reaction is used in each real-time assay, therefore, a total of 8 to 10 assays could be performed with this small amount of RNA. Because of the small amounts of sample that can be used for accurate analysis, it could be suggested that this method is sensitive enough to detect RNA from nearly any sample that might be identified as biological material.

Table 2-6. Sensitivity of RNA Detection using RiboGreen Analysis. Absorbance was converted to ng of RNA by comparison to a standard curve. Standard error is included in table. ND (not detected) means that the reading could not be distinguished from that of background.

Amount of Sample	Concentration using RiboGreen
10 μl blood	80 ng
2-fold	40 ng
4-fold	15 ng
6-fold	ND
10-fold	ND
100-fold	ND
1000-fold	ND
5 μl blood	35 ng
2-fold	12 ng
4-fold	ND
6-fold	ND
10-fold	ND
100-fold	ND
1000-fold	ND
1 μl blood	15 ng
2-fold	ND
4-fold	ND
6-fold	ND
10-fold	ND
100-fold	ND
1000-fold	ND
1 hair follicle	70 ng
2-fold	20 ng
4-fold	ND
6-fold	ND
10-fold	ND
100-fold	ND
1000-fold	ND

Discussion

Analysis of the cDNAs from the first dried blood trials with the human specific primer/probe set 18S (60 bp)/BA (60 bp) suggested that different types of RNA and different sized regions of RNA influence the ability to detect RNA from *ex vivo* aged blood samples. (Figure 2-2 and 2-3). Integration of these concepts should enhance precision associated with the technique described in this dissertation. The larger the region amplified, the shorter the period of detection of RNA in dried blood stains due to a more rapid loss of signal. Analysis of RNA using 18S (180 bp)/BA (180 bp) yields a greater change in the difference in Ct value over a 50-day period when compared to the analysis of the same RNA using 18S (60 bp)/BA (60 bp). The decreased detection can be explained by the failure or reduced ability to amplify the 180 bp region of β -actin and 18S when compared to a 60-bp region on the same molecule. Examination of 2 regions on the same RNA could decrease intra-person variability because the 2 regions examined will initially exist in equimolar amounts because they are present on the same molecule.

Analysis of cDNAs with different combinations of primer/probe sets, many provide different ranges of time lines. Multiplexing the primers/probes that recognize small PCR products (60 bp) for both 18S and β -actin may allow detection over an extended period of time, with detection above background levels suggested at time points up to a year or more (data not shown). To increase precision of this technique, the same cDNA can be analyzed with a variety of primer/probe sets that have different time lines of detection of RNA in dried blood samples. The developed primer/probe sets were designed based on amplicon size, human-specific polymorphisms contained within the RNA sequence, and preferences of the Primer Express software.

Real-time analysis of RNA isolated from dried blood stains by different investigators using TRI Reagent BD supports the RiboGreen results presented in Chapter One (Table 1-4). The results presented in Chapter Two showed little technique variability for all investigators (Figure 2-6). TRI Reagent BD is an organic extraction which does not use spin columns to recover RNA and because of this can potentially yield more total RNA. Often, small fragments of RNA are not recovered because they are too small to have affinity for the spin column and thus get discarded during the procedure. The spin columns provided with the QIAamp RNA Blood Kits only bind RNAs larger than 200 nucleotides, but small RNAs (< 200 nucleotides) make up 15-20% of the total RNA [42]. TRI Reagent BDE can recover RNAs greater and less than 200 nucleotides in size.

Based on real-time detection, RNA in blood appears to be stable once rehydrated with TRI Reagent BD. This could aid in sample recovery from crime scenes. Sometimes it is difficult to immediately transport a sample to the lab due to the location of a crime scene. Rehydration in TRI Reagent BD would prevent further RNA decay of the samples (Figure 2-7). The storage of the RNA in the 75% ethanol at -20 °C during the isolation procedure provides greater flexibility in the isolation protocol (Figure 2-8). Due to the lengthy protocol, it is sometimes difficult to completely process the samples through to the end. Storage of the RNA in ethanol provides an intermediate stopping point. Along with many of the developed primer/probe sets being human-specific, DNA isolated using TRI Reagent BD after prior removal of RNA can be genotyped (Figure 2-10). DNA isolated from blood deposited on both blood and plastic was successfully genotyped and the specific gender was identified. If a statistically significant gender and/or gender by day interaction can be identified the possibility exists to generate a gender specific curve that could reduce the

variability associated with the confidence intervals. STR analysis should eventually be repeated using a smaller rehydration volume for hair and saliva/buccal cells. This may help to increase the sensitivity and allow for DNA detection and genotyping of these other biological samples.

To investigate the human-specificity of the primers and probes used, each one was tested using a variety of cDNAs template from species other than human and many different types of natural fibers (Figure 2-3 and Figure 2-4). Only the commercially available 18S and 18S (180 bp) detected RNA in the various fabrics but at low levels (Ct value >35). All of the primers and probes for β -actin and the primers and probe for 18S (60 bp) appeared to be human-specific. Primers and probes for the commercially available 18S and 18S (180 bp) generated the greatest detection of RNA for all species examined (Figure 2-4). This is not surprising because of the high degree of conservation and the large amounts of 18S present in the cell. If contamination of evidence is an issue, the PCR product could be sequenced to determine and confirm exact origin of sample.

This method discussed in this chapter has been shown to be very sensitive with detection of RNA from 1 μ l of blood diluted 100-fold using 18S (60 bp)/BA (60 bp) (Table 2-5). Assuming proportional recovery, this would be theoretically equivalent to 0.01 μ l of blood which is an amount that cannot be identified by the human eye. RNA isolated from blood that had aged up to 15 months was successfully detected using several of the primer/probe sets (data not shown). This system has also been shown to generate the same results (ratio/differences) for RNA isolated from different volumes of blood (Figure 2-9). The inability to control for starting sample amount was one of the major flaws with previous techniques developed to estimate age of blood stain. Because this method has overcome

some of the problems associated with the earlier developed technique, it has the potential to become a more accurate and precise means of dating biological samples.

RNA has also been recovered from blood deposited on a variety of surfaces other than fabrics, including newspaper, brick, plastic, etc. Of these different surfaces, RNA isolation from blood deposited on polyester yielded the greatest amount of RNA (Table 2-3). This may be explained by the manner in which the blood dries and becomes embedded in the fabric. RNA isolated from blood deposited on all surfaces appears to generate similar detection of RNA decay, based on changes in Ct value over time, even though different amounts of RNA were recovered from each surface. Previous work has shown that different amounts of DNA are recovered from blood deposited on different surfaces [43]. These results suggest that the difference in DNA recovery is due to the different textures of the stain carriers and the differing degrees of DNA loss during extraction. When examining RNA recovered from different surfaces, one will need to keep in mind that the time line of detection may be different for each surface because of how that specific surface might interfere with RNA recovery or contribute to RNA degradation. RNA isolated from hair and saliva/buccal cells could be recovered and detected using real-time PCR (Table 2-4). The technique described in this dissertation has the potential to be applied to biological evidence other than blood that may be found at a crime scene. Hair and saliva gave similar values for detection of the isolated RNA based on the real-time results from the surface experiment. The predicated concentrations based on RiboGreen analysis of these RNAs identified saliva as having the greatest recovery of RNA which contradicted real-time results. Because the values suggested by RiboGreen analysis were not realistic and extremely high, contamination by factors contained within the saliva/buccal cells may be the explanation for the misleading

RiboGreen results. The results could also be explained by the inhibition of the real-time reaction used for the analysis of the RNA isolated from saliva/buccal cells due to the presence of certain compounds. The predicated RiboGreen values suggest μg concentrations of RNA, which based on sample size, would be almost impossible. In this chapter, several experiments were performed to further refine the technique developed for estimating the age of a biological sample. The results suggest that this is a very sensitive yet robust method to isolate RNA from dried blood stains. Analysis of cDNAs with different primer/probe sets may further enhance the sensitivity of this technique and hopefully allow for smaller confidence intervals.

Chapter Three- Environmental Effects on RNA Stability in Dried Blood Stains

Introduction

The results presented in the previous 2 chapters were generated using RNA isolated from blood stains stored at constant environmental conditions. Samples found at crime scenes can often be exposed to a variety of environmental conditions. This needs to be taken into consideration because specific environmental conditions may alter the stability of RNA in dried blood samples. In this chapter, the effects of light, temperature, and humidity on RNA stability in dried blood samples are examined.

Methods

Blood Collection

For the environmental studies, 10 ml blood was collected from 1-2 individuals in plain coated BD vacutainers on three separate occasions (Chapter One). Thirty aliquots (10 µl) of blood were spotted onto a single piece of cotton fabric and placed at the specific conditions discussed below.

RNA Processing

Full Spectrum Light

Half of the total blood stains from the same blood draw were exposed to 12 hours of full spectrum light a day and the other half were not exposed to any light source. For this

study, all samples were kept inside an environmental chamber at a constant temperature of 25 °C and 50% humidity. The chamber was divided by Aluminum foil covered shelves that separated the exposed samples from the non-exposed samples. The bulbs used for the light exposure were manufactured by GE and designed to mimic natural sunlight (Sunshine, full spectrum light bulb). The average initial lumens of the bulbs was 620, the color temperature was 5000 °K, and the CRI was 90. RNA was simultaneously isolated from 3 blood stains exposed to each treatment at 0, 30, 60 and 90 days after deposition.

Temperature

Half of the total blood stains from each blood draw were subjected to a storage temperature of 5 °C and the other half were subjected to a storage temperature of 40 °C (42 °F and 103 °F). All samples were exposed to a constant humidity of 50% inside an environmental chamber. RNA was simultaneously isolated from 3 blood stains exposed to each treatment at 0, 30, 60 and 90 days after deposition.

Constant Humidity

Half of the total blood stains from the same blood draw were subjected to a constant humidity of 50% and the other half were subjected to a constant humidity of 95%. All samples were exposed to a constant temperature of 25 °C inside an environmental chamber. RNA was simultaneously isolated from 3 blood stains exposed to each treatment at 0, 30, 60 days after deposition.

Varied Humidity

Half of the total blood stains from each blood draw were subjected to an initial humidity of 50% and the other half were subjected to a constant humidity of 95%. All samples were exposed to a constant temperature of 25 °C inside an environmental chamber

for the duration of this experiment. After 1 day, all blood stains exposed to 50% humidity were placed at 95% humidity for the remainder of the experiment. RNA was simultaneously isolated from 3 blood stains exposed to each treatment at 0 (same humidity for both), 1 (half at 95% and half at 50%), and 7 (both at 95%) days after deposition.

Real-time PCR Analysis

All RNA isolated from the dried blood stains examined in this chapter was converted to cDNA using previously described methods (Chapter One) and stored at -80 °C until all samples were processed for the time frame examined. Real-time analysis using 18S (60bp)/BA (60 bp), 18S (180 bp)/18S (500bp), and BA (60 bp)/BA (300bp), was performed and each cDNA was analyzed using duplicate reactions. Results are based on the averaged value for triplicate isolates, triplicate blood draws, and duplicate assays for a total of 18 measurements. Controls were included as described in Table 1-2.

Environmental studies: paired Student's t-test

A paired Student's t-test was performed on the measurements taken between the 2 sets of samples exposed to full spectrum light, temperature, and humidity [35]. Three total measurements were compared for each time point between experimental and control groups representing 3 separate blood draws for the same subject. All measurements (triplicate RNA isolates and duplicate assays) were averaged to generate a single value for each blood draw. Significance was demonstrated based on a P-value lower than 0.01.

Results

Full Spectrum Light

Over the 90-day period examined, analysis using all primer/probe sets, except 18S (60bp)/BA (60bp) show a decrease in detection of either 18S (500 bp) [18S (180 bp)/ 18S (500 bp)] or BA (300 bp) [BA (60 bp)/ BA (300 bp)] over time resulting in an increased difference in Ct value (Figures 3-2 and 3-3). Real-time analysis using 18S (60bp)/BA (60bp) did not show a change in the difference in Ct value over time (Figure 3-1). A statistically significant difference was not observed for any time point within a primer/probe set between RNA isolated from blood stains exposed to full spectrum light compared to RNA isolated from blood stains not exposed to full spectrum light. These results show that full spectrum light does not influence RNA stability in dried blood over the time frame examined.

Temperature

An increased difference in Ct value was observed for the RNA isolated from the samples exposed to 40 °C compared to the RNA isolated from the samples exposed to 5 °C for all time points except 0 day when analyzed using all primer/probe sets (Figure 3-4, 3-5, 3-6).

Figure 3-1. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp). Results show the mean difference in Ct value generated by exposure to full spectrum light or control for time points indicated. Standard error bars are including on graph. N=3.

Figure 3-2. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp). Results show the mean difference in Ct value generated by exposure to full spectrum light or control for time points indicated. Standard error bars are included on graph. N=3.

Figure 3-3. The Effects of Full Spectrum Light on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp). Results show the mean difference in Ct value generated by exposure to full spectrum light or control for time points indicated. Standard error bars are included on graph. N=3.

This suggests a decrease in detection of 18S (500 bp), BA (300 bp) and BA (60 bp) when compared to 18S (180 bp), BA (60 bp) and 18S (60 bp), and isolated from blood stains exposed to 40 °C. Very little change in individual Ct values were observed when all regions of RNA isolated from blood exposed to 5 °C were analyzed with all primer/probe sets (Figure 3-4, 3-5, 3-6). No changes in the difference in Ct values were observed based on the analysis with any primer/probe set over the indicated time frame. This suggests a stabilization of RNA in dried blood stains at low temperatures. Statistical analysis of the RNA isolated from the samples exposed to the higher temperature verses the lower temperature indicates that temperature has an effect on RNA detection in dried blood stains. Analysis using all primer/probe sets resulted in statistically significant differences between the RNA isolated from the samples exposed to 5 °C compared to the RNA isolated from the samples exposed to 40 °C at time point's 30, 60 and 90 days for both individuals examined (3-4, 3-5, 3-6). Significant differences are indicated on the graphs using an asterisk (*). Real-time analysis of the RNA isolated from the samples exposed to the higher temperature generated Ct values at or above backgrounds level for many of the time points examined after 0 day, suggesting that the decrease in detection is due to total degradation of the RNA. Differences in the starting Ct values of β -actin and 18S were observed between the 2 individuals examined. This is further investigated in Chapter Five.

Constant Humidity

Over the 60-day period examined, a greater loss in detection of 18S (500) compared to 18S (180 bp) is observed when RNA isolated from blood exposed to both 50% and 95% humidity was analyzed using 18S (180 bp)/18S (500bp) (Figure 3-8). A similar trend was observed when BA (60 bp)/BA (300bp) was used to analyzed the same RNA (Figure 3-9). Analysis using 18S (60bp)/BA (60 bp) shows a change in the difference in Ct value over time for the RNA isolated

from the samples exposed to 95% humidity but not from the RNA isolated from the samples exposed to 50% humidity (Figure 3-7). Statistical analysis of the RNA isolated from the samples exposed to 95% humidity versus the RNA isolated from the samples exposed to 50% humidity indicates that humidity does have an effect on RNA detection over time in dried blood stains. Real-time analysis using BA (60 bp)/BA (300bp) resulted in statistically significant differences between the RNA isolated from the blood stains stored at 50% humidity and the RNA isolated from the blood stains stored at 95% humidity for time points 30 and 60 days (Figure 3-9). Analysis of the same RNAs using 18S (180 bp)/18S (500bp) resulted in statistically significant differences for at 60 days and analysis using 18S (60bp)/BA (60 bp) resulted in statistically significant differences at 30 and 60 days (Figure 3-7). The experiment was terminated after 60 days due to mechanical failure of the environmental chamber.

Varied Humidity

Real-time analysis of RNA isolated from blood stains exposed to varied humidity using 18S (180 bp)/18S (500bp) (Figure 3-11), and BA (60 bp)/BA (300bp) (Figure 3-12), indicates that varied humidity has an effect on RNA detection. RNA isolated after 7 days from the samples stored at 95% humidity was identified to be statistically different than the RNA isolated from the samples initially stored at 50% humidity and then moved to 95% humidity 1 day after deposition. There were no statistically significant differences found between RNAs of both exposure groups when 18S (60bp)/BA (60 bp) (Figure 3-10) was used to analyze the RNAs. Visually it appeared that blood stains stored at 50% humidity dried within a half of an hour but blood stains stored at 95% humidity never completely dried. These results suggest that exposure to varied humidity may cause rehydration of blood stains which could influence RNA detection.

Figure 3-4. The Effects of Temperature on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp). Results show the mean difference in Ct value generated by exposure to increased or decreased temperatures for the time points indicated. Standard error bars are included. A and B represent the two individuals examined. N=3.

Figure 3-5. The Effects of Temperature on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp). Results show the mean difference in Ct value generated by exposure to increased or decreased temperatures for time points indicated. Standard error bars are included. A and B represent the two individuals examined. N=3.

Figure 3-6. The Effects of Temperature on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp). Results show the mean difference in Ct value generated by exposure to increased or decreased temperature for time points indicated. Standard error bars are included. A and B represent the two individuals examined. N=3.

Figure 3-7. The Effects of Humidity on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp). Results show the mean difference in Ct value generated by exposure to 95% or 50% humidity for time points indicated. Standard error bars are included. N=3.

Figure 3-8. The Effects of Humidity on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp). Results show the mean difference in Ct value generated by exposure to 95% or 50% humidity for time points indicated Standard error bars are included. N=3.

Figure 3-9. The Effects of Humidity on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp). Results show the mean difference in Ct value generated by exposure to 95% or 50% humidity for time points indicated. Standard error bars are included. N=3.

Figure 3-10. The Effects of Rehydration on RNA Detection in Dried Blood Stains using 18S (60 bp)/BA (60 bp). Day 1 shows results based on one day exposure at either 95% or 50% humidity. Day seven is based on results after the samples stored at 50% humidity were moved to 95% humidity. Standard error bars are included in graph. N=3.

Figure 3-11. The Effects of R-hydration on RNA Detection in Dried Blood Stains using 18S (180 bp)/18S (500 bp). Day 1 shows results based on one day exposure at either 95% or 50% humidity. Day seven is based on results after the samples stored at 50% humidity were moved to 95% humidity. Standard error bars are included in graph. N=3.

Figure 3-12. The Effects of Rehydration on RNA Detection in Dried Blood Stains using BA (60 bp)/BA (300 bp). Day 1 shows results based on one day exposure at either 95% or 50% humidity. Day seven is based on results after the samples stored at 50% humidity were moved to 95% humidity. Standard error bars are included in graph. N=3.

Discussion

Full spectrum Light

In 1988, McNally showed that when dried blood stains were exposed to ultraviolet light for time periods up to 5 days, the integrity of DNA was not altered based on the lack of false restriction fragment-length polymorphisms [44]. These results suggest that ultraviolet light does not influence DNA stability in dried blood stains. Upon desiccation, RNA and DNA degradation may function in a similar manner in dried blood stains. Other work also suggests that UV has no effect on RNA isolated from dried blood over a 60-day period when specific mRNAs were analyzed using semi-quantitative duplex and competitive RT-PCR [45]. Based on all of these observations, full spectrum light can be excluded as an environmental condition influencing RNA stability in dried blood stains over the time period examined.

Temperature

Our results indicate that temperature has an effect on the detection of 18S rRNA and β -actin mRNA in dried blood samples. RNA isolated from blood exposed to 2 very extreme temperatures was examined to identify an initial effect but intermediate temperatures will still need to be examined in future experiments. RNA isolated from the samples exposed to the cooler temperature was more stable than the RNA isolated from the samples exposed to the higher temperature. The Ct values obtained from analysis using most of the primer/probe sets were at background levels for the RNA isolated at 60 and 90 days from the samples exposed to the higher temperatures. Based on the results, it appears there were differences in

the initial difference in Ct value for the subjects examined although statistical analysis was not performed to establish the specific effect due to the limited sample size. The 2 subjects were of the same race and ethnicity, different gender and about 20 years apart in age. Chapter Five addresses this point by examining inter-person variability of RNA detection using a large population.

Humidity

Based on the results presented in this chapter, humidity has been identified to effect RNA detection in dried blood samples. Although no moisture analysis experiments were performed, the samples exposed to 95% humidity appeared to be in a wet or hydrated state while the samples exposed to 50% humidity appeared to dry within the first few hours after deposition. Because the results indicate an effect of humidity on RNA detection in dried blood stains, RNA isolated from blood stains stored at other percentages of humidity between 50-90% and below 50% will need to be examined. Real-time analysis of the RNAs isolated from blood exposed to 50% and 95% humidity using 18S (180 bp)/18S (500bp) (Figure 3-8) indicated a statistically significant difference between exposures for the 60-day time point. Analysis with all other primer/probe sets showed a statistically significant difference between 50% and 95% for both the 30 and 60 day time points. This suggests that the regions amplified by 18S (180 bp)/18S (500bp) are less influenced by humidity than the other regions examined in this experiment. This could be due to increased protein protection of the regions amplified by 18S (180 bp)/18S (500bp). This experiment will need to be repeated to examine the effects of humidity on RNA in dried blood stains at time points greater than 60

days. This experiment ended at 60 days because both experimental and control environmental chambers lost power and went to 100% humidity.

Varied humidity was identified to effect RNA detection in dried blood stains. Consistent with the above results, this suggests that hydration of the blood stains may make the RNA more susceptible to degradation. If samples collected from a crime scene are found outside, they could be exposed to varied environmental conditions, including humidity. After a 7-day period, the RNA isolated from samples that were initially exposed to 50% humidity and then moved to 95% humidity was identified to be more stable than the RNA isolated from the same samples stored at 95% humidity for the duration of the experiment. After 7 days, analysis using BA (60 bp)/BA (300 bp) and 18S (180 bp)/18S (500 bp) show statistically significant differences between the RNA isolated from the samples constantly stored at 95% humidity and the RNA isolated from the samples initially dried at 50% humidity and then moved to 95% humidity (Figures 3-11 and 3-12). Rehydration of blood stains due to increased percentages of humidity may reactivate enzymatic processes resulting in decreased levels of RNA recovered from those stains.

In summary, both temperature and humidity have an effect on RNA detection in dried blood stains. More work needs to be done in this area but an initial effect was identified. Consistent with our results, in 2004 Bauer suggested an effect of temperature and humidity on the stability of β -actin and cyclophilin mRNAs isolated from dried blood stains [45]. RNA suitable for RT-PCR could not be isolated from samples stored at 37 °C and 100% humidity after 7 days. Using the method discussed in this dissertation, a reduction in the detection of RNA over time is observed when blood stains are exposed to these environmental conditions but RNA suitable for real-time analysis can still be recovered from

blood stains that have aged up to 60 days. This shows the increased sensitivity of the techniques described in this dissertation over traditional RNA analysis methods including the method used in the previously mentioned publication. Chapter Two suggested that the RNA isolated from blood deposited on different surfaces may result in differences in detection (Table 2-4A and B). Exposure of blood stains to various environmental conditions may also generate differences in the detection of RNA when recovered from different surfaces. The combined effects will need to be examined.

Chapter 4- DNA Microarray analysis of *ex vivo* Blood Stains

Introduction

The focus of the studies done in this chapter are aimed at identifying changing RNA profiles that can be used to establish an early time line (0-24 hours) for estimating the age of *ex vivo* biological material. Previous research has shown that certain genes, such as interleukins, have increased expression over a 2-hour period in response to skin wounds. Postmortem skin samples were shown to have up-regulated gene expression of interleukin-10 [46]. Gene chip analysis of 0-24 hour dried blood samples has been performed to identify mRNAs whose detectable levels are altered after the blood leaves the body. It is possible that certain genes may elicit a stress response as a result of the *ex vivo* cellular environment. The analysis and identification of these RNA species could establish a time line for dating blood stains that spans several hours to days. This would provide a time line of RNA detection that is shorter than the previously developed time lines utilizing β -actin mRNA and 18S rRNA. Practical use of this time line could be for analyzing violent crimes that were discovered soon after they were committed.

Methods

Blood Collection and Sampling

Blood was collected from a single individual in 10-ml plain vacutainers by standard venipuncture for microarray analysis and real-time confirmation.

RNA isolation for Microarray

For the first microarray, 10 aliquots of blood (200 µl) were deposited into 1.5-ml tubes and stored at room temperature and 50% humidity. RNA was immediately isolated from 6 of the aliquots prior to deposition (0 hours) and 6 of the aliquots 4 hours after deposition according to the standard TRI Reagent BD isolation procedure mentioned in Chapter One. For the second microarray experiment, 10 aliquots of blood were placed on Petri dishes (100 µl) and stored at room temperature and 50% humidity. RNA was immediately isolated from 6 of the aliquots prior to deposition (0 hours) and 6 of the aliquots 1 hour after deposition. Specific procedures were followed for both wet and dry blood samples. If the samples were completely dried, the tip of a moistened pair of tweezers was used to collect the sample which was then placed in 750 µl of TRI Reagent BD, 200 µl water and 3 µl of polyaracyl carrier. The normal protocol (Chapter One) was then followed. If the samples were still moist, a P-1000 (Eppendorf) Micropipettor was used to draw up as much liquid as possible. The remainder of the blood stain was scraped onto a spatula and placed into a tube containing the aspirated blood, 750 µl of TRI Reagent BD, 200 µl of water and 3 µl of polyaracyl carrier. From this point, the standard protocol (Chapter One) was then followed.

DNA Microarray

For each trial, the RNA samples isolated from blood that had aged for 0, 1, or 4 hours were combined. Approximate concentrations were determined by 260/280 and the samples were frozen at -80 °C until they were sent to Paradigm Genetics for DNA Microarray

analysis. The procedure conducted is described in A-3 of the Appendix. Three candidate RNAs were selected from the first trial and 4 from the second trial. Real-time PCR primer and probes were designed for each identified RNA using Primer Express software from Applied Biosystems. Primers for the candidate RNAs amplified a 100 bp region and all probes were labeled with FAM. All identified RNAs were multiplexed with primers and probes for a universal 18S (Chapter One). Sequences for all of the primers and probes are provided in Table 4-1 and optimal concentrations are presented in Table 4-2.

Real-time Confirmation

The mRNAs identified by the microarray were examined using real-time PCR. RNA was isolated from triplicate 200- μ l blood aliquots stored in both 1.5-ml tubes and on Petri dishes at 0, 2, 4, and 24 hours after deposition according to the procedure mentioned above. RNA was also isolated from triplicate samples of different volumes of blood dried (30 μ l and 1 ml) on cotton fabric at 0, 2, 4, and 24 hours after deposition according to procedures mentioned in Chapter One. To confirm the results for the second microarray trial, RNA was isolated from triplicate 100 and 200 μ l blood aliquots that were dried on Petri dishes according to the procedure mentioned above at 0, 1, 2, 4, 24 hour, 5 days, and 11 days after deposition. Duplicate real-time assays were performed on each cDNA using primers and probes for the identified mRNAs of both trials according to the procedures described in Chapter One. Results are based on the averaged value of 3 isolates, 3 blood draws and duplicates assays for a total of 18 measurements. Controls were included as described in Table 1-2.

Table 4-1. Primer and probes sequences required for PCR amplification of each of the identified mRNAs.

RNA	FORWARD PRIMER	REVERSE PRIMER	PROBE
GOS1	GCACGTGCTGTTTTTTATTGGA	CAAAACTCAATCCCAAACCTCTTT	AAACCGCTGACATCTAGAACTGACCTACCACAA
EGR1	GCCTGCGACATCTGTGGAA	CCGAAGAGGCCACAACACTT	ACCAAGATCCACTTGCGGCAGAAGG
RGPS	GGAAGGACTGTGACCTGCCATA	CCTGATTCACTACCCAACCTTGAA	TGGGTGTTCAAGAAACATCACTCAGAACTATTG
PRAME	CAGACCCTGAAGGCAATGGT	AAGGAGCACATCAAGTCCATCA	TTCACCTGCCTCCCTCTGGGAGTG
MNDA	TAGGACTAACTACAAAAATGCAAGAGGAA	GTGATGGCATATCTTTGGCAAGT	AAAAAAAAGTTCCAAGGCGTTGCCTGTCTAG
S100	CCGAGTGTCTCAGEATATCAGGAA	CTGCCACGACCATCTTTATC	AGACGTCTGGTTCAAAGAGTTGGATATCAAACTG
DSC3	TGTTAGTCAAATCCCGGAGAGAA	GATGCCAGGAACACCCTTGT	TGAAAACATTGGCAGAATTTGTCTCAGACG

Table 4-2. Optimal primer and probes concentrations required for PCR amplification of each of the identified mRNAs.

	Forward Primer	Reverse Primer	Probe
GOS1	250 nM	250 nM	250 nM
EGR1	500 nM	500 nM	500 nM
RGPS	500 nM	500 nM	250 nM
PRAME	250 nM	250 nM	250 nM
MNDA	250 nM	250 nM	250 nM
S100	500 nM	500 nM	250 nM
DSC3	500 nM	500 nM	250 nM

* Concentrations for 18S were kept constant at 50 nM for multiplexes with each of the above mentioned genes

Additive Experiment

Blood was collected from 1-2 individuals by standard venipuncture in 10-ml plain, ethylenediaminetetra-acetic acid (EDTA), and acid citrate dextrose (ACD) coated BD Vacutainers on 3 occasions. Fifteen aliquots of blood (200 μ l) were deposited into uncapped 1.5-ml tubes and stored at room temperature and 50% humidity for the duration of the experiment. RNA was isolated from 3 aliquots of blood containing each additive at 0 and 2 hours after deposition according to the standard TRI Reagent BD isolation procedure mentioned in Chapter One. Real-time analysis using primers and probes for the identified RNAs was performed according to the procedures described in Chapter One.

Results

DNA Microarray-Trial 1

Comparison of the RNAs isolated from 200 μ l of blood stored in 1.5-ml tubes at time points 0 and 4 hours identified about 1,100 genes that were significantly altered. Figure 4-1 illustrates the altered genes, with blue representing the RNAs that remain unchanged from 0-4 hours, red representing the RNAs that have increased levels from 0-4 hours and green representing the RNAs which undergo a decrease in levels from 0-4 hours. The 3 most up-regulated mRNAs in blood 1 hour after deposition were the early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS), and have been selected for further analyses. EGR1 was up-regulated 66-fold, GOS1 was up-regulated 96-fold and RGPS was up-regulated 49-fold. P-values for each of the selected RNAs were less than 0.001. Due to the price and variability associated with microarrays, future examinations of these RNAs were performed using real-time PCR. Real-time analysis supports the increased RNA levels suggested by the array results, but the values for fold increase are inconsistent. Figure 4-2 shows the results comparing mRNA levels of the 3 candidate genes isolated from 200 μ l of blood stored in 1.5-ml tubes over a 24 hour period. Figure 4-3 identifies the changes in mRNA levels isolated from the 30- μ l blood samples that dried on cotton over a 24 hour period. For all mRNAs, the greatest increase in mRNA levels was observed 2 hours after deposition in the 200 μ l samples (stored in uncapped 1.5 ml tubes). Although this supports the results from the microarray, the fold increase is different than that obtained from the array. No changes in mRNA levels were

observed for any of the mRNAs isolated from the 30- μ l dried blood samples over the time frame examined. Analysis of EGR1 shows a very dramatic change in mRNA levels in the 200- μ l samples from 0 to 2 hours but no change in levels were observed in the mRNA isolated from the 30- μ l samples. Analysis of GOS1 also shows increased mRNA levels from 0 to 4 hours for the 200- μ l samples but no change was observed for the 30- μ l samples. Consistent with the array results, GOS1 was the most abundant mRNA and RGPS was the least abundant mRNA based on the real-time results. Comparisons of EGR1 mRNAs isolated from 0 and 2 hour old 200- μ l blood samples showed a 10 Ct value difference between these 2 time points. Analysis of mRNA levels of all 3 candidate mRNAs was examined for another individual and similar results were obtained for the 200- μ l blood samples (data not shown).

Figure 4-1. Scatter plot analysis identifying the results from the first DNA microarray. Altered mRNA levels from 0-4 hours in 200 μ l *ex vivo* human blood samples. Red represents the mRNAs that have increased from 0-1 hour, green represents the mRNAs that have decreased from 0-1 hours and blue represents the mRNAs that have remained unchanged from 0-1 hours.

Figure 4-2. Messenger RNA levels in 200 μ l of aqueous blood. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). Standard error bars are included on graph. N=3.

Because no change in EGR1, RGPS, and GOS1 mRNA levels was observed in the 30- μ l blood samples, the effect of sample storage on mRNA levels in *ex vivo* blood stains was the next objective. EGR1, RGPS, and GOS1 mRNAs isolated from 200- μ l blood samples stored on Petri dishes were compared to RNAs isolated from 200- μ l samples stored in 1.5-ml tubes. Figure 4-4 shows that the levels of the candidate mRNA are not altered in the samples aliquoted onto the Petri dish over the time frame examined. A small increase in mRNA levels is observed when the RNA isolated from the samples stored in the 1.5-ml tubes were analyzed using primers and probes for GOS1 and EGR1 but not as much as suggested by the earlier experiment. A change in mRNA levels is not observed in the RNA isolated from either sample when primers and probes for RGPS are used for analysis. The candidate mRNAs were next examined in larger volume dried blood samples deposited on fabric. RNA was isolated from blood samples at 1 and a half and 2 hours after deposition. The samples were cut (1 inch X 1 inch) from the central region of a 1 ml spot of blood deposited on fabric. The blood stains used had not dried at the time of analysis. No change was observed in mRNA levels for the 3 mRNA examined (Figure 4-5)

Figure 4-3. Messenger RNA levels in 30 μ l of dried blood. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). Standard error bars are included on graph. N=3.

Figure 4-4. Messenger RNA levels in Blood Deposited on Different Surfaces. RNA was extracted from 200 μ l samples of blood aged in 1.5 ml tubes and on Petri dishes. Standard error bars are included on graph. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). N=3.

Figure 4-5. Messenger RNA levels in 1 ml of dried blood. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). . Standard error bars are included on graph. N=3.

Additive Experiment

To determine if coagulation of blood is responsible for the enhanced mRNA levels of GOS1, EGR1, and RGPS isolated from the 200 μ l samples of blood stored in 1.5-ml tubes, mRNA levels in *ex vivo* blood collected with different additives were examined. Analysis of EGR1 did not show a difference in mRNA levels over the time frame examined (Figure 4-6). Increased mRNA levels were observed for all genes examined 2 hours after deposition when isolated from blood supplemented with each additive. Examination of GOS1 mRNA levels showed an increase in mRNA levels from 0 to 2 hours when isolated from blood collected with different additives. Messenger RNA levels for all candidate genes were altered the least in the blood containing EDTA (Figure 4-7). Consistent with previous results, RGPS mRNA levels in blood collected in plain, EDTA and ACD solution B vacutainers changed the least over the 2 hour period examined. No change was observed in RGPS mRNA levels in the blood samples collected with EDTA or ACD solution B from 0-2 hours after deposition. A small change in mRNA levels was observed for all mRNAs in the blood collected in plain vacutainers from 0-2 hours after deposition (Figure 4-8).

Figure 4-6. EGR1 mRNA levels in Blood Collected with Different Additives. Results shown indicate the change in mRNA levels of EGR1 over the time period indicated in 200 μ l of blood supplemented with EDTA, ACD, or nothing. Standard error bars are included on graph. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). N=3.

Figure 4-7. GOS1 mRNA levels in Blood Collected with Different Additives. Results shown indicate the change in mRNA levels of GOS1 over the time period indicated in 200 μ l of blood supplemented with EDTA, ACD, or nothing. Standard error bars are included on graph. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). N=3.

Figure 4-8. RGPS mRNA levels in Blood Collected with Different Additives. Results shown indicate the change in mRNA levels of RGPS over the time period indicated in 200 μ l of blood supplemented with EDTA, ACD, or nothing. Standard error bars are included on graph. RNAs examined include: Early growth response gene (EGR1), putative G0/G1 switch gene (GOS1), and the regulator of G-protein signaling (RGPS). N=3.

DNA Microarray-Trial 2

Four mRNAs were selected from the second DNA microarray based on comparisons of the RNAs isolated from 100 μ l of fresh blood to the RNAs isolated from 100 μ l of blood aged for 1 hour on a Petri dish (Figure 4-9). The 2 most up-regulated and the 2 most down-regulated mRNAs were identified and used for real-time analysis. Preferentially expressed antigen in melanoma (PRAME) was up-regulated 6.18-fold and desmocollin type 3 (DSC3) was up-regulated 5.65-fold from 0-1 hour. S100 calcium binding protein A8 (S100) decreased 4.76-fold and myeloid cell nuclear differentiation antigen (MNDA) decreased 4.06-fold from 0-1 hour. All identified RNAs had P-values less than 0.001. Due to the price and variability associated with this assay, future examinations of these RNAs were performed using real-time PCR. Messenger RNA levels of PRAME and DSC3 were analyzed in different volumes and ages of blood using real-time PCR. Figure 4-10 shows the changing mRNA levels of PRAME and DSC3 isolated from 200 μ l blood samples over a 24-hour period. An increase in mRNA levels for both PRAME and DSC3 1 hour after deposition is suggested. Consistent with the array results, mRNA levels for PRAME were altered the most compared to DSC3 mRNA levels. Figure 4-11 shows the same experiment described in Figure 4-10 but RNA was isolated from 100- μ l samples of blood instead of 200- μ l samples. No changes in mRNA levels were observed for either mRNA between 0 and 4 hours. Based on the results, it appears that levels of both RNAs examined declined over the time frame examined. Figure 4-12 shows real-time analysis of the same cDNAs using primers and probes for MNDA and S100. The trend suggested by the array results are not confirmed using real-time analysis for either of the mRNAs isolated from 100- μ l samples of

Figure 4-9. Scatter plot analysis identifying the results from the second DNA microarray. Altered mRNA levels from 0-1 hours in 100 μ l *ex vivo* human blood samples. Red represents increased mRNA levels from 0-1 hour, green represents decreased mRNA levels from 0-1 hour, and blue represents unchanged mRNA levels from 0-1 hour.

blood. No increase or decrease in mRNA levels is observed for the time period indicated. In Figure 4-13, 200- μ l samples of blood do show a slight decline in MNDA levels after 24 hours but this will need to be further investigated with an extended time line.

DISCUSSION

The RNAs examined in the first microarray were isolated from 200 μ l samples of blood stored that had been stored in uncapped 1.5-ml tubes. Large volumes of blood were used because RNA of sufficient quality is required to perform microarray analyses. Comparisons of Figure 4-1 and 4-9 identify more alterations in RNA levels between fresh and aged blood for the samples stored in the 1.5-ml tubes compared to the RNAs isolated from 100- μ l dried samples. Because the mRNAs from the first array did not show altered levels between 0 and 2 hours in the dried blood samples, other avenues were pursued. Based on these results and assuming viability of the cells throughout the duration of this experiment, the altered mRNA levels in the blood stored in the 1.5-ml tubes was hypothesized to be a result of the surface volume in which the blood was contained. The identification of altered mRNA levels of each candidate mRNA isolated from 0-4 hour old blood containing EDTA or ACD does not support clotting as a possible explanation. If clotting of the blood were responsible for the increased mRNA levels, then the RNA isolated from the blood containing the anticoagulants should not show the same trend. When blood is deposited on flat surfaces it tends to disperse as much as the initial volume allows, but blood deposited into 1.5-ml tubes can only disperse to the area provided. The close contact of blood cells in the 1.5-ml tubes raises the possibility that the cells are stored in a more

Figure 4-10. Messenger RNA levels in 200 μ l blood stains. RNAs examined include: Preferentially expressed antigen in melanoma (PRAME) and desmocollin type 3 (DSC3). The value plotted is based on the average of all measurements. . Standard error bars are included on graph. N=3.

Figure 4-11. Messenger RNA levels in 100 μ l blood stains. RNAs examined include: Preferentially expressed antigen in melanoma (PRAME) and desmocollin type 3 (DSC3). The value plotted is based on the average of all measurements. Standard error bars are included on graph. N=3.

Figure 4-12. Messenger RNA levels in 100 μ l blood stains. RNAs examined include: S100 calcium binding protein A8 (S100) and myeloid cell nuclear differentiation antigen (MNDA). The value plotted is based on the average of all measurements. Standard error bars are included on graph. N=3.

Figure 4-13. Messenger RNA levels in 200 μ l blood stains. RNAs examined include: S100 calcium binding protein A8 (S100) and myeloid cell nuclear differentiation antigen (MNDA). The value plotted is based on the average of all measurements. Standard error bars are included on graph. N=3.

aqueous or hydrated state. The storage state of the blood could influence the transport of oxygen or other molecules. This could be an explanation for the altered mRNA levels, either prompting transcription or reducing the amounts of degradation of mRNA.

Examination of RNA levels in blood collected with different additives showed varying levels of the mRNAs associated with blood collected with different additives. This concept may be critical in determining how blood should be stored prior to analysis. The effects that different additives have on specific RNA levels may need to be identified prior to downstream RNA applications.

Although the second round of array results were supported using real-time PCR to analyze PRAME and DSC3 mRNA levels, the volume and state of blood appears to play a critical roll in determining RNA levels. Increased RNA levels of PRAME and DSC3 were only observed in the 200 µl blood volume from 0-1 hour after deposition. This could be a result of how long the blood samples take to dry. The samples of smaller volume appeared to dry faster than the samples of larger volume. Consistent with previous results discussed in this dissertation, RNA present in dried blood stains appears to act more stable upon desiccation and as long as the blood is in an aqueous state it is speculated that enzymatic activity will continue resulting in the increased mRNA levels. Consistent results were obtained when the RNA isolated from the 200-µl samples (dried on Petri dish) was analyzed, but very inconsistent results were associated with the RNA isolated and analyzed from the 100-µl samples (dried on Petri dish). Blood samples for each round of experimentation were kept at constant environmental conditions inside the lab but these conditions could have changed from blood draw to blood draw. The room temperature (23-25 °C) and humidity

(19-50%) inside the lab have been noted to change from day to day depending on the outside conditions. Increased temperature and humidity have both been suggested to enhance RNA degradation potentially through increased enzymatic activity (Chapter Three). Increased transcription resulting in the decreased Ct values for the above RNAs or a decrease in degradation could explain the altered mRNA levels. Before this technique could be applicable, there are many issues that will need to be investigated to ensure reproducibility and practicality. PRAME and DSC3 mRNAs were undetectable in 200 µl samples of blood after 7 days based on real-time PCR analysis (data not shown). Very similar to enzyme tests, a sample of a certain volume could be determined to be older or younger than 7 days based on the presence of these mRNAs. A day by day analysis will need to be performed to identify the exact day when the RNA is no longer detectable using real-time PCR. Because the mRNAs in question are inducible, PRAME and DSC3 mRNA levels in *ex vivo* blood of many individuals will need to be examined and the variation between individuals and within individuals will need to be accessed and controlled for.

Levels of PRAME and DSC3 mRNA in fresh blood were identified to be very low, almost at that of background levels, but after 1 hour outside of the body, the levels of the mRNA increased by several Ct values. Consistent with the array result, PRAME mRNA levels increased the most (6 Ct cycles) while DSC3 levels increased by 3 Ct cycles. Messenger RNAs levels of both S100 and MNDA were examined but the results did not show a decline in RNA levels over the time frame examined for the 100 µl samples. A slight decline in MNDA mRNA levels isolated from the 200-µl samples of blood may exist but further validation is necessary. Further investigations of each of these mRNAs will need to be done before this can become a valid and practical method. These include: examination of

a longer time line of detection, analysis of mRNAs in different blood volumes, different surface deposition of blood, and analysis of mRNAs from different individuals.

Chapter 5- A Method to Determine the Age of a Biological Sample – An Extended Survey

Introduction

The focus of the studies done in the final chapter is aimed at analyzing RNA isolated from dried blood stains using the newly developed primer/probe sets. To control for inter-person variability, a larger population size was used for analysis with more narrow age ranges for both males and females. Although the results presented in Chapter One suggest potential for the technique described in this dissertation, the confidence intervals associated with the change in RNA ratio over time were too large to be of practical use. The results from this chapter show that the integration of the confidence intervals obtained from analysis using each primer/probe set can minimize the ultimate range of age associated with a given blood stain making the technique described in this dissertation of practical use.

Methods

Blood Collection and Sampling

Ten-ml of blood was collected in plain vacutainers by standard venipuncture from 15 males and 15 females of European ancestry on 3 separate occasions. Males and females of similar ages were recruited to avoid age as a potential variable. Males ranged in age from

21-27 (median=24), and females ranged in age from 21-31 (median =26). Thirty aliquots of blood (10 µl) were distributed on plastic Petri dishes and stored at 25 °C and 50% humidity in an EC22560 Environmental chamber (Lab-Line, Melrose Park, IL). For each blood draw and individual, a separate Petri dish was used. Blood was drawn 3 times a week for 2 individuals. 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 0, 6, 15, 30, 45, 60, 90, 120, and 150 days according to the standard RNA isolation procedure for TRI Reagent BD described in Chapter One. Information about all subjects tested for is provided in Table 5-1.

Real-time Analysis of Dried Blood for Second Trial

Real-time PCR was performed on cDNA from select time points for all individuals using 3 sets [18S (180 bp)/18S (500 bp), BA (60 bp)/BA (300 bp), 18S (180 bp)/BA (180 bp)] according to standard procedure (Chapter One). Duplicates of each cDNA from each time point for each individual and blood draw were assayed. This totaled 18 measurements for each time point per individual which resulted in a total of 540 measurements for all 30 individuals (3 blood draws X 3 RNA samples X 2 assay X 30 individuals=540).

Experimental set up is identical to that described in Figure 1-1 except the time points for RNA isolation and the numbers of subjects are different. Due to the cost of real-time PCR, specific primer/probes sets and time points were selected for analysis. A preliminary real-time analysis of all time points for 1 blood draw for 1 individual using all 4 of the previously mentioned primer/probe sets was performed [the 3 mentioned above and 18S (60 bp)/BA (60 bp)]. Of these 4 sets, the 3 that yielded the most dramatic change in the difference in Ct

value over the 150-day period were selected for future analyses. The 5 time points that showed the most dramatic change in the difference in Ct value over time for each primer/probe set were selected based on the preliminary run. Complementary DNAs from 5 time points and 3 blood draws from the same individual were analyzed in the same real-time run. Because 3 amplicon sets were used to analyze every cDNA, 3 real-time runs were performed on the same cDNA from every individual. A control cDNA (same cDNA aliquot used in all 90 real-time runs) was assayed in each real-time reaction to control for machine, primer and probe, and master mix variability. All results were adjusted based on this value. All other controls included are described in Table 1-2. Thresholds were adjusted for all amplicon combinations (Table 1-1) before the Ct values were exported into Excel. Ct values for each component of the multiplex were analyzed by looking at the difference between the less detectable region amplified and the more detectable region amplified [BA (300 bp)-BA (60, bp), 18S (500 bp)-18S (180 bp), BA (180 bp)-18S (180 bp)].

Table 5-1. **Subjects involved in final population study.**

ID	SEX	AGE	Birth Control
SA	F	25	Ortho tri cyclin
NS	F	29	Apri
HM	F	24	No
BH	F	22	Microgestin
MA	F	25	Ortho tri cyclin
AW	F	21	No
SW	F	26	Loestrin1/20
KK	F	26	No
RR	F	25	No
MF	F	31	Ortho novum tricyclin
SS	F	31	No
SM	F	27	Levlen 28
AS	F	23	Ortho novum 777
JR	F	26	Lo ovral
WS	F	27	No
JT	M	23	
BB	M	27	
RF	M	20	
MS	M	25	
NK	M	26	
BR	M	22	
HL	M	25	
CBO	M	22	
BL	M	23	
BLO	M	22	
BS	M	21	
MG	M	22	
CB	M	48	
CS	M	27	
SK	M	21	

Statistical Analysis for Second trial Population Study

Analysis was performed using a nested analysis of variance using the method of restricted maximum likelihood. 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 sex and age of blood were taken to be random. These include, intra-person (draw/ID), inter-person (ID), RNA isolate, duplicate assays, and residual variability. The response variable is the difference in Ct value for the more detectable region amplified subtracted from the less detectable region amplified. In addition non-linear regression models were fit to ratio and age of blood for this trial [33]. Calibration confidence intervals were determined from those results [34].

Results

Selection of Amplicon Sets for Analysis

Figures 5-1, 5-2, 5-3, and 5-4 show the real-time results of RNA isolated from aged dried blood stains. All cDNAs were analyzed using 4 amplicon sets [18S (60 bp)/BA (60 bp), 18S (180 bp)/18S (500 bp), BA (60 bp)/BA (300 bp), 18S (180 bp)/BA (180 bp)]. All results shown are based on the measurements of 1 subject, 1 blood draw, 3 isolates per time point, and replicate assays. The average difference in Ct value is graphed for all measurements and standard error bars are included. Consistent with other results, cDNAs analyzed with 18S (60 bp)/BA (60 bp) showed no change in the difference in Ct value over

Figure 5-1. Preliminary Analysis using 18S (60 bp)/BA (60 bp). Results graphed are based on the mean difference in Ct value over the time frame indicated. Standard error bars are included on graph. N=1.

Figure 5-2. Preliminary Analysis using 18S (150 bp)/18S (500 bp). Results graphed are based on the mean difference in Ct value over the indicated time frame. Standard error bars are included on graph. N=1.

Figure 5-3. Preliminary Analysis using BA (60 bp)/BA (300 bp). Results graphed are based on the mean difference in Ct value over the indicated time frame. Standard error bars are included on graph. N=1.

Figure 5-4. Preliminary Analysis using 18S (180 bp)/BA (180 bp) Results graphed are based on the mean difference in Ct value over the indicated time frame. Standard error bars are included on graph. N=1.

the 150-day period. Because no change is observed, this primer/probe set (Figure 5-1) was not selected for future analysis. Analysis of the cDNAs using BA (60 bp)/BA (300 bp), showed a plateau in the difference in Ct value after 90 days (Figure 5-3). It is reasonable to assume that the plateau is a result of the 300-bp region of β -actin reaching background levels of detection. The 5 time points selected for future analysis were: 0, 6, 15, 30, and 90 days. This primer/probe set shows the greatest change in the difference in Ct value from 0-90 days. Figures 5-3 and 5-4 show analysis of cDNAs using 18S (180 bp)/BA (180 bp) and 18S (180 bp)/18S (500 bp). Both primer/probe sets still amplify the specified cDNA at 150 days but the Ct values for BA (180 bp) and 18S (500 bp) appear to plateau after 120 days. Due to this observation, the time points selected for future analysis were: 0, 6, 30, 90, and 120 days.

Real-time Analysis of Variance

Figures 5-5, 5-6, and 5-7 show the compiled results for the cDNAs from the 30 individuals analyzed with BA (60 bp)/BA (300 bp), 18S (180 bp)/18S (500 bp), 18S (180 bp)/BA (180 bp), respectively. In the first dried blood trials, a ratio between the Ct values for the 2 regions amplified was used for the statistical analysis but the difference in Ct value was examined in these experiments. Analysis by either method is acceptable. The values obtained for the statistical analysis using all 3 primer/probe sets are presented in Table 5-2.

Figure 5-5. **One Way Analysis of Mean (ratio) by Age of *Ex Vivo* Blood Under Constant Conditions using BA (60 bp)/BA (300 bp).** Standard error bars are included. N=30. 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 0.95.

Figure 5-6. **One Way Analysis of Mean (ratio) by Age of *Ex Vivo* Blood Under Constant Condition using 18S (180 bp)/18S (500 bp)**. Standard error bars are included. N=30. 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 0.86.

Figure 5-7. **One Way Analysis of Mean (ratio) by Age of *Ex Vivo* Blood Under Constant Conditions using 18S (180 bp)/BA (180 bp).** Standard error bars are included. N=30. 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 0.93.

RNA isolation and duplicate assays were not found to contribute to the random variability and were therefore, factored out of the analysis. The combined percentages for all random effects [intra-person (draw/ID), inter-person (ID/), RNA isolate, duplicate assays, and residual variability] add up to 100%. The effects category examines the effects of gender, gender by day, age of the blood, subject and gender, and draw and gender. Analysis of the RNAs isolated from dried blood stains using all of the primer/probe sets gives a linear relationship between age of blood and the difference in Ct value. The R^2 values based on the analysis using each primer/probe set were high with the greatest value (0.95) obtained for BA (60 bp)/BA (300 bp). Almost all of the variability is contributed by gender and subject based on analysis using BA (60 bp)/BA (300 bp) and most of the variability is residual based on analysis using 18S (180 bp)/18S (500 bp) and 18S (180 bp)/BA (180 bp). The sums of squares value indicate that age of blood contributes the largest effect for each of the primer/probe sets, at least 10-fold higher than any of the other factors. Although a significant effect, based on an F-value lower than 0.05, was identified for gender by day interaction in all primer/probe sets, the sum of squares value suggest that the extent of this effect is very minimal (data not shown). Age of blood was identified to be significant in all sets amplified based on the F-value and Sum of Squares value. All factors except for age of blood and gender were zeroed out for the sum of squares analysis for 18S (180 bp)/BA (180 bp). Based on the zeroed values, it might appear that this primer/probe set is highly significant but actually is a result of the data set being problematic. A zeroed/negative value suggests not enough variance exists between the factors analyzed. This supports this primer/probe set yielding the worst results. The variance contributed by males and females were examined based on the results from all primer/probe sets. The only set that suggested a difference in

gender contribution was 18S (180 bp)/18S (500 bp). Based on the results for this set, females contributed 60% of the gender variance while males only contributed 40% (data not shown). This effect was investigated because of the results generated for the first dried blood trials (Chapter One) suggested that females contributed the majority of the intra-person variance. Table 5-1 identified which female subjects were taking birth control pills because it was hypothesized that the greater female variance observed in trial 1 was a result of hormonal regulation. The results in this final study do not support greater variability in females.

Table 5-2. Statistical Analysis of Final Population Study

		BA (60 bp)/BA (300 bp)	18S (180 bp)/18S (500 bp)	18S (180 bp)/BA (180 bp)
Random Effects Effects (SS)	R ²	0.95	0.86	0.93
	MSE	0.236	0.417	0.253
	Inter-person	85%	23.8%	19.4%
	Intra-person	1.4%	16.3%	12.4%
	residual	13%	59.8%	68%
	Gender	0.18	131	0
	ID/Gender	949	544	0
	Age of Blood	9625	5958	9561
	Gender by Day	4.57	93	7.9
	Draw/Gender	44.4	237	0

Confidence Intervals

The confidence intervals for each primer/probe set are presented in Tables 5-3, 5-4, and 5-5. Initial observation shows a decrease in the ranges of these values compared to the first analysis (Chapter One). Analysis using BA (60 bp)/BA (300 bp) yields the smallest range of values. By analyzing the same cDNA with each primer/probe set, the confidence intervals can be reduced. Three separate values will give 3 specific ranges, which could

narrow the associated age of blood sample and confirm the other results generated. In an attempt to integrate confidence intervals for all 3 primer/probe sets into a predictable model, statistical analysis was explored but found to be inconclusive. Due to the variability between the slopes of each of the primer/probe sets, the results could not be integrated. Further investigation may identify another statistical model that may be useful and this concept should still be explored.

Table 5-3. Confidence Intervals associated with 18S (180 bp)/BA (180 bp)

Difference in Ct Value	Confidence Intervals (Days)
7.4	0-3.5
7.9	0-6.3
8.4	0-10.8
8.9	0.37-18
9.4	1.26-30
9.9	2.71-49
10.4	5-79
10.9	9-129
11.4	15-208
11.9	25-337

Table 5-4. Confidence Intervals Associated with 18S (180)/18S (500 bp)

Difference in Ct Value	Confidence Intervals (Days)
10	0-19
10.5	0-31
11	0-47
11.5	0-72
12	0-106
12.5	4-156
13	9-226
13.5	17-326
14	28-471
14.5	45-677

Table 5-5. Confidence Intervals Associated with BA (60 bp)/BA (300 bp)

Difference in Ct Value	Confidence Intervals (Days)
2.5	0-3.5
3	0-5.8
3.5	0-9
4	0.86-13.4
4.5	2.13-19.5
5	3.91-28
5.5	6.37-40
6	10-56
6.5	15-79
7	21-111

Chapter 5 Discussion

The cDNAs from the second population study have been analyzed with a variety of human-specific primers and probes to try and reduce the confidence intervals associated with the technique described in this dissertation. The preliminary trials have identified unique behavior for each of the primer/probe sets. The time line associated with analysis using BA (60 bp)/ BA (300 bp) appears to be the shortest of all primer/probe sets examined (Figure 5-3). Accurate detection is achieved for RNA aged up to 90 days but after 90 days the Ct values for BA (300 bp) becomes near that of background levels and not reliable for analysis. The time lines associated with analysis using 18S (180 bp)/18S (500 bp) and 18S (180 bp)/BA (180 bp) act in a similar fashion with accurate detection of RNA that has aged up to 120 days. After 120 days the Ct values become near that of background level for BA (180 bp) and 18S (500 bp) and the associated standard errors become very high. It is interesting to note that when blood on cotton was analyzed, accurate results were obtain from RNA that had aged up to 150 days (Figure 1-5). This would suggest that RNA stability may also be dependant upon the surface on which the blood was deposited or on how quickly the blood stain dried. This is consistent with previous results discussed in this dissertation, and the speculation that the longer the blood remains in an aqueous or hydrated form the more cellular/enzymatic activity can take place. Blood deposited on plastic may not dry as fast as blood deposited on cotton because cotton is a porous surface which absorbs liquids. This may cause the blood to dry faster thus stabilizing the RNA. Analysis using the primer/probe set, 18S (60 bp)/BA (60 bp) did not show any change in RNA levels over the 150-day period examined (Figure 5-1). This is also consistent with previous experiments conducted (Figure 2-2). Although not selected for this experiment, this primer/probe set may be useful for

analysis of RNA isolated from blood that had aged longer than 150 days. These results show how very different time lines can be generated from the analysis of the same RNA.

The statistical analysis of the final population study is consistent with the analysis for the previous experiments. All primer/probe sets display a linear relationship between age of blood and difference in Ct value indicating a decreased amount of 1 region amplified compared to the other. The R^2 value for the analysis with all primer/probe sets was high, supporting this relationship. Although some of the other effects were found to be statistically significant, the variability contributed by age of blood was at least 10-fold higher than any of effect making these factors minimal contributors of variability. In this round of experimentation statistical analysis examining the differences in Ct values was selected instead of relative ratio because of the quality of the results obtained. In summary, BA (60 bp)/BA (300 bp) yielded the highest R^2 value and the most narrow confidence interval of the 3 primer/probe sets used for analysis. If a blood stain of unknown age was determined to have a difference in Ct value of 3.5 when the RNA was analyzed using BA (60 bp)/ BA (300 bp) this would establish an expected age range of 0-9 days (Table 5-5). Although the confidence intervals associated with analysis using 18S (180 bp)/18S (500 bp) and 18S (180 bp)/BA (180 bp) are not useful on their own, integration of the confidence intervals for all 3 primer/probe sets will narrow the time frame of detection. For example, if the same blood stain was determined to have a difference in Ct value of 9.9 when the cDNA was analyzed with 18S (180 bp)/BA (180 bp), then would generate an age range of 2.71-49 days (Table 5-3). Integration of these results yields a reduced confidence interval range of 2.71-9 days.

In this final chapter the precision of the developed technique was further evaluated by analyzing cDNAs with a variety of human specific primers and probes that have

characterized time frames of RNA detection in dried blood stains. Integration of the time line will provide an ultimate time frame of detection that may be of practical use.

Final Discussion

In summary, the results presented in this dissertation indicate that the different types of RNA and different sized regions of RNA decay at different rates in *ex vivo* dried blood stains. Figure 1-5 shows the rate of decay of two RNA species in dried blood over a 150-day period. The Ct value of 18S did not change over the course of 150 days but the Ct values for β -actin appeared to be reduced as a function of time thus the relative ratio of 18S rRNA to β -actin mRNA increased over time. Different primer/probe sets were used to examine different sized regions on or between either β -actin and 18S rRNA isolated from blood stains that had aged up to 120 days (Figures 5-5, 5-6, and 5-7). All results indicate that the larger the region amplified the less detectable it becomes over time and that β -actin degrades more rapidly than 18S in dried blood stains. Confidence intervals were generated based on cDNA analysis using each of the primer/probe sets. Integration of these intervals has been shown to reduce the age ranges associated with the results obtained using a single primer/probe set for analysis. Preliminary results indicate that the RNA isolated from dried blood stains exposed to high temperatures and humidity is less stable than the RNA isolated from dried blood stains exposed to lower temperatures and humidity. Full spectrum light was not identified to affect RNA degradation in dried blood over the 90-day period examined.

After examining several housekeeping mRNAs (Figure 1-2), β -actin, which, although less abundant in the cell than the ribosomal species it is one of the 2 most abundant mRNAs present in aged dried blood samples as identified by the housekeeping gene expression panel. Both 18S and β -actin are considered “housekeeping genes” which are present in all cell types

at relatively high levels, thus their RNA products would likely be recovered from crime scene evidence [40]. 18S functions during protein synthesis and there is now evidence that rRNA interacts with mRNA or tRNA at each stage of translation, and that the proteins are necessary to maintain the rRNA in a configuration in which it can perform the catalytic functions [47]. Cytoskeletal β -actin is one of the most abundant cellular proteins found in mammalian cells. It is the major component, together with cytoskeletal γ -actin, of the microfilamentous structures found in these cells. Functionally, cytoskeletal actin has been implicated in intracellular movement of organelles, cytokinesis, and cell motility [48, 49]. Like most mRNAs, it is not permanently incorporated into a protective protein complex and thus, as the results suggest, degrades more rapidly than 18S. It was our initial belief that protein protection would influence RNA stability and the results presented in this dissertation support this concept, although this is only a speculation because the concept was never directly tested. Because β -actin mRNA and 18S rRNA are present in all cell types, the analysis described in this dissertation could potentially be applied to tissue types other than blood. Along with blood, RNA was recovered from both hair and saliva and analyzed using RiboGreen and real-time PCR (Table 2-3 and 2-4).

Analysis of cDNAs from the first dried blood trials using 18S (60 bp)/BA (60 bp) suggested amplicon size as a factor contributing to RNA detection (Figure 2-2 and 2-3). Results in Chapter Two prompted the idea that the larger the region amplified, the more likely an event will occur within that region which will hinder RNA detection. This idea was validated in Chapter Five. Two different sized regions on the same RNA were analyzed, potentially controlling for intra-person variability. Of these 2 regions, one is small and the other is large. The small serves as the control because there it will be targeted for decay less

than the larger region as the age of the sample increases. For example, two regions on the 18S rRNA molecule can be multiplexed with 1 of the regions being of small amplicon size (~50 bp) and the other being of larger amplicon size (<300 bp). Because initially the target sequences will be present in equimolar amounts, there should be no initial variation associated within an individual.

After amplicon size was suggested to have an effect on RNA detection in dried blood stains, 4 custom designed primer/probe sets amplify different sized regions of RNA. Analysis of cDNAs using BA (60 bp)/BA (300 bp) showed the most change in the difference in Ct value over a short period of time (Figure 5-3). Analysis of cDNAs using 18S (180 bp)/18S (500 bp) showed a more gradual change with RNA detection of 120-day old sample of blood deposited on plastic (Figure 5-2). Analysis of cDNAs using 18S (180 bp)/BA (180 bp) showed very distinct changes in the difference in Ct value over the first 30 days, but then appeared to plateau because increased differences in Ct values were no longer observed (Figure 5-4). Analysis of cDNAs using 18S (60 bp)/BA (60 bp) showed no change in the difference in Ct value over a 5 month period. This primer/probe set may be useful in future applications that examine RNA isolated from blood stains that are 1 year or older (Figure 5-1).

Comparisons of differences in Ct values from RNAs isolated from the same individual and between different individuals suggest relatively constant levels of the housekeeping genes selected for analysis (Table 5-2). Recent publications have suggested that the once thought invariant steady state levels of GAPDH actually vary in certain breast cancers. It is thought that estrogen affects the steady state levels of GAPDH [50]. To identify potential variation in the steady state levels of β -actin and 18S, 3 blood draws were

performed on each individual. RNA levels of β -actin and 18S within the same person were not found to be major contributors to variability for all studies examined (Table 5-2). For all experiments, age of blood contributed the largest amount of variability as shown in Table 5-2. Both BA (60 bp)/BA (300 bp) and 18S (180 bp)/18S (500 bp) amplify 2 regions on the same RNA and statistical analysis of the results indicate that the least amount of variability is contributed by blood draw. There is no potential for varying levels of 2 different RNAs when these primer/probes set are used for analysis because both regions analyzed are initially present in equimolar amounts.

There are several options to further enhance precision and accuracy of the technique described in this dissertation. First, if the identity of the person whose bloodstain was found at the crime scene was known, then several additional procedures could be followed that may help to more accurately establish the age of that bloodstain. Blood from the individual could be freshly collected and processed in a mechanism similar to that described in this dissertation to establish a standard curve of RNA ratios/differences in Ct value over time specific for that individual. The ratio/difference in Ct value of RNA from the blood found at the crime scene could then be compared to a standard curve specific for that individual. Environmental conditions similar to those present at the crime scene could be mimicked in the laboratory. This application would help to enhance the accuracy and specificity of the method described in this dissertation. If the identity of the bloodstain cannot be determined, this method will still be applicable based on comparison to a universal standard curve. The confidence intervals associated with the data for a single subject based on the results of the first dried blood trials are narrower than those calculated for all subjects of the study. Given a ratio of 1.52, the confidence intervals for the age of the bloodstain for a single individual

ranges from 0-53 days and 0-68 days for all subjects from the first population study, suggesting the potential for increased precision when analyzing results from a single individual (Table 1-6). Second, integration of the confidence intervals established based on analysis using all primer/probe sets has been shown to reduce the age range of a blood stain of unknown age when compared to analysis using a single primer/probe set. Third, All statistics performed on the results described in this dissertation were based on 95% confidence levels. Another option is to reduce the confidence level to 85%. This method is often used to analyze results in the forensic community (TSG-EG kickoff meeting, February 2004, Morgantown WV). Prior to this research, 1 day old blood stains could not be distinguished from 6 month old blood but this research provides a foundation to elucidate an accurate means of dating a blood stain [3].

Environmental and Array Experiments

Results of both the environmental and array studies support a theme that has been apparent throughout this dissertation. It seems that when the blood remains fluid or hydrated RNA degradation occurs more rapidly presumably due to the continued presence of enzymatic activity. Once the blood stain dries, the cells become desiccated and the RNA present within the cells appears to be more stable than RNA present in the hydrated samples. This observation has prevailed in the analysis of blood stains exposed both to high temperatures and humidity. Blood stains did not appear to dry as quickly when present at increased temperatures and humidity and RNA detection was greatly reduced. The array studies also support this concept. The larger volumes of blood consistently had increased levels of the mRNAs identified by the microarray from 0-1 hour after deposition (Figure 4-

10) while the smaller volumes of blood had very inconsistent mRNA levels for the time period examined (Figure 4-11). General observations of the blood during this experiment suggest variability in the drying process and this could have an indirect effect on mRNA levels in the blood cells. At this point it is unknown if the increase in mRNAs levels is a result of increased transcription or decreased mRNA decay.

The RNAs identified by the microarrays function in a variety of ways. Early growth response gene (EGR1) has been shown to be up-regulated in response to hypoxia and may also regulate genes involved in DNA repair, cell survival and apoptosis [51]. Much less is known about regulator of G-protein signaling (RGPS) and putative lymphocyte G0/G1 switch gene (GOS2) but they both regulate entry into the G1 phase of the cell cycle [52, 53]. These mRNAs could participate in the transcriptional activation of genes needed for growth regulation of the cell. Desmocollin type 3 (DMS3) is involved in cell to cell communication and it would be logical that this mRNA would be up-regulated once the cells exit the body in an attempt to communicate with the neighboring cells [54]. Preferentially expressed antigen of melanoma (PRAME) has been shown to contain a leucine rich motif which has been shown in other genes to be required for function in cellular trafficking and cell adhesion [55]. PRAME probably functions in a similar way to DSC3 in the *ex vivo* blood cells.

Real-time variability-review

What does the future hold for real-time PCR analysis and how does real-time PCR stand up to the critics? According to the critics, 2 problems still remain with the use of real-

time PCR and will most likely contribute variability, template preparation and the dispensing of reagents. The use of standardized competitor templates allows comparison between experiments and the use of internal standards solves problems of variation in template starting amounts and operator loading errors. The problems of template preparation have been addressed and it has been shown that in our lab there is little variability in RNA isolations by different investigators (Figure 2-6). Individual variability may still be an issue when other people utilize the technique described in this dissertation. Applied Biosystems has recently developed and launched the 6100 Nucleic Acid PrepStation which can isolate RNA and DNA from up to 96 biological specimens in less than 30 minutes to try and overcome this problem. Critics have also found that probes synthesized by different manufacturers have different stability upon storage, with the most consistent probes being those obtained from ABI. To control for this a standard cDNA was included with every real-time PCR run. Technology has advanced rapidly in the last 2 years and the latest real-time thermal cyclers have been developed with multiplexing and high throughput applications in mind. All of them aim to minimize the variability inherent in conventional real-time PCR, and monitor fluorescent signals as they are generated, recording a threshold cycle where fluorescence rises above the background but before the reaction reaches a plateau. Critics have termed ABI 7700 the least flexible because it relies on single excitation lasers and the range is very narrow (488-514 nm) and can not excite efficiently the wide range of fluorophores available today. New machines have been developed to absorb a greater wavelength range (350-830 nm) to achieve greater spectral compensation between the reporter and quencher. Until recently, quencher, usually FAM and TAMRA, suffered from a number of drawbacks, including either poor spectral overlap between the fluorescent dye

(FAM) and quencher molecule (TAMRA) or inherent fluorescence of the quencher resulting in a relatively poor signal to noise ratio. A new class of quenchers has become available [e.g. Black Hole Quenchers (BHQ)], that have an absorption maximum of 474 nM and they can quench fluorescence over the entire visible spectrum and into the infrared. Several critics have compared signals generated using 2 different quenchers with the same reporter in a real-time PCR reaction designed to detect insulin-like growth factor II (IGF-II) mRNA levels in colonic biopsies. Comparisons between 1 probe labeled with FAM and TAMRA and the other with FAM and a BHQ, showed that the TAMRA quenched probe performed better, both in terms of reproducibility as well as sensitivity [56]. This technique may or may not enhance the results but has the potential to be examined. In summary, real-time PCR is a fast growing technology with a lot of advances being made since the execution of the experiments mentioned in this dissertation. The technologies may help to increase the accuracy and precision of the technique described in this dissertation.

Advantages of Our Technique

The described method of dating blood stains provides several features that make it more reliable than the previously mentioned methods. Nucleic acid polymorphisms present in the coding region of the gene can be used to ascertain that the blood came from humans. This is an advantage over the older techniques because it excludes the potential for false signals due to contamination. By examining the RNA ratio or difference in Ct value, the analysis is independent of sample size (Figure 2-9). This trend should remain the same over time but this concept still needs to be further investigated. There is also potential for application to tissues other than blood, such as hair and saliva, with tissue-specific mRNA profiles to determine the tissue from which the sample is derived [57]. Recent research has

identified RNA species specific for both spermatozoa and menstrual blood [58, 59]. A downfall of the previously mentioned techniques was that they could only be applied to blood. The method described in this dissertation provides simultaneous isolation of both RNA and DNA from the same sample, with the isolated DNA being of sufficient quality for human identification by STR analysis (Figure 2-10). Additionally, all of the experimental manipulations occur simultaneously to both target RNAs in the same tube thus eliminating potential issues such as pipetting errors or differences in enzyme efficiency. Both RNAs are isolated using the same RNA isolation procedure, both RNAs are reverse transcribed in the same tube, and both RNAs are multiplexed in the same tube for real-time analysis. Blood stains can be incubated for at least 7 days in TRI Reagent BD at room temperature without compromising the detection of the RNAs examined in the experiments described in this dissertation. This makes the technique described in this dissertation favorable for the transportation of biological samples to crime labs (Figure 2-7). Little investigator technical variability was identified with the method described in this dissertation (Figure 2-6) and RNA has been recovered and analyzed from blood stains deposited on a variety of different surfaces making this technique very robust (Table 2-5). The time period over which useful estimates can be made using RNA isolated from blood dried on fabric, is at least on the order of 150 days (Figure 1-5). Finally, only relatively small samples are required for the test, with samples as small as 1 μ l of blood yielding ample RNA for analysis (Table 2-5).

By examining 18S and β -actin, 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 material that does not correspond to the time when a crime was committed. For example, if a bloodstain is determined to be over

2 months old and the crime was committed only 2 weeks ago, that particular bloodstain could 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 blood stain is known to have been deposited at the time a crime was committed, then comparisons of RNA ratios/difference in Ct values could be used to estimate when the sample was deposited. The ultimate goal for this research project is to develop a 96-well plate with many combinations of primer/probe sets, including genes identified by array analysis. Eventually, array genes may be able to exclude blood from being younger than a certain age or identify very young samples. Both PRAME and DSC3 are totally undetectable using real-time PCR after being isolated from 7-day old 200 µl dried blood stains but have a peak in mRNA levels 1 hour after deposition. Analysis of the same cDNAs using several combinations of these time lines will reduce the confidence intervals associated with a given dried blood stain.

Future

The results that have been presented in this dissertation support the hypothesis that there are differences in *ex vivo* RNA decay rates. β -actin mRNA has been shown to be less stable and to decay more rapidly than 18S rRNA in *ex vivo* blood stains. Amplicon size has also been shown to play an important role in RNA detection. The larger the region amplified the more likely an event will occur within the transcript rendering it non-functional for real-time PCR analysis. Using real-time PCR with the detection of various combinations of both 18S rRNA and β -actin mRNA the relative RNA ratios/differences can be used to estimate the time of blood deposition.

Present work is being conducted to expand the study population to look at the specific effects of sex, age, and ethnicity and to also apply the technique to other biological samples such as saliva and hair. Primers and probes are being designed for housekeeping genes other than β -actin and 18S that were identified at relatively high levels in dried blood samples (Figure 1-1). Because of unique *in vivo* feature of RNA decay, primers specific to the 3' and 5' ends of mRNA will be explored on these RNAs. In addition to randomly priming RNA, Oligo dT will also be used for the analysis of primer/probe sets not including 18S rRNA. With the integration of all of the above mentioned factors, there is potential for the development of a technique more sensitive and precise than the previously described technique that may be of practical use in the near future.

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Appendix

A-1. The existing blank plate is to be closed and a new one created, selecting under the 'Run' pull-down menu 'Plate Read' instead of the default 'Real-time'. Under 'Sample-Type' pull-down, 'Sample Type Setup' is to be selected and the unknown should be changed to SYBR using the pull-down menu. The wells of interest should be highlighted using SYBR, under the 'Sample Type' menu. Post-PCR Read needs to be selected to initiate the read. The read will then be analyzed and fluorescence can be viewed by selecting 'Rn/ Δ Rn' under 'Analysis. The actual well reading is displayed and the results can be exported into Excel by selecting 'File/Export/Experimental Report'

A-2 All DNA samples were rehydrated overnight with 75 μ l of nuclease free water. Three μ l, 10 μ l and 1 μ l of the rehydrated DNA from each sample was added to the standard identity PCR reaction. Human allelic ladders D3S1358, vWA, D16S539, D2S1338, D8S1179, D21S11, D18S51, D19S433, TH01, and FGA were used to genotype the samples. An additional primer set was used to determine gender. These primers were provided by Applied Biosystems STM Plus kit (part# 4309589). DNA with a known genotype was included with every reaction as a control. PCR reaction were run in an ABI 9700 and then analyzed in an ABI 3100 (Applied Biosystems, Foster City, CA).

A-3. Cy5/Cy3 dyes were incorporated into the RNA. Aliquots from the control and the aged blood were labeled with Cy5 or Cy3 respectively, were simultaneously hybridized to a custom glass microarray containing over 16,000 genes specific for total human RNA. To correct for differential Cy5/Cy3 dye labeling efficiencies, the control and aged sample were

labeled with Cy3 or Cy5, respectively, and again hybridized to a microarray. Inferences were made for each gene, demonstrated by a significant change in levels detected (increase or decrease) based on a P-value of less than 0.001 and a fold different greater than five for the first trial and three for the second trial.

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West Virginia University B. A. (Biology) 1998

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COURSES TAUGHT AT WEST VIRGINIA UNIVERSITY:

Teaching Assistant Biology 115 (Principals of Biology-lab section) 1998
Basic biology laboratory experiments including genetics and .
Teaching Assistant Biology 117 (Introduction to Physiology-lab section) 1999
Basic biology laboratory experiments including reproduction and
Biology 219 (The Living Cell-lab section) 1999, 2000
Examined many basic Molecular Biology techniques including gel electrophoresis,
plasmid rescue, restriction enzyme digest, lac operon, etc.
Teaching Assistant Biology 104 (Introduction to Biology) 1999
Biology lab for non-biology majors, taught very basic biology lab experiments.
Teaching Assistant Biology (Recombinant DNA Technology) 2000
Very intense lab based Molecular Biology course focusing on techniques including
Southern analysis, PCR genomic libraries, etc.

PEER REVIEWED PUBLICATIONS

Anderson S, Karandikar U, Mason N, Trott RL, Bishop CP, Bidwai AP. The Drosophila
SSL gene is expressed in larvae, pupae, and adults, exhibits sexual dimorphism, and mimics
properties of the beta subunit of casein kinase II. Biochem Biophys Res Commun. 2003 Feb
21;301(4):941-7.

INVITED TALKS

NIJ's DNA Grantee Workshop
June 23-25, 2003
Washington, D.C.

Fifth Annual National Conference on Science and the Law

March 14-17, 2004
Tampa, FL

WORK IN PROGRESS

A Method for Determining the Age of a Bloodstain. Accepted for publication in Forensic Science International (04/04)

SUMMARY OF Ph.D RESEARCH PROJECT

Polymerase Chain Reaction (PCR) allows for the unambiguous identification of the person from whom a biological sample was derived. PCR can be used to indicate that a suspect had visited a given location sometime prior to the collection of evidence. The ability to determine the age of a blood or biological sample would be of significant benefit to the forensic science community. It would provide a temporal linkage between the blood of the perpetrator and the commission of a crime or it could be used to exclude evidence that does not correspond to the time a crime was committed. We have used Real-time Reverse Transcriptase PCR to show that the ratio between different types of RNA (rRNA vs. mRNA) changes over time in both aqueous and dried blood. Under the conditions examined the ratio changes in a consistent and reliable way. Although other approaches have been used in the past to estimate the age of a biological sample, our approach offers the following advantages: DNA and RNA can be co-isolated from the same sample, probes can be made species specific, relatively small samples can be assayed, and the window of time estimates for dried blood is at least 0 to 150 days. Research Funded by a grant from the National Institute of Justice.

SUMMARY OF ADDITIONAL RESEARCH PROJECTS

Identification of *grooved*, a novel developmental gene in *Drosophila melanogaster*. I have utilized various molecular biology techniques to try and clone the *grooved* gene. Based on the mutant phenotype, *grooved* is believed to be involved in cell to cell communication and nervous system development involving the Notch Pathway. Because of the remarkable similarities between gene regulation in *Drosophila* and human development, the identification of *grooved* may give more insight into this advanced process.

LAB EXPERIENCE

Real time PCR, PCR, RT-PCR, gel electrophoresis, *Drosophila* genetics (P-element insertions, γ mutagenesis), Southern Blot, RNA/DNA isolations, DNA microarray, Primer design, Genomic libraries, Restriction enzyme digests, Plasmid Rescue.

COMMITTEES

Co-Chair of Search Committee for Biology Department Seminar Series.

Additional Experience

Microsoft Office 2000 and XP, familiar with both PC and MAC and various computer applications/internet.