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Project Title: Transcriptome sequencing of forensically relevant biological fluids and tissues to optimize degradation analysis for sample age estimation.

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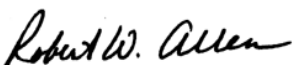
Semi-annual Report Special Report; Final overview **Signature of Submitting Official:** 

ACCOMPLISHMENTS

1) What are the major goals of the project?

Goal 1 – Use whole transcriptome sequencing data from fresh and aged body fluid stains to identify mRNA markers that exhibit degradation patterns that closely correlate with sample age.

Goal 2 – Develop real-time, quantitative assays (qPCR) for the mRNA markers identified from sequencing and begin the process of developing a valid method for estimating the age of evidentiary samples recovered from a crime scene.

2) What was accomplished under these goals?

One of the goals for the project was to characterize the transcriptomes of forensically relevant body fluid stains and tissues that have been stored for periods of up to one year in a laboratory environment. As will be summarized in the final report, and is also published in Weinbrecht et.al. 2017, our studies identified a host of mRNA transcripts in blood, semen, saliva, vaginal secretions, and teeth that undergo degradation during storage. The degradation profiles of transcripts can vary creating an opportunity for the correlation of degradation rates with sample age. Among the thousands of transcripts detected by RNA sequencing, a cohort was selected for further analysis using bioinformatics tools with the sequencing data as well as using quantitative PCR assays developed as part of this project. In the final progress reports for this project, a table itemizes the benchmarks proposed in achieving the goals stated above. Suffice it to say that all these benchmarks were completely or largely achieved over the course of support.

The first half of support for this project was spent developing transcript "databases" composed of degradation profiles of thousands of transcripts, some common to all or subgroups of body fluid types, or transcripts restricted to individual body fluids or tissues (Weinbrecht et.al. 2017). This data was produced with RNA sequencing (RNA-seq) on an Ion Torrent PGM platform using a method that allows individual transcripts to be quantified. These experiments not only revealed the overall characteristics of mRNA degradation in dried stains, but also allowed for the identification of individual transcripts whose degradation kinetics with time in storage suggested the feasibility of using RNA degradation as a measure of elapsed time. We proposed and accomplished obtaining RNA-seq data from aged stains made with blood, semen, saliva, vaginal secretions, and also with teeth.

We have investigated the transcriptomes of the different tissues during the course of this project, but have focused the majority of our bioinformatic analyses on blood and semen stains. One graduate student worked on characterizing the transcriptome in semen stains (both aspermic and normo-fertile) (Habib, 2014) and another studied the transcriptome in un-diseased teeth aged for periods of up to 6 months (Jorgenson, 2014). A third student further investigated the transcriptome produced from aged teeth and presented this data at the annual meeting of the International Association for Identification (IAI) in Atlanta in 2017 (Mullaney, 2017). Thus, we have achieved one of the goals originally proposed for this project. Since this work occurred early during the course of support, details of our findings can be found in semi-annual and annual reports submitted previously.

Goal 2 was to use the information obtained from the RNA-seq studies to examine the degradation of specific mRNA transcripts using qPCR, a technology largely available in crime laboratories. The annual report filed in December 2016 summarized our progress in developing qPCR technology that would reliably estimate the age of dried semen and bloodstains. Our first approach to applying qPCR technology for transcript abundance was to try and use commercially available kits targeting some of the transcripts identified using RNA-seq. A graduate student also experimented with a qPCR assay developed in-house that strived to reliably quantify the *SEMG1* and *PRM1* transcripts (present in semen and spermatozoa respectively) in dried semen stains aged for periods of up to 6 months (Sherier, 2016). The results of these efforts were discouraging because of the high degree of variability seen in quantitation estimates for these markers. After much investigation, the variability observed was traced largely to stochastic effects during reverse transcription. In other words, cDNA libraries prepared from replicate stains of the same age, when reverse transcribed into cDNA, yielded differences the quantities of cDNA actually produced. To address this, we examined other reverse transcription kits on the market and found one that greatly improved the reproducibility of the cDNA library produced and then subjected to qPCR quantitation of selected transcripts (Table 1).

Table 1. Variability in cDNA composition in libraries prepared with two RT-PCR kits.

RT Kit	No. Reactions	Avg SD*
Hi-Capacity	39	1.89

Superscript	32	0.78

*The average SD reflects the variation in Ct values among a cohort of mRNA transcripts quantified in semen and blood stains aged for varying periods. The differences are significant at $p<0.0001$.

Results from our studies using qPCR have also shown that, at least for blood and semen stains prepared from unrelated random donors, there was no appreciable person-to-person difference in the abundance of any transcript quantified using qPCR. This is important because if the abundance of a transcript were to vary among male or female donors, or unrelated random donors, it would, at a minimum, complicate and possibly even eliminate the viability of our proposal to estimate elapsed time using RNA degradation followed using qPCR.

Early work using qPCR and Taqman technology also showed that the transcripts like 18S rRNA or the *GAPDH* "housekeeping" gene transcript serving as the "unchanging control" for delta Ct calculations also degrade over a period of 6 months of storage (Sherier, 2016). Thus, the Taqman approach for quantitative PCR did not appear promising for the development of a reliable qPCR assay for developing degradation profiles useful for estimating time.

During our analysis of RNA-seq results, it was noticed that a number of transcripts demonstrated a preferential disappearance of the sequencing read depth from the 5'end of the transcript. This observation for a number of transcripts suggested an alternate way to follow transcript degradation as an indicator of time. If degradation proceeds from the 5' to 3' end in a predictable way, one could correlate that with the passage of time and, rather than comparing the abundance of one transcript with that of a second (housekeeping gene or ribosomal RNA species), the relative abundance of the 5' and 3' ends of a **single** transcript could be compared. With this approach, stochastic effects during cDNA synthesis should be reduced because of better normalization of qPCR reactions since a single transcript is assayed. The strategy of this approach was to create oligonucleotide primers targeting the 5' and the 3' ends of several transcripts of interest (Figure 1). The abundance of ~90 bp amplicons produced from the 5' and 3' ends of the transcripts (expressed as Ct values) were produced using qPCR with SYBR Green intercalating dye. ΔCt values could thus be calculated by subtracting the Ct from the amplicon produced from the 3' end of the transcript from the Ct of the amplicon produced from the 5' end of the same transcript.

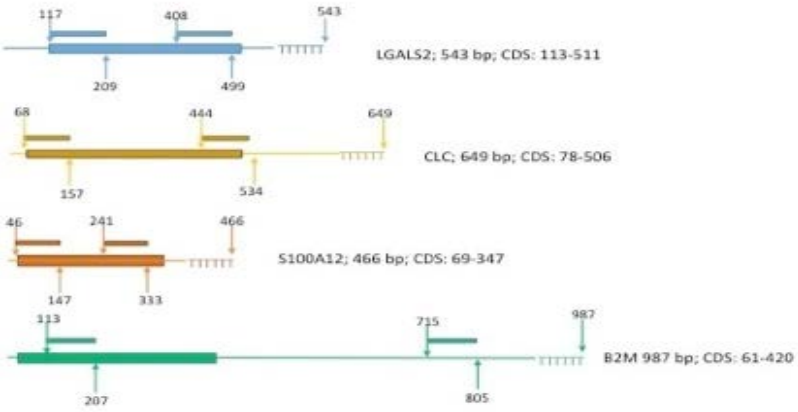


Figure 1. Diagram of primer binding sites targeting transcripts using the 5'-3' qPCR assay in bloodstains stored for up to one year. Heavy solid lines are coding sequence or primers while thin lines represent non-coding regions.

The kinetics of transcript degradation assessed over a storage period of 1 year using the 5'-3' qPCR assay for four transcripts analyzed in detail are shown in Figure 2.

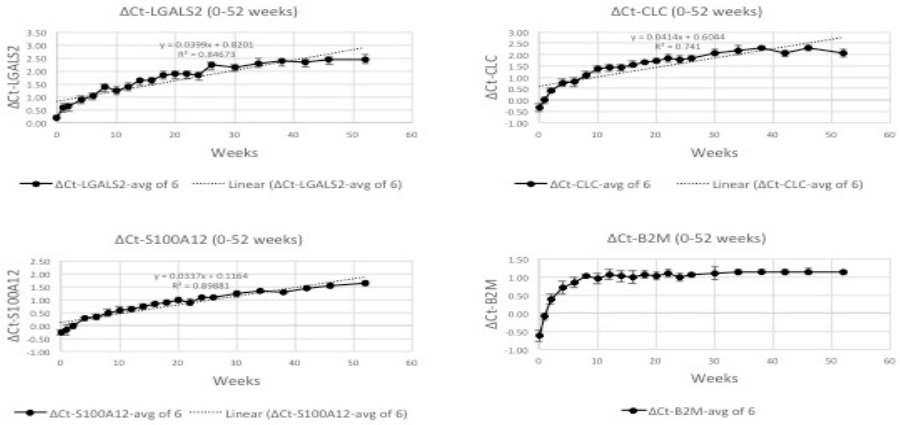


Figure 2. Degradation kinetics for transcripts (expressed as ΔCt) from the *LGALS2, CLC, S100A12*, and *B2M* genes during storage of blood stains for up to 52 weeks. The 5'-3' qPCR assay was used to quantify the abundance of transcripts in dried bloodstains aged at room temperature for up to 52 weeks. Stains from three males and females were studied and shown are the average ΔCt values with standard deviations for each storage time point.

We have explored the degradation kinetics for *LGALS2* in multiple storage experiments and find degradation kinetics are very reproducible from assay to assay as shown in Figure 3 in which Ct values representing numerous data points obtained from experiments performed on different dates are shown.

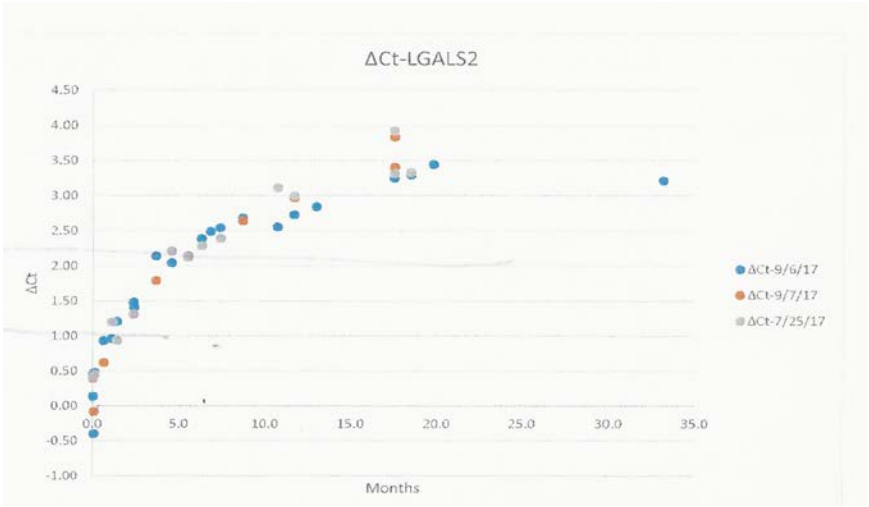


Figure 3. Ct values for *LGALS2* produced using the 5'-3' assay with cDNA prepared from aged stains from different donors and assayed on different dates.

The results in Figure 2 for the *LGALS2* and *S100A12* transcripts are reasonably linear over the year of storage and could therefore possibly be used as a "standard curve" to estimate the age of a blood stain recovered from a crime scene. Statistical analysis of each time point compared to its neighbors was performed using one-way ANOVA with Tukey's post-hoc treatment of the data. Results of that analysis for the 4 markers shown in Figure 2 are summarized in Table 2.

Table 2. Table of significance* among pairwise comparisons of ΔCt values for *LGALS2, CLC, S100A12*, and *B2M* transcripts analyzed using one-way ANOVA with Tukey's post-hoc data treatment.

ΔCt-LGALS2 weeks	0	1	2	4	6	8	10	12	14	16	18	20	22	24	26	30	34	38	42	46	52
0		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
1	.000		1.000	.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
2	.000	1.000		.016	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
4	.000	.001	.016		.801	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
6	.000	.000	.000	.801		.001	.205	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
8	.000	.000	.000	.000	.001		.991	1.000	.067	.010	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
10	.000	.000	.000	.000	.205	.991		.829	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
12	.000	.000	.000	.000	.000	1.000	.829		.280	.062	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
14	.000	.000	.000	.000	.000	.067	.000	.280		1.000	.082	.040	.020	.309	.000	.000	.000	.000	.000	.000	.000
16	.000	.000	.000	.000	.000	.010	.000	.062	1.000		.339	.205	.121	.739	.000	.000	.000	.000	.000	.000	.000
18	.000	.000	.000	.000	.000	.000	.000	.000	.082	.339		1.000	1.000	1.000	.000	.007	.000	.000	.000	.000	.000
20	.000	.000	.000	.000	.000	.000	.000	.000	.040	.205	1.000		1.000	1.000	.000	.016	.000	.000	.000	.000	.000
22	.000	.000	.000	.000	.000	.000	.000	.000	.020	.121	1.000	1.000		1.000	.001	.032	.000	.000	.000	.000	.000
24	.000	.000	.000	.000	.000	.000	.000	.000	.309	.739	1.000	1.000	1.000		.000	.001	.000	.000	.000	.000	.000
26	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.001	.000		1.000	.998	.786	.968	.228	.106
30	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.007	.016	.032	.001	1.000		.671	.154	.403	.014	.004
34	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.998	.671		1.000	1.000	.985	.917
38	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.786	.154	1.000		1.000	1.000	1.000
42	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.968	.403	1.000	1.000		.999	.989
46	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.228	.014	.985	1.000	.999		1.000
52	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.106	.004	.917	1.000	.989	1.000	

This resource was prepared by the author(s) using Federal funds provided by the U.S. Department of Justice. Opinions or points of view expressed are those of the author(s) and do not necessarily reflect the official position or policies of the U.S. Department of Justice.

ΔCt-CLC

weeks	0	1	2	4	6	8	10	12	14	16	18	20	22	24	26	30	34	38	42	46	52
0		.033	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
1	.033		.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
2	.000	.001		.017	.008	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
4	.000	.000	.017		1.000	.016	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
6	.000	.000	.008	1.000		.033	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
8	.000	.000	.000	.016	.033		.486	.086	.106	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
10	.000	.000	.000	.000	.000	.486		1.000	1.000	.558	.037	.008	.000	.001	.000	.000	.000	.000	.000	.000	.000
12	.000	.000	.000	.000	.000	.086	1.000		1.000	.971	.294	.095	.001	.012	.002	.000	.000	.000	.000	.000	.000
14	.000	.000	.000	.000	.000	.106	1.000	1.000		.956	.251	.077	.001	.009	.001	.000	.000	.000	.000	.000	.000
16	.000	.000	.000	.000	.000	.000	.558	.971	.956		1.000	.984	.241	.725	.340	.000	.000	.000	.000	.000	.000
18	.000	.000	.000	.000	.000	.000	.037	.294	.251	1.000		1.000	.951	1.000	.982	.001	.000	.000	.008	.000	.005
20	.000	.000	.000	.000	.000	.000	.008	.095	.077	.984	1.000		.998	1.000	1.000	.007	.000	.000	.040	.000	.025
22	.000	.000	.000	.000	.000	.000	.000	.001	.001	.241	.951	.998		1.000	1.000	.328	.020	.000	.711	.000	.600
24	.000	.000	.000	.000	.000	.000	.001	.012	.009	.725	1.000	1.000	1.000		1.000	.059	.002	.000	.231	.000	.163
26	.000	.000	.000	.000	.000	.000	.000	.002	.001	.340	.982	1.000	1.000	1.000		.231	.011	.000	.586	.000	.472
30	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.001	.007	.328	.059	.231		1.000	.831	1.000	.711	1.000
34	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.020	.002	.011	1.000		1.000	.990	.999	.997
38	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.831	1.000		.458	1.000	.572
42	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.040	.711	.231	.586	1.000	.990	.458		.328	1.000
46	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.711	.999	1.000	.328		.431
52	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.005	.025	.600	.163	.472	1.000	.997	.572	1.000	.431	

ΔCt-S100A12

weeks	0	1	2	4	6	8	10	12	14	16	18	20	22	24	26	30	34	38	42	46	52
0		.994	.008	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
1	.994		.448	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
2	.008	.448		.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
4	.000	.000	.001		1.000	.028	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
6	.000	.000	.000	1.000		.213	.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
8	.000	.000	.000	.028	.213		.978	.613	.015	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
10	.000	.000	.000	.000	.001	.978		1.000	.732	.069	.001	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
12	.000	.000	.000	.000	.000	.613	1.000		.993	.409	.010	.000	.008	.000	.000	.000	.000	.000	.000	.000	.000
14	.000	.000	.000	.000	.000	.015	.732	.993		1.000	.530	.016	.468	.000	.000	.000	.000	.000	.000	.000	.000
16	.000	.000	.000	.000	.000	.000	.069	.409	1.000		.998	.390	.996	.008	.003	.000	.000	.000	.000	.000	.000
18	.000	.000	.000	.000	.000	.000	.001	.010	.530	.998		.998	1.000	.353	.213	.000	.000	.000	.000	.000	.000
20	.000	.000	.000	.000	.000	.000	.000	.000	.016	.390	.998		.999	.997	.981	.025	.000	.001	.000	.000	.000
22	.000	.000	.000	.000	.000	.000	.000	.000	.008	.468	.996	1.000	.999		.409	.255	.000	.000	.000	.000	.000
24	.000	.000	.000	.000	.000	.000	.000	.000	.000	.008	.353	.997	.409		1.000	.653	.005	.144	.000	.000	.000
26	.000	.000	.000	.000	.000	.000	.000	.000	.000	.003	.213	.981	.255	1.000		.818	.011	.255	.000	.000	.000
30	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.025	.000	.653	.818		.929	1.000	.025	.000	.000
34	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.005	.011	.929		1.000	.929	.059	.001
38	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.001	.000	.144	.255	1.000	1.000		.240	.001	.000
42	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.025	.929	.240		.984	.270
46	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.059	.001	.984		.999
52	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.001	.000	.270	.999	

ΔCt-B2M

weeks	0	1	2	4	6	8	10	12	14	16	18	20	22	24	26	30	34	38	42	46	52
0		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
1	.000		.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
2	.000	.000		.003	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000	.000
4	.000	.000	.003		.847	.002	.055	.000	.003	.010	.000	.001	.000	.009	.000	.000	.000	.000	.000	.000	.000
6	.000	.000	.000	.847		.629	.996	.122	.715	.901	.174	.486	.045	.892	.322	.045	.009	.005	.017	.004	.021
8	.000	.000	.000	.002	.629		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	.976	.948	.994	.927	.996
10	.000	.000	.000	.055	.996	1.000		.954	1.000	1.000	.980	1.000	.807	1.000	.998	.807	.451	.352	.593	.307	.647
12	.000	.000	.000	.000	.122	1.000	.954		1.000	.999	1.000	1.000	1.000	.999	1.000	1.000	1.000	1.000	1.000	1.000	1.000
14	.000	.000	.000	.003	.715	1.000	1.000	1.000		1.000	1.000	1.000	.999	1.000	1.000	.999	.954	.911	.985	.881	.991
16	.000	.000	.000	.010	.901	1.000	1.000	.999	1.000		1.000	1.000	.982	1.000	1.000	.982	.821	.731	.911	.681	.935
18	.000	.000	.000	.000	.174	1.000	.980	1.000	1.000	1.000		1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000
20	.000	.000	.000	.001	.486	1.000	1.000	1.000	1.000	1.000	1.000		1.000	1.000	1.000	1.000	.994	.982	.999	.973	.999
22	.000	.000	.000	.000	.045	1.000	.807	1.000	.999	.982	1.000	1.000		.985	1.000	1.000	1.000	1.000	1.000	1.000	1.000
24	.000	.000	.000	.009	.892	1.000	1.000	.999	1.000	1.000	1.000	1.000	.985		1.000	.985	.834	.747	.919	.698	.942
26	.000	.000	.000	.000	.322	1.000	.998	1.000	1.000	1.000	1.000	1.000	1.000	1.000		1.000	.999	.997	1.000	.995	1.000
30	.000	.000	.000	.000	.045	1.000	.807	1.000	.999	.982	1.000	1.000	1.000	.985	1.000		1.000	1.000	1.000	1.000	1.000
34	.000	.000	.000	.000	.009	.976	.451	1.000	.954	.821	1.000	.994	1.000	.834	.999	1.000		1.000	1.000	1.000	1.000
38	.000	.000	.000	.000	.005	.948	.352	1.000	.911	.731	1.000	.982	1.000	.747	.997	1.000	1.000		1.000	1.000	1.000
42	.000	.000	.000	.000	.017	.994	.593	1.000	.985	.911	1.000	.999	1.000	.919	1.000	1.000	1.000	1.000		1.000	1.000
46	.000	.000	.000	.000	.004	.927	.307	1.000	.881	.681	1.000	.973	1.000	.698	.995	1.000	1.000	1.000	1.000		1.000
52	.000	.000	.000	.000	.021	.996	.647	1.000	.991	.935	1.000	.999	1.000	.942	1.000	1.000	1.000	1.000	1.000	1.000	

*Shaded cells in the table represent ΔCt values that are not significant from their neighbors.

Results of the statistical analysis indicate that the "window" of possible error in estimating the age of a bloodstain is about 2-4 weeks depending upon the marker used for the estimate and the length of time a sample has been stored. For stains that have been stored longer, the error associated with an estimate could be greater for markers like *LGALS2* or *S100A12* where the window of possible error would increase to about 6 weeks (Table 2).

The kinetics for *B2M* are especially interesting in that the degradation of the transcript occurs rapidly over the first 6-8 weeks of storage and then levels off for the remainder of the year (Figure 2, Table 2). The reason for these results is unclear inasmuch as there remains in the RNA extract levels of the transcript that are well above the limit of detection for the qPCR assay and the Ct values for both the 5' and 3' ends of the transcript are stable over one year of storage (not shown).

The degradation curve shown in Figure 2 for the LGALS2 transcript was used as a standard curve to estimate the age of blood stains whose age was known but not made available to the scientist conducting the experiment. Age estimates were made assuming a linear model of degradation. Estimated and actual ages for a series of bloodstains are shown in Table 3.

Table 3. Age estimates

Unknowns	Actual age (weeks)	Estimated age with LGALS2 R2=0.8798	Estimated age with CLC R2=0.8007	Estimated age with S100A12 R2=0.8958	Estimated age with B2M
1	5	5	1	9	3
2	20	17	12	20	Not estimated
3	20	18	12	20	Not estimated
4	30	31*	28	36	Not estimated
5	40	34*	30	38	Not estimated
6	40	35*	36	44	Not estimated
7	40	31*	27	41	Not estimated
8	49	38*	33	46	Not estimated

* Recall that by 24 weeks the degradation curve for the *LGALS2* transcript has begun to level off indicating that mRNA fragments from the 5' end of the transcript are sufficiently low in abundance to challenge the detection threshold for qPCR technology. The window of possible error in age estimates will therefore be higher for more aged stains.

As is evident in Table 3, the accuracy of age estimates based upon the degradation kinetics of the different markers varies significantly. For example, *LGALS2* estimates the age of stains up to about 24 weeks of storage with reasonable accuracy, but thereafter becomes less accurate because the slope of the degradation curve begins to decrease. However, the *S100A12* marker, which appears less accurate at shorter storage times, produces more accurate estimates of sample age in older stains. The utility of the *B2M* marker may be in estimating the age of stains stored for very short times (perhaps in days or even hours). Thus a multiplex qPCR assay of the 5'-3' type utilizing Taqman technology with probes fluorescing different colors may afford a crime laboratory the greatest dynamic range for estimating the age of evidentiary stains accurately.

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We have adapted Taqman methods for use with the 5'-3' qPCR assay in comparison to the SYBR Green intercalating dye method used to produce the results discussed thus far. Shown in Figure 4 are degradation curves for the LGALS2 transcript produced using the two methods.

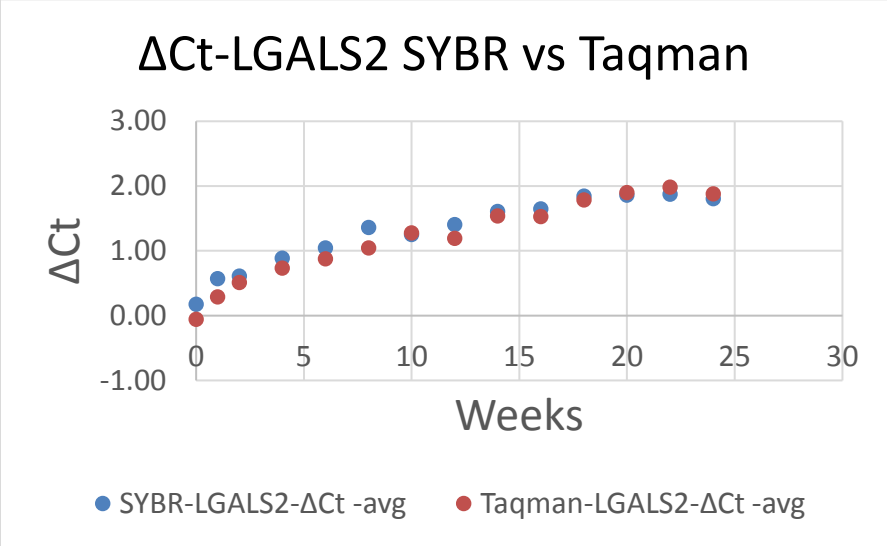

Figure 3. Kinetics of degradation of the LGALS2 transcript produced using the Taqman methodology or fluorescence associated with SYBR Green dye intercalation. The 5'-3' qPCR assay was used to produce degradation kinetics for the *LGALS2* transcript. Shown are ΔCt values representing the relative abundance of the 5' amplicon minus the abundance of the 3' amplicon produced using either SYBR green dye or fluorescence associated with Taqman probes designed to hybridize to the 5' or 3', ~90 bp amplicon produced during qPCR.

Results show little if any difference in the curves produced using the two methods and suggest the feasibility of designing a Taqman assay in which multiple transcript markers could be assayed simultaneously using the 5'-3' qPCR methodology.

Proposed studies for the future.

The two proposed goals of this project have been completed. Now that we have a good understanding of mRNA degradation in body fluid stains stored under ideal conditions, future research will investigate the effects of the environment on this process. In future experiments, stains will be stored under conditions in which temperature, humidity, sunlight exposure, and oxygen tension will all be varied. In addition, creating stains on different substrates will also be performed to assess the substrate effects on degradation kinetics. A project proposal to

continue these and other studies is planned for 2018 in hopes of securing support. Ultimately, we believe it will be possible to use mRNA degradation in forensic stains to estimate their age. Moreover, we believe it will be possible to develop a qPCR kit available commercially that can be used for this purpose in a crime laboratory. Questions concerned with how long evidence has been at a crime scene do occur during the investigation of a crime and the 5'-3' assay promises to be able to provide answers. This assay may also be able to help estimate the age of human remains.

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