THE DEGRADATION OF BETA-ACTIN IN AGING BLOOD AND SEMEN STAINS

By

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THE DEGRADATION OF BETA-ACTIN IN AGING

BLOOD AND SEMEN STAINS

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Abstract: Piecing together a crime scene is one of the most significant components of any forensic investigation and estimating the age of biological stains at a scene can be crucial to the case. Several studies have demonstrated a correlation between the degradation kinetics for mRNA transcripts present in dried body fluid stains aged for varying amounts of time. The relationship between the age of a sample and the state of degradation of many transcripts can be exploited to estimate the age of an unknown crime scene stain. In this laboratory, preliminary work has demonstrated that the 5' and 3' ends of many mRNA transcripts degrade at different rates during aging of body fluid stains. The purpose of this study was to map the degradation of the entire *ACTB* transcript (~1800 nucleotides long) in dried blood and semen stains and to determine if variances in degradation sensitivity are uniformly distributed along the length of the molecule. We also evaluated the kinetics of *ACTB* mRNA degradation in dried blood and semen stains to determine whether there are any tissue-specific differences in transcript degradation. The findings of this study will contribute to the knowledge that will be needed to apply this technology to estimate the age of biological evidence recovered from a crime scene.

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INTRODUCTION

The estimation of the age of stains is a novel technique developed to resolve the question of time since deposition (TsD) for biological evidence. The molecular analysis of degradation of messenger RNAs (mRNAs) encoding housekeeping genes, ribosomal RNA species, and even microRNAs, has increased in relevance to criminal investigations and suggests that the estimation of the age of crime scene stains is feasible and will sometimes establish or refute the importance of a biological sample of evidence to the crime that was committed [1, 5-13, 15-23]. Not only has the state of mRNA degradation aided in determining the age of a stain, but also in estimating the age of the stain's donor [14]. Another application of RNA analysis of crime scene stains and tissues is to identify tissue-specific RNA that will characterize the nature of the biological evidence recovered [15-19, 21]. RNA is more abundant in cells than DNA and is more susceptible to degradation. This degradation could be due to high pH levels, RNases, or even UV light [1]. The take home lesson from these studies is that RNA analysis has a place in a forensic laboratory that engages in forensic genetics. It is no longer all about DNA.

There is a lack of consensus on how best to determine the age of biological evidence recovered from a crime scene or what tissue specific mRNAs to use to identify the nature of a sample, and this field is continuously evolving [2]. It is known that the degradation state of many mRNAs and the age of the stain are correlated; as the age of the stain increases, mRNA will

become increasingly fragmented. The problem is determining what is the best way to study this degradation pattern. Previous studies have compared the ratio of the abundance of ribosomal RNA (rRNA) to the abundance of mRNA transcripts such as the housekeeping gene beta-actin (*ACTB*) [1]. Presumably, the repeated quantification of the transcript whose abundance is unchanging (i.e., rRNA) allows for the normalization of the abundance of the transcript that is changing with the age of the sample. Fu and Allen [8] took a different approach for assessing the state of RNA degradation after discovering that the 5' end of several transcripts degraded faster than the 3' end of the transcript. Subtracting the abundance of RNA fragments mapping to the 3' end of a transcript (expressed as Cq) from that exhibited by the 5' end of the transcript allowed for the calculation of a ΔCq value that, when plotted versus elapsed time, created a regression that rises steadily as body fluids age during storage [8]. In the study reported here, the degradation kinetics of the housekeeping gene beta-actin (*ACTB*) mRNA were compared in dried blood and semen stains aged for periods of up to 24 weeks. The study has two goals: first, to compare the characteristics of *ACTB* degradation in blood and semen stains stored under the same environmental conditions and second, to map the sensitivity of the *ACTB* transcript to fragmentation along its length to determine if there are areas in the transcript that are especially sensitive or resistant to those chemical reactions that catalyze the degradation of the molecule as stains age. In other words, the study was seeking to answer if the degradation kinetics exhibited by beta-actin are the same in blood stains as they are in semen stains and what areas of the transcript are the best indicators of sample age in comparison to the extreme 5' and 3' ends of the molecule.

CHAPTER II

LITERATURE REVIEW

Beta-actin is a protein encoded by the Actin Beta (*ACTB*) gene. It is part of a family of actin proteins that make up the structural framework of all the cells throughout the body and is a member of a class of constitutively expressed genes known as housekeeping genes [4]. The actin family of proteins are an essential component of the cytoskeleton of cells that maintains the cell's integrity and structure. They are able to form filaments that are specialized to the specific needs of the cell [3]. The interest of the forensic community in the beta-actin gene comes from the fact that the *ACTB* gene is widely expressed in virtually all tissues in the body making the study of the transcript possible regardless of the biological nature of the evidence under study. Bauer et al. [5] developed an assay, focusing on the degradation of the transcripts encoding beta-actin and cyclophilin, using reverse transcription-polymerase chain reaction (RT-PCR) to evaluate 106 blood stains stored up to 15 years. His team found that as the age of the samples increased, the abundance of beta-actin mRNA decreased. Another time-dependent study by Zubakov et al. [20] tested blood stains stored up to 16 years and found that over time, the transcripts began to degrade. They focused on blood specific mRNA markers that could be used in forensic investigations for tissue identification [20]. These studies and others suggest defining the kinetics of degradation of beta-actin's messenger RNA over time may allow for estimating the age of

different body fluid stains using the qPCR approach developed in this laboratory.

Determining the age of stains can be important in criminal investigations in order to piece together the sequence of events associated with a crime. Numerous studies have investigated strategies and approaches to determine time since deposition by observing the degradation of specific transcripts and/or housekeeping genes. Weinbrecht et al. [6] utilized next-generation RNA sequencing to characterize changes in the transcriptome in dried stains prepared from one of four different body fluids (blood, semen, saliva, and vaginal secretions) aged in the laboratory for periods of up to one year. They found that *ACTB* mRNA abundance in blood and semen dropped by 50% over 180 days of storage. Weinbrecht et al. [6] also established that there is a relationship between the age of stains and the state of degradation of the transcriptome. As the age of the stain increases, the total number of transcripts detected by RNA sequencing decreases. However, the disappearance of the transcripts between blood, semen, and vaginal secretions each displayed different degradation kinetics. Qi et al. [7], used reverse transcription PCR to study the ratio of 18S rRNA and beta-actin mRNA in blood stains over a 28-day period. The cycle threshold (Ct) values for beta-actin increased over time whereas 18S Ct values did not change, meaning that beta-actin degraded as a function of time. There was no significant difference in the RNA abundance between male and female donors. Anderson et al. [1] also studied the abundance ratio of 18S and beta-actin RNAs of blood stains over a period of 150 days. They also found that beta-actin mRNA degrades as a function of time in aging stains when compared with the fairly stable levels of 18S rRNA. Anderson et al. [1] suggests that the degradation of beta-actin mRNA can be useful in estimating the age of stains. They suggested that there could be a difference in degradation based on gender, but the sample size used was too small to make a conclusion. Anderson et al. [11] followed up their earlier study by quantifying qPCR amplicons from beta-actin mRNA and 18S rRNA targets that varied in their transcript length. The rationale for the approach taken by Anderson et al. [11] was that the abundance ratio of long versus short PCR

amplicons might be more informative as the age of the sample increases. A different approach was taken by Fu and Allen [8] where multiple transcripts such as beta-2-microglobulin (B2M), galectin-2 (LGALS2), charcot-leyden crystal galectin (CLC), and S100 calcium binding protein A12 (S100A12) were analyzed to determine the degradation patterns in aged blood stains. More importantly, Fu and Allen [8] discovered the 5' end of the transcripts degraded faster than the 3' end of the transcript with the passage of time. By choosing qPCR amplification targets mapping to the 5' and 3' ends of the same transcript, the qPCR reactions could be internally normalized (as opposed to targeting a second transcript altogether) and stochastic effects would be reduced leading more reproducible data. Thus, the abundance ratio of RNA fragments mapping to the 5' and 3' ends of the RNAs reflected the state of degradation for a transcript at any point in time and produced degradation kinetics useful for estimating the age of a sample [8].

Heneghan et al. [9] extended the work of Fu and Allen [8] to the next stage by studying the effect(s) of environmental conditions on the rate of degradation of 3 of the 4 transcripts studied by Fu and Allen [8] in samples stored at different temperatures and relative humidities. The higher the temperature and relative humidity, the faster the degradation occurred for all transcripts studied [9]. Conversely, degradation of the transcripts occurred very slowly or not at all in blood stains stored in a cold and dry environment (4˚C/10% relative humidity). Like Heneghan et al. [9], Salzmann et al. [10] studied time since deposition of biological stains and exposed them to indoor and outdoor conditions for up to 1.5 years. The samples that were indoors were more stable than the outdoor samples. Salzmann et al. [10] also reported that stable transcripts in the samples were typically significantly shorter in length than those that tended to degrade quickly. Similarly, Anderson et al. [11] found that shorter segments of beta-actin and 18S rRNA resisted degradation for longer periods of time than longer transcripts that degraded more quickly. However, Weinbrecht et al. [6] did not find any correlation between transcript length and its degradation rate.

Among the many previous studies analyzing the correlation of the degradation of a diverse group of mRNAs with elapsed time, most studies have concentrated on estimating the age of blood stains [1, 6-13, 20-23]. The fact that the study presented here examines the kinetics of degradation of a transcript common to both blood and semen adds to that literature. The research presented here will focus solely on the degradation kinetics of a single transcript (*ACTB*) in aging blood and semen stains stored under identical conditions. The study will also map the sensitivity of the *ACTB* transcript to strand breakage along the length of the RNA molecule. Based on the study by Fu and Allen, [8] degradation of many RNA transcripts exhibit a 5' bias in the breakdown rate; degradation kinetics suitable to use for TsD estimates can be obtained by targeting qPCR primers to sites at the extreme 5' and 3' ends of the molecules. The degradation kinetics for *ACTB* suggest that targeting qPCR sites in the middle areas of the transcript yield better degradation kinetics than qPCR targets closer to the 5' map points. The improved degradation curves would allow *ACTB* to be used to make TsD estimates in both blood and semen stains.

CHAPTER III

MATERIALS AND METHODS

Preparation of Blood and Semen Stains

This project was reviewed and approved by the Institutional Review Board of Oklahoma State University Center for Health Sciences. About 7-10 mL of blood was collected by phlebotomy from three volunteers (two female and one male) into collection tubes lacking anti-coagulant and then 100 µL of blood was spotted onto Whatman Human Identification Bloodstain cards (Boondocks Medical, Copperhill, TN). The cards were labeled with the individual's number (female one, female two, male one) and the collection date and were left to dry overnight in a fume hood. Semen stains were prepared from about 5 mL of semen deposited into 50 cc conical bottom centrifuge tubes from two male donors. For male one's semen, 50 µL was spotted onto a sterile polyester tipped applicator (PurWraps, Thermo Fisher Scientific, Waltham, MA) whereas for male two's semen, 100 µL was spotted onto a sterile polyester tipped applicator. The reason for these two different amounts was due to early test spotting of male one. 50 µL was spotted onto collection cards, but the semen kept spreading causing it to increase in size. Due to semen being clear in color, it was difficult to determine exactly where the spotted stain was in relation to the other stains, so we moved to the tipped applicator. Since the stains spread on the collection cards, it was originally thought that 100 µL of semen on the sterile polyester tipped applicator would oversaturate the cotton tip, but that was not the case. We continued spotting 50 µL for male

one and 100 µL for male two and when it came time for extraction, we used two applicator tips from male one that way we had 100 µL of semen total for both males. The wrappings of the swabs were labeled with the individual's number and collection date and were left to dry overnight in a fume hood. All samples were placed into a -80˚C freezer to prevent aging until the experiment was ready to be initiated. Once the time trial began, the samples were taken out of the freezer and stored at room temperature in a dry and dark cabinet to begin the aging process. Stains were stored for periods of up to 24 weeks.

RNA extraction

RNA extraction began by cutting the blood stains with clean scissors from the collection cards into 5-7 pieces which were then placed into a microfuge tube. Two swab tips from the 50 µL semen stains and one swab tip from the 100 µL semen stain were clipped from the applicator stick and each placed into a 2 mL microcentrifuge tubes as well. RNA was extracted from both sample types in 1000 µL of TRIzol (Life Technologies, Carlsbad, CA). Samples were vortexed for 30 minutes at room temperature in the TRIzol to disrupt cell structures and liberate nucleic acids. To help the organic and aqueous layers to separate following the extraction step, 200 µL of chloroform was added to the TRIzol solution and shaken for 15 seconds by hand, incubated at room temperature for 3 minutes, and then centrifuged at 12,000 g for 15 minutes at 4˚C. Once the organic and aqueous layers were separated, the aqueous layer was collected. The Zymo RNA Clean and Concentrator kit (Zymo Research, Irvine, CA) was used to capture and concentrate the cleaned RNA following the instructions from the manufacturer. A second purification step was added to remove any inhibitors that may have been lingering within the semen stains as semen tends to have more inhibiting products than blood. The procedure from the supplier of the Zymo OneStep PCR Inhibitor Removal kit was followed (Zymo Research, Irvine, CA).

Once the samples were purified and concentrated, RNA was quantified in each extract using fluorescence with the Qubit RNA H.S. Assay kit (Thermo Fisher Scientific, Waltham, MA). RNA yield from the three 100 µL bloodstains averaged 645 ng from fresh stains of the three donors and the RNA yield from the two 100 µL semen stains averaged 1,693 ng at time zero. All RNA extracts underwent treatment with ezDNase following instructions from the supplier (Thermo Fisher Scientific, Waltham, MA) to digest any contaminating DNA that might still be in the samples. RNA samples were reverse transcribed into a complimentary DNA (cDNA) library using the SuperScript IV VILO Master Mix kit following instructions from the supplier (Thermo Fisher Scientific, Waltham, MA) The samples were stored at -20˚C until specific transcripts in the library were quantified using quantitative polymerase chain reaction (qPCR) methods.

Primer design

The degradation of beta-actin (*ACTB*) mRNA was chosen for analysis primarily because it is available in abundance in both blood and semen. *ACTB* is also a large transcript (~1800 nucleotides) and one of the goals of this project was to map the sensitivity of a transcript to degradation as stains age. This laboratory has determined that *ACTB* degrades with time in storage. This study compares the degradation kinetics of multiple map points within the transcript length during an aging experiment. qPCR efficiencies for our designed primers were determined using a five-fold serial dilution of the cDNA that was created from time zero bloodstain and semen stains through RT-PCR as noted in Table 1. Efficiency values were between 0.94 – 1.08 which are all in an acceptable 90%-110% range for our assays.

		99 bp	94 bp	91 bp	97 bp	86 bp	92 bp	95 bp	92 bp	103 bp	88 bp	93 bp	90 bp
cDNA	Log(cDNA)	32-130	145-238	279-369	421-517	520-605	743-834	819-913	1004-1095	1167-1269	1393-1480	1486-1578	1701-1790
10	1	20.032	19.078	18.870	19.336	17.926	18.563	19.263	17.361		17.156	16.172	16.729
10	1	20.094	18.821	18.678	19.097	17.932	18.544	19.201	17.292	17.182	17.147		16.802
10	1	19.950	19.114	18.912	19.166	18.081	18.625	19.308	17.357	17.180	17.192	16.283	16.954
2	0.30	22.219	21.334	21.315	21.349	20.218	21.076	21.473	19.630	19.494	19.402	18.507	19.076
2	0.30	21.935	20.950	21.097	21.353	20.038	20.996	21.480	19.542	19.603	19.344	18.605	19.181
2	0.30	22.280	21.185	21.375	21.418	20.178	21.002	21.463	19.599	19.478	19.254	18.446	19.148
0.4	-0.40	24.472	23.450	23.741	23.647	22.508	23.192	23.783	21.989	21.582	21.543	20.739	21.322
0.4	-0.40	24.189	22.923	23.517	23.664	22.567	23.237	23.779	21.719	21.832	21.478	20.805	21.326
0.4	-0.40	24.291	23.424	23.790	23.784	22.485	23.135	23.748	21.871	21.838	21.735	20.740	21.450
0.08	-1.10	26.779	25.883	26.188	26.161	24.404	25.742	26.100	24.188	24.132	23.853	22.979	23.704
0.08	-1.10		25.618	25.925	26.135	24.764	25.649	26.049	24.228	23.932	23.862	23.029	23.645
0.08	-1.10	26.569	25.883	26.176	26.140	24.683	25.617	26.085	24.211	24.156	23.466	22.989	23.685
	slope	-3.15	-3.22	-3.47	-3.31	-3.19	-3.35	-3.26	-3.27	-3.27	-3.14	-3.22	-3.26
	R2	0.997049	0.99361398	0.99817755	0.99874582	0.99802091	0.99906874	0.99980244	0.99933828	0.99864495	0.99783565	0.9995983	0.99938513
	efficiency	1.08	1.05	0.94	1.00	1.06	0.99	1.03	1.02	1.02	1.08	1.04	1.03

Table 1: qPCR assay efficiencies. The highlighted cells indicate the lowest efficiency value (0.94) and the highest efficiency value (1.08) which are in the acceptable 90%-110% range for our assays.

qPCR assays

qPCR assays were used to map out the *ACTB* gene from the 5' end to the 3' end and to quantify the transcript abundance (expressed as Cq) for each RNA fragment amplified with each primer set in both blood and semen stains. qPCR reactions contained 3 µL of water, 5 µL of the PowerUp SYBR green Master Mix (Thermo Fisher Scientific, Waltham, MA), and 1 µL of our 10x designed primer set previously mentioned (5 µM for all twelve primers) for a total of 9 µL. The total qPCR reaction after the addition of 1 µL of our cDNA sample (diluted to ~5ng/µL) was 10 µL. Through the use of the Quant Studio 5 Real Time PCR System (Thermo Fisher Scientific, Waltham, MA), samples underwent qPCR using the standard cycling program of 50˚C for 2 min and 95˚C for 2 min followed by 40 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The threshold was set to 0.2 for recording of Cq values that the abundance of an RNA fragment harboring the qPCR target was produced.

Data Analysis

The Cq values at each of the 12 map points in the *ACTB* transcript for the 24-week storage period were produced from duplicate reactions and averaged. The greater the Cq value, the more degraded the samples, so rising Cq values in stored samples reflect the disappearance of those RNA fragments through degradation over time. The changes in Cq values for the different qPCR amplicons were plotted in Excel which allowed visualization of the state of *ACTB* degradation as a sensitivity map in blood and semen stains for each storage time point. The state of degradation for a transcript can also be displayed as a rising regression reflecting the changing quantities of RNA fragments mapping to the 5' and 3' qPCR targets (expressed as ΔCq calculated by subtracting the Cq value for a 3' target from the Cq value produced for a target mapping to a 5' region).

The ΔCq of the data underwent statistical analysis through the single factor analysis of variance (ANOVA) to determine if there was any significant difference in Cq values among the three blood donors or between the two semen donors. When determining significance, the p-value was calculated; any value ≤ 0.05 was considered to be statistically significant.

CHAPTER IV

RESULTS

It was shown in earlier publications from this laboratory that many mRNA transcripts exhibit a 5' bias in degradation as a blood stain ages. The degradation process is revealed graphically as a rising ΔCq curve. There has not been extensive investigation into the degradation kinetics of a single transcript in two different tissues aged under identical conditions. The *ACTB* mRNA is expressed at roughly the same levels in both blood and semen. In preliminary studies with primers mapping to the 5' end of the *ACTB* transcript, we observed that selecting a combination of qPCR targets mapping to the middle of the transcript [(nucleotide 819 (nt819) or nucleotides 1486 (nt1486)] combined with a 3' target mapping to nucleotide 1701 (nt1701) produced degradation kinetics more useful for estimating sample age. As samples are aging, the qPCR target mapping tot the 3' end of the transcript (nt1701) remains quite stable, so this target was paired with others moving in a 5' direction to develop and compare degradation kinetics.

All primers designed for *ACTB*, have similar melting temperatures, and display similar efficiencies for promoting amplification of qPCR targets about 90 basepairs in length. Twelve sets of primers were designed through BLAST from the National Center for Biotechnology Information (NCBI). A map of amplicon primer pairs is illustrated in **Figure 1**.

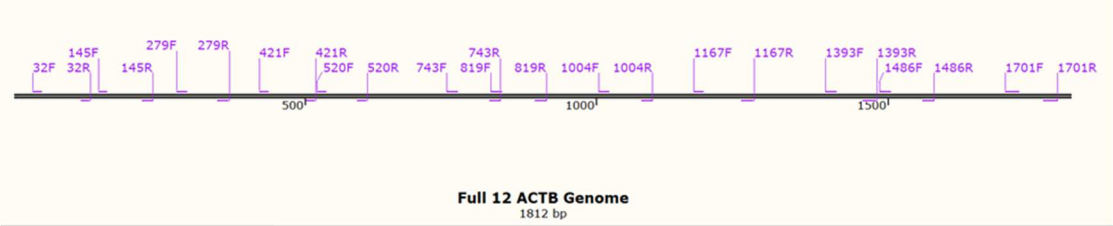
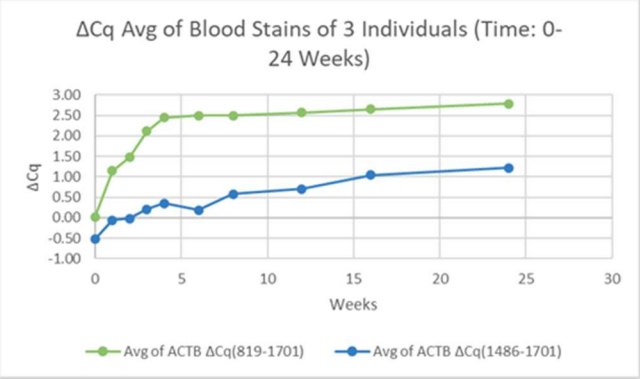


Figure 1: Map of the *ACTB* transcript (~1800 nucleotides) with each ~90 basepair primer set spanning the length of the transcript

The degradation kinetics for the *ACTB* transcript in blood and semen stains are displayed in **Figure 2**. Amplicon abundances for the two 5' qPCR targets (nt819 and nt1486) were compared with the abundance for the 3' qPCR target mapping to nt1701 creating ΔCq regressions (**Figure 2**). The degradation kinetics of both blood and semen stains are reflected in the rising curve of the ΔCq values. If one examines the slopes of the linear portions of the curves in blood stains, it is apparent that the ΔCq increases more rapidly for the nt819 primer pair than for the primer pair mapping to nt1486. The data supports the notion that there is a 5' bias in *ACTB* degradation in blood stains even for the middle regions of the 1800 nucleotide transcript.

There is also an apparent 5' bias in *ACTB* degradation in semen stains, but the bias appears to be less severe. As both curves rise in a similar fashion either the nt819 or nt1486 targets could be useful for estimating TsD.



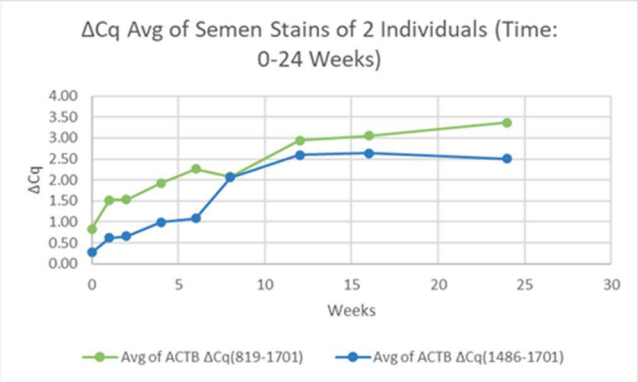
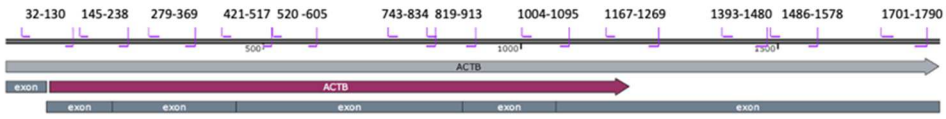


Figure 2: The kinetics at which the *ACTB* transcript degrades in blood and semen stains that have been stored for up to 24 weeks (20˚C; 35% relative humidity). qPCR primer pairs mapping to positions nt819 or nt1486 (for 5' qPCR targets) were used to produce the degradation kinetics of the *ACTB* transcript in semen stains versus a 3' qPCR target mapping to nt1701. Plots showing the values of ΔCq (Cq of 5' product minus Cq of 3' product) vs storage time are displayed.

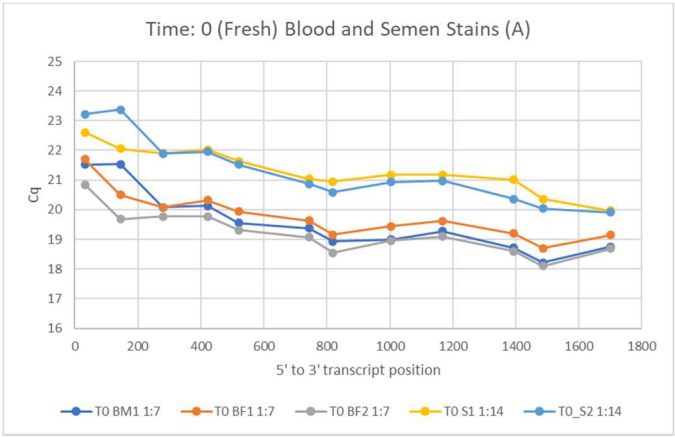
The ΔCq values increase steadily at each storage timepoint (week) for both blood and semen stains, which is consistent with earlier findings using different transcripts in blood stains and suggest it may be possible for useful estimates for time since deposition (TsD) to be made using the *ACTB* transcript in both blood and semen stains. Given our experience with other mRNAs we have studied, the question arose as to why qPCR targets in the middle to final third of the *ACTB* transcript produce more useful degradation kinetics than regions mapping at the transcript's 5' end. "Mapping the sensitivity" of the complete *ACTB* transcript (~1800 nucleotides) to degradation during storage of blood and semen stains would be one approach to analyze in detail the degradation characteristics for the *ACTB* transcript as a stain ages. To develop the sensitivity map of the *ACTB*, a panel of qPCR primer pairs were created, each of which directed the amplification of ~90 basepair products (**Figure 3**).

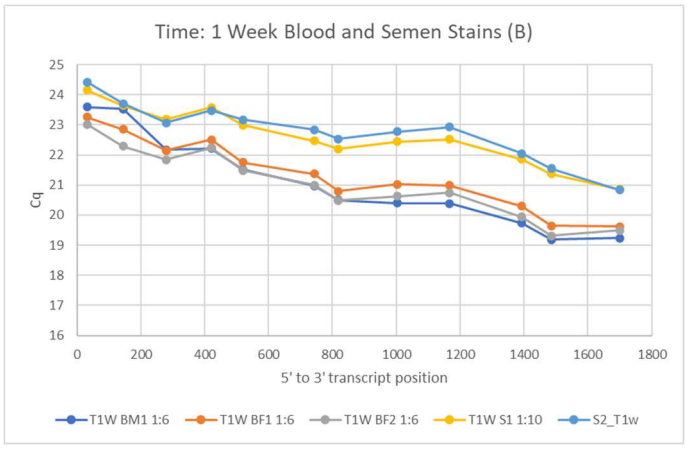


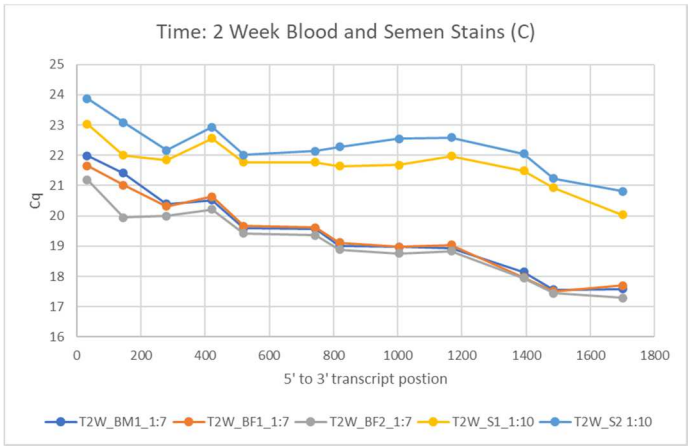
	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt	5′ nt
NM_001101.5	32	145	279	421	520	743	819	1004	1167	1393	1486	1701
length	99 bp	94 bp	91 bp	97 bp	86 bp	92 bp	95 bp	92 bp	103 bp	88 bp	93 bp	90 bp
PCR position	32-130 (cross exon)	145-238 (cross exon)	279-369	421-517 (cross exon)	520-605	743-834	819-913 (cross exon)	1004-1095 (cross exon)	1167-1269	1393-1480	1486-1578	1701-1790

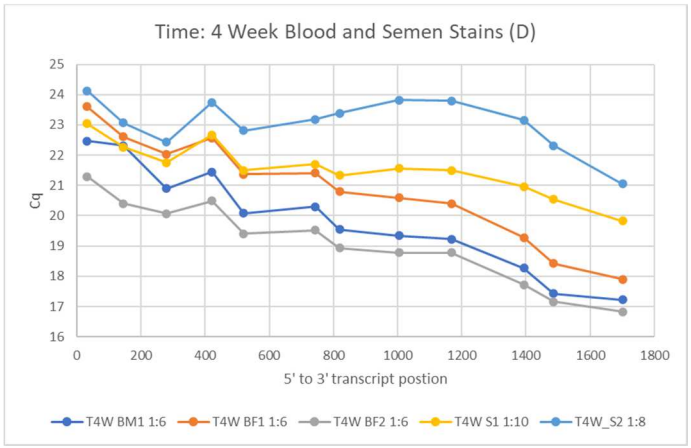
Figure 3: Locations of qPCR primer pairs that span the entire *ACTB* transcript are shown on the map above. qPCR primer pairs were synthesized that directed amplification of RNA targets at the indicated positions. Primer pairs that cross exons and the size of the qPCR product are also displayed.

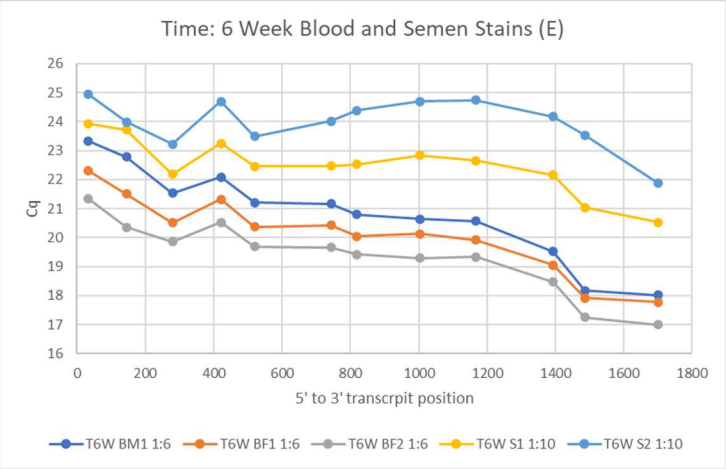
The abundance of RNA fragments harboring these targets would reflect the state of RNA degradation at each of these map locations in aging blood and semen stains (expressed as the Cq value). **Figure 4** displays the sensitivity maps of *ACTB* mRNA in blood and semen stains for stains aged for periods of up to 24 weeks (20˚C/35% relative humidity).

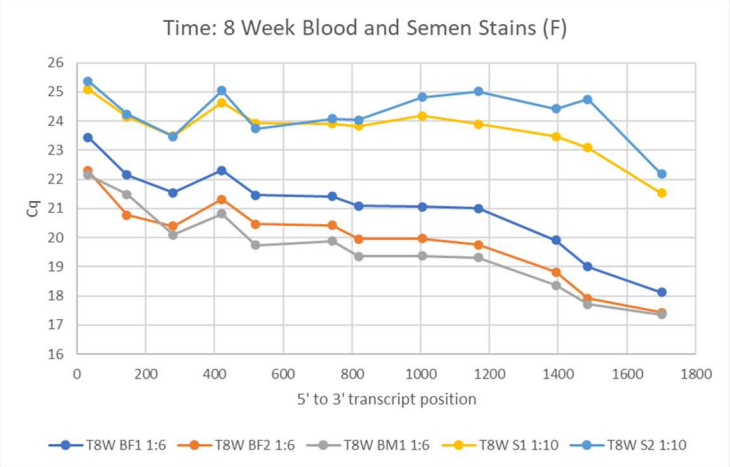


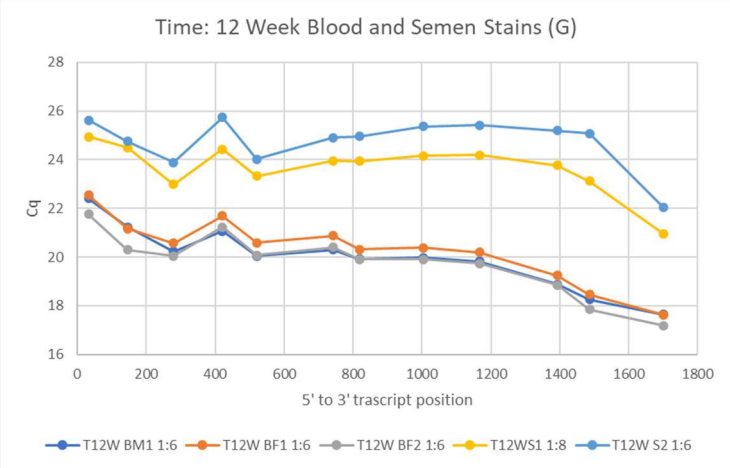

Time: 1 Week Blood and Semen Stains (B)
Cq
5' to 3' transcript position
T1W BM1 1:6
T1W BF1 1:6
T1W BF2 1:6
T1W S1 1:10
S2_T1w

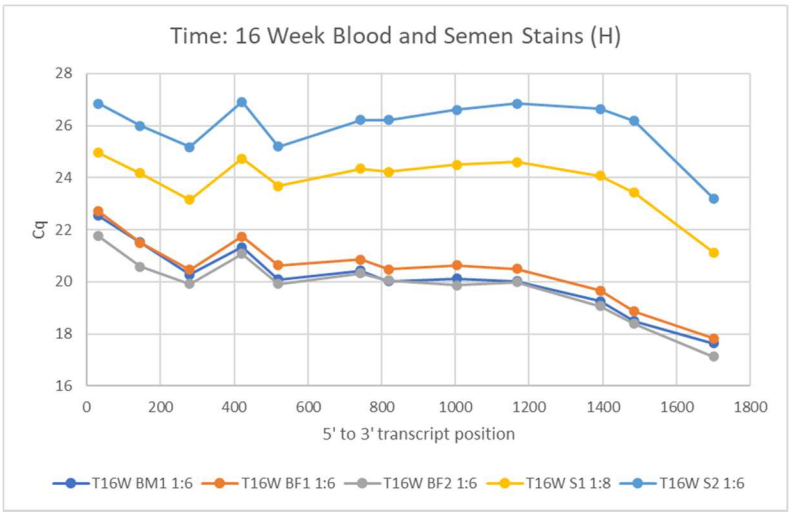

Time: 2 Week Blood and Semen Stains (C)
Cq
5' to 3' transcript postion
T2W_BM1_1:7
T2W_BF1_1:7
T2W_BF2_1:7
T2W_S1_1:10
T2W_S2 1:10


Time: 4 Week Blood and Semen Stains (D)
Cq
5' to 3' transcript postion
T4W BM1 1:6
T4W BF1 1:6
T4W BF2 1:6
T4W S1 1:10
T4W_S2 1:8


Time: 6 Week Blood and Semen Stains (E)
Cq
5' to 3' transcrpit position
T6W BM1 1:6
T6W BF1 1:6
T6W BF2 1:6
T6W S1 1:10
T6W S2 1:10


Time: 8 Week Blood and Semen Stains (F)
Cq
5' to 3' transcript position
T8W BF1 1:6
T8W BF2 1:6
T8W BM1 1:6
T8W S1 1:10
T8W S2 1:10


Time: 12 Week Blood and Semen Stains (G)
Cq
5' to 3' trascript position
T12W BM1 1:6
T12W BF1 1:6
T12W BF2 1:6
T12WS1 1:8
T12W S2 1:6



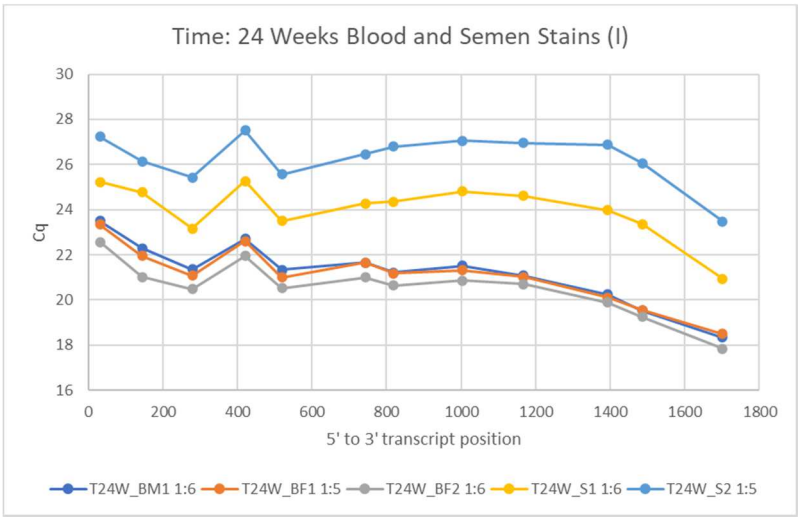


Figure 4A-I: Sensitivity mapping of beta-actin mRNA in blood and semen stains aged up to 24 weeks (20˚C; 35% relative humidity). The graphs read from left (the 5' end of the transcript) to right (the 3' end of the transcript) with each point indicating the quantitation of RNA. The dark blue curve indicates the blood stain from male 1, the orange curve from female 1, and the grey curve from female 2. The yellow curve indicates the semen stain from male 1 and the light blue curve from male 2. The week the samples were extracted is also noted as well as the dilution ratio.

At time zero, the Cq value decreases as the primer pairs move from the left end of the transcript (the 5' end) to the right end of the transcript (the 3' end) (**Figure 4A**). The falling Cq regression indicates an increasing abundance of RNA fragments harboring qPCR targets as primers move from the 5' to the 3' end of the molecule. This finding confirms the 5' bias in RNA degradation reported for other mRNAs that have been studied in dried blood stains [8 & 10].

Both blood and semen stains exhibit the 5' bias in freshly prepared samples. Keep in mind that samples from time zero are actually up to one day old since the stains were dried in a fume hood overnight. Cq values for the semen stains from two donors were not significantly different ($p = 0.88$) nor were the Cq values at any time point in blood stains significantly different ($p = 0.95$).

After 6-8 weeks of storage, the 5' bias in semen stains appears to change (**Figure 4F**). The middle two-thirds of the *ACTB* transcript starts to become similarly vulnerable to fragmentation after 8 weeks. In blood stains of comparable age, the flattening of the Cq curve in the central region of the *ACTB* transcript is not as prominent as it is in aged semen stains (**Figure 4**). The 5' bias appears to be preserved in blood stains throughout the course of the 24-week storage period whereas the bias changes in semen stains. In other words, sensitivity to degradation in semen stains changes with time, losing the 5' bias character in the middle of the molecule, unlike blood stains where it consistently diminishes as one proceeds from the 5' to the 3' end of the transcript. The characteristics of *ACTB* degradation therefore differs in blood and semen stains perhaps due to differences in the *ACTB* transcript itself in blood or semen perhaps through of tissue specific differences in how the RNA is degraded.

From this study, we collected a detailed map of the sensitivity of the *ACTB* transcript to degradation as blood and semen stains aged in the laboratory. It appears that during the degradation process across the 24-week period, the 5' bias in *ACTB* degradation in blood stains

persists through the storage term but the character of degradation changes as semen stains age. In both blood and semen stains, the 5' bias theory predicts that the 5' end of the *ACTB* transcript [(and many other transcripts we have studied (data not shown)] [8] will degrade at a faster rate with time in comparison to the 3' end of the transcript. This theory must be modified in the case of *ACTB* degradation in semen stains. Nonetheless, the transcript degradation kinetics produced with RNA from semen stains (i.e., the ΔCq values) still rise with aging if one compared Cq values for map points at nt819 or nt1486 with values from the nt1701 target. Simply stated, if one simply moves the primer pair directing amplification of the 5' qPCR product to middle areas of the transcript, the 5' bias useful in degradation remains in semen stains and therefore kinetics useful for TsD estimates can be produced.

CHAPTER V

DISCUSSION/CONCLUSION

In forensic investigations, the possibility of estimating the age of blood and semen stains is important since it may establish the relevance of a piece of biological evidence recovered from a scene to the estimated time the crime occurred. This would allow investigators to only focus on samples that fit the time-interval [11]. Currently, estimating the age of biological fluids recovered from crime scenes is not widely utilized in crime scene investigations, nor is it used to estimate the post-mortem-interval (PMI) by forensic pathologists [14]. However, several studies have suggested that there is a relationship between RNA degradation and the aging of dried body fluid stains. This laboratory studied the degradation of mRNA transcripts in various body fluid stains representing typical types of evidence recovered from a crime scene. Degradation of several transcripts were found to correlate well with the passage of time and thus be useful for estimating the age of blood stains. The effect of environmental conditions on RNA degradation in blood stains have been studied as well [6, 8, 9]. Much of the literature focuses on RNA degradation in dried blood stains but fails to describe how mRNA targets present in both blood and semen degrades comparatively. RNA is less stable than DNA since it is only single stranded whereas DNA contains a double helix making it much more stable. The susceptibility to degradation makes it a useful tool in estimating the age of stains. This si perhaps the first such study of RNA degradation in both blood and semen stains analyzed side-by-side during storage over 24 weeks.

The purpose of this study was to analyze the degradation characteristics of beta-actin mRNA in blood and semen stains aged up to 24 weeks. Another aim of this study was to identify optimal map point(s) on the *ACTB* transcript for the 5'-3' qPCR assay to produce the most useful degradation kinetics for TsD estimates. The *ACTB* transcript was chosen for this study due to the widespread expression of the beta-actin gene in nearly all cells of the body [4]. Several conclusions can be drawn from our results: First, RNA present in both blood and semen stains appears to degrade with characteristics that differ, especially after the stains have aged 4-8 weeks. Throughout the 24 weeks of storage, the blood stains maintain the 5' bias during degradation, however, the 5' bias changes with the semen stains. After being stored for around 8 weeks, the middle two-thirds of the *ACTB* transcript in semen stains becomes equally vulnerable to RNA fragmentation. Another finding is that; choosing a 5' qPCR target that maps to the middle of the *ACTB* mRNA creates degradation kinetics that are more informative for the estimation of TsD than qPCR targets mapping to the 5' end of the molecule. Using 5' qPCR targets mapping to the middle area of the *ACTB* transcript enables this mRNA to be useful as an indicator of aging both in blood and semen stains. As the 5' target moves closer to the 5' end of the transcript, the severity of the 5' bias in the degradation rate increases and the rapid change in the ΔCq regression reduces the utility of *ACTB* degradation kinetics for TsD estimates.

One possible explanation for why blood and semen degrade differently could be due to the process of methylation (or some other modification) of the 2' position of the ribose sugar. The 2' position is occupied by an -OH group which is highly reactive making it more chemically liable [24]. The 2'OH side group can attack the phosphodiester linkage forming the backbone of the RNA molecule resulting in a hydrolysis reaction that breaks the RNA [25]. If the -OH group were to be methylated (O-CH3) the hydrolysis reaction would be prevented for that nucleotide and perhaps for other neighboring nucleotides in the RNA molecules. It is known that methylation of RNA stabilizes the molecule in vivo and there is no reason to suggest methylation

might stabilize RNA molecules ex vivo as well [26]. It is also possible that the methyl group attached to the ribose sugar could dissociate from the oxygen removing protection for the phosphodiester linkage causing the RNA molecule to become more susceptible to degradation after a period of aging. Methylation is known to vary among differentiated tissues in a lineage specific manner and blood and semen represent distinct end points for differentiation which could explain why blood and semen degrade so differently.

These samples were also stored in favorable conditions that led to steady degradation. However, crime scenes can vary significantly in environmental conditions which Heneghan et al. [9] showed can have substantial effects on degradation kinetics of RNA in blood stains. We do not have data on how the storage environment affects degradation of the *ACTB* transcript in blood or semen, and moreover, do not know how the environment might affect the change in sensitivity of the transcript in semen stains. More research must be done in this area to develop this knowledge in aging semen stains.

Since beta-actin is generally expressed at high levels in most tissues, it would be beneficial for future researchers to continue to study the degradation of the beta-actin RNA in other tissues. It has recently been confirmed that the transcript degrades very little and without a 5' bias during aging based on the breakdown of *ACTB* RNA in dried saliva stains. Therefore, it appears that, at least *ACTB* mRNA, degrades in a tissue-specific manner. This finding will complicate the process of creating a qPCR assay using ACTB as the target that can predict TsD estimates for any biological sample. However, it should be mentioned, that our research on RNA degradation in various body fluid stains involved studying the *ACTB* transcript. In addition to the thousands of additional transcripts found in body fluids, blood, semen, saliva, and vagina secretion stains all share 1901 distinct transcripts, according to Weinbrecht et al. [6]. Other transcript may yield degradation characteristics that are identical in all tissues. Possible future studies could also include a longer aging process and the effect of environmental factors such as

temperature, sunlight exposure, humidity, or even exposure to microorganisms [1]. As more research becomes published and added to the literature, RNA could become more utilized in forensic laboratories around the world. RNA may become the new DNA.

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APPENDICES

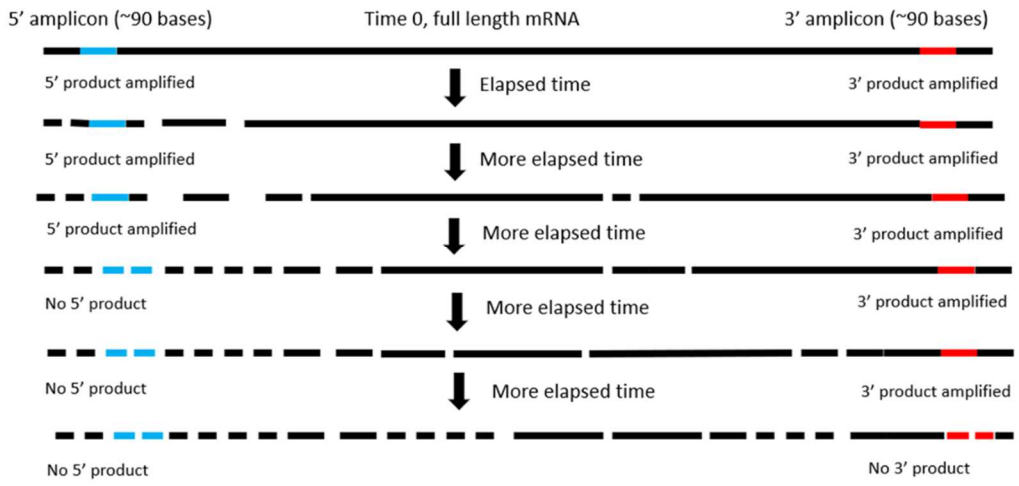

Figure 1: Example of 5’ Bias Breakdown

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Blood Male 1	10	19.85827	1.985827	0.669997
Blood Female 1	10	20.84121	2.084121	0.896498
Blood Female 2	10	19.65184	1.965184	0.926427

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.08078	2	0.04039	0.048606	0.95264	3.354131
Within Groups	22.43629	27	0.830974			
Total	22.51707	29				

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Semen Male 1	9	19.23916	2.137685	0.743446
Semen Male 2	9	19.7864	2.198489	0.719117

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.016637	1	0.016637	0.022751	0.881993	4.493998
Within Groups	11.7005	16	0.731281			
Total	11.71714	17				

Figure 2: Results of ANOVA. The p-values of both blood and semen are above our alpha value of 0.05 meaning that we accept our null hypothesis that there is no statistical significance between each blood donor nor a statistical significance between each semen donor.

VITA

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