Quantitative PCR analysis of bloodstains of different ages

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ABSTRACT

An accurate method to estimate the age of a stain or the time since deposition (TsD) would represent an important tool in police investigations for evaluating the true relevance of a stain. In this study, two laboratories reproduced an mRNA-based method for TsD estimation published by another group. The qPCR-based assay includes four transcripts (B2M, LGALS2, CLC, and S100A12) and showed preferential degradation of the 5′ end over the 3′ end. In this study, the blood-specific marker ALAS2 was added to examine whether it would show the same degradation pattern. Based on our qPCR data several elastic net models with different penalty combinations were created, using training data from the two laboratories separately and combined. Each model was then used to estimate the age of bloodstains from two independent test sets each laboratory had prepared. The elastic net model built on both datasets with training samples up to 320 days old displayed the best prediction performance across all test samples (MAD=18.9 days). There was a substantial difference in the prediction performance for the two laboratories: Restricting TsD to up to 100 days for test data, one laboratory obtained an MAD of 2.0 days when trained on its own data, whereas the other laboratory obtained an MAD of 15 days.

1. Introduction

A DNA profile provides information on the identity of the donor of a biological stain detected at a crime scene, but it is not informative on how or when the stain was deposited. The victim and suspect may have had previous contacts and several opportunities for the deposition of biological material. In these cases, a method to determine the time since deposition (TsD) of a biological stain would be of great assistance. Over the years several approaches have been tested in search of suitable methods for TsD estimation, such as spectroscopic measurements of bloodstains of different ages [1], changes in the microbiome composition over time [2–4], and time-dependent degradation of RNA [5–7].

Although mRNA is known for its rapid postmortem and in vitro decay because of the ubiquitously present RNAses [8], several studies found surprisingly high stability of RNA molecules under controlled conditions. For example, one study reported successful RNA analysis from a 23-year-old blood stain [9]. The use of mRNAs as markers to determine the presence of a specific body fluid in a crime scene stain has been thoroughly investigated for the past decades [10–13]. Several forensic laboratories are implementing these techniques in their forensic casework [14]. Already in the early investigations of RNA analysis in

forensics, Bauer et al. [15] demonstrated the temporal degradation of RNA as a potential method to determine TsD. In crime scene stains, the amount of biological material originally deposited is not known; hence measuring the presence of a target where the amount is expected to decrease over time will not by itself provide useful information. Instead, Bauer et al. [15] proposed analyzing two different targets, one that is expected to be stable over time and one that is degrading. By analyzing two different targets within the housekeeping gene β-actin, one located at the 3′ end and one located at the 5′ end, they were able to detect a time-related difference [15]. The method also has the advantage that it reduces inter- and intra-personal variance as it is expected that the same amounts of both targets were present when the stain was deposited at time point 0. This strategy has later been developed further by other groups. Anderson et al. examined the relative degradation of β-actin mRNA and 18 S rRNA by qPCR in blood samples [5]. A time frame of 150 days was inspected, and a linear degradation of both of these housekeeping genes was observed, whereas 18S rRNA was more stable than β-actin mRNA. This proof of concept was refined [16] in that the authors investigated the temporal degradation of two differently sized targets within the same RNA molecules and found that the larger fragment disappeared faster than the shorter fragment. More recently, Fu and

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Allen [17] suggested four RNA transcripts for TsD estimation (LGALS2, CLC, S100A12, and B2M). They reported that the targets located at the 5′ end of the transcripts degraded faster than the ones at the 3′ end. They hypothesize that chemical hydrolysis reactions might be responsible for the breaking of RNA molecules and consequently lead to different rates of degradation at the 5′ and the 3′ ends [18]. Thus, a ratio between the 5′ and the 3′ target was used to correlate with the age of the stain. By statistically analyzing the degradation curve of each marker, the authors determined that the TsD of bloodstains could be estimated within 2–4 weeks for stains less than six months old and within 4–6 weeks for older bloodstains. Approaches to adjust for changes in temperature and humidity for this panel have been investigated [19]. Another strategy to estimate TsD was to analyze a ratio for temporal changes in two different RNAs (e.g. β-actin and 18S rRNA) [5,6,20–22]. In addition, some miR-NAs [6,20,23,24] and circRNAs [25] have been shown to degrade at a slower rate than mRNA targets. Moreover, the whole transcriptome has been analyzed for time-dependent changes in different body fluids [7, 26,27]. So far, no reliable method for TsD estimation in any of the forensically relevant body fluids has come forth, but promising approaches exist.

This study is a cooperation between two laboratories, the Section of Forensic Genetics at Oslo University Hospital (Laboratory 1) and the Zurich Institute of Forensic Medicine (Laboratory 2). We investigated if the method to estimate TsD of blood stains presented by Fu and Allen [17] could be reproduced, and if a similar pattern would be observed by targeting the 5′ and 3′ ends of the blood-specific marker Aminolevulinate synthetase 2 (ALAS2). ALAS2 was chosen since it is a generally recognized blood marker, well-described in the literature, and relatively stable. ALAS2 is moderately expressed in human blood, but not as highly expressed as the genes HBB or HBA encoding the beta/alpha hemoglobin chains, which would be challenging to include in a multiplex. The protocols have been adapted from Fu and Allen [17], with in-house modifications regarding sample collection, storage, extraction methods, and instrumentation. In addition, we used machine learning modeling to predict the age of bloodstains.

2. Materials and methods

This study was approved by by the data protection officer at Oslo University Hospital (case number 22/05747) and by the local ethics committee in Zurich (declaration of no objection KEK-No. 24-2015). All participants gave informed consent prior to participating in the study. The MIQE guidelines [28] were followed for qPCR experiments when possible.

2.1. Sample collection

In laboratory 1, blood samples were collected by finger prick from five volunteers. Parallels of 10 μl blood were pipetted directly from the finger onto clean (washed at 60 °C and UV irradiated on each side for 30 min) pieces of fabric (1.5 ×1.5 cm). The sample collection was repeated once from the same participants to obtain more samples. The samples were stored in ventilated plastic containers, at room temperature (approx. 22 °C) and exposed to some artificial light during daytime until further processing. Extractions were performed on fresh stains (1–2 h after deposition), and then after 2, 16, 28, 42, 54, 210, and 310 days (only 4 samples at 210 and 310 days), in total 38 samples. Test samples not included in the training data were extracted from the same sample pool at different time points (74, 277, and 357 days). In addition, samples from a separate participant extracted at 1, 21, 1100, and 1200 days were included.

Laboratory 2 used RNA extracts from a former study on the degradation of human mRNA transcripts over time as an indicator of TsD [7]. For this study, blood had been obtained by venipuncture and collected in EDTA-coated tubes. Blood was collected from three different individuals. Samples were prepared by pipetting 50 μl of blood directly on

sterile cotton swabs (Milian) and the blood stains were subjected to two different environmental conditions. Indoor samples were placed in a dark dry space at room temperature, while outdoor samples were positioned on a flat rooftop, where they faced sun, wind, etc., but were protected from rain. Fresh samples (day 0) were directly analyzed upon collection. For the other samples, analysis proceeded after one day, 7 days, 4 weeks (28 days), 6 months (182 days), one year (364 days), and 1.5 years (551 days). In total, 42 RNA extracts (21 for indoor conditions, and 21 for outdoor conditions) were analyzed that had been stored for approx. 2 years at -80 °C. In addition, eleven test samples were collected and aged for a variety of TsDs (1, 1, 3, 14, 21, 24, 25, 32, 32, 85, and 2310 days). The test samples were prepared by pipetting 50 μl of blood, collected from different persons that have not contributed to the long-term time series experiment on a sterile swab. The samples were stored at room temperature (approx. 22 °C) without direct UV-light exposure until further processing.

2.2. RNA extraction and reverse transcription

In Laboratory 1, RNA was extracted with the ReliaPrep™ RNA Tissue Miniprep System (Promega) using the Non-Fibrous Tissue protocol and the following pretreatment: the stains were placed in Eppendorf tubes and 250 μl of LBA+TG buffer was added and incubated at 25 °C for 10 min at 600 rpm. The fabric and liquid were transferred to a DNA IQ™ spin basket, placed in a new Eppendorf tube, and centrifuged at 15,000 rpm for 1 min. One negative control was included in each extraction.

In Laboratory 2 the RNA from the long-term time series experiment had been extracted as stated by Salzmann et al. [7]. Briefly, the RNA was extracted using the ReliaPrep™ RNA Cell Miniprep kit (Promega) according to the protocol for > 5×10^5 to 2×10^6 cells with the following modifications: Stains were incubated in BL + TG buffer for 2–3 h and transferred to NAO™ Baskets before the protocol was followed. The final elution volume was 30 μl. The test samples were extracted either with the ReliaPrep™ RNA Tissue Miniprep System (Promega) with the same adaptation as mentioned above (samples B1- B5, B10-B11) or with the mirVana™ miRNA Isolation kit (Invitrogen) (samples B6 – B9). The manufacturer's protocol was followed (using 450 μl of lysis buffer initially) with the following modifications: For the first washing step, 500 μl of miRNA wash solution 1 was applied to the filter cartridge, and the RNA was eluted in 60 μl of DPEC H_2O.

All RNA extracts from laboratory 2 and the samples at the first 6 timepoints from laboratory 1 were quantified using the QuantiFluor® RNA HS System (Promega) on a Quantus™ Fluorometer (Promega) according to the manufacturer's instructions. However, the RNA quality and integrity were not checked, and no testing for PCR-inhibiting factors was carried out. If the samples were extracted using the ReliaPrep™ RNA Cell Miniprep kit (Promega), a DNase treatment was already performed during the extraction because it is part of the protocol. In addition, all RNA extracts were treated with a second DNase treatment employing the TURBO DNA-free Kit (Thermo Fisher Scientific) according to the protocol. The reverse transcription of all RNA extracts was performed using SuperScript™ IV VILO™ (Thermo Fisher Scientific) according to the protocol, with 10 μl of the RNA extract added to the reaction in a total volume of 20 μl. RT minus controls were included in Laboratory 1 to detect possible genomic DNA contamination: 2 μl of RNA were combined with 4 μl of SuperScript IV VILO No RT control in a total volume of 20 μl. No RT minus controls for the time series samples of Laboratory 2 could be obtained since there was not enough RNA extract available. However, Laboratory 2 included RT minus controls for the test sample set. cDNA and RT minus controls were then stored at -80 °C in Laboratory 1 and at -20 °C in Laboratory 2.

2.3. qPCR assay

To measure mRNA transcript quantity and the relative abundance of the 5′ and 3′ targets for each transcript a qPCR assay was set up. The

qPCR primer sequences for the transcripts LGALS2, CLC, S100A12, and B2M were taken from Fu and Allen [17]. For ALAS2 new primer pairs located at the 3′ and 5′ ends of the transcript were designed and their specificity was checked using the NCBI primer-blast tool. The reverse primers of both the 3′ and the 5′ targets spanned an exon-exon junction. All primer sequences are listed in Table 1.

Real-Time qPCR was performed with the PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific) on a 7500 Real-Time PCR System (Thermo Fisher Scientific). The qPCR reaction was done according to the protocol in a 10 µl reaction, with 5 µl PowerUp™ SYBR™ Green Master Mix, 1 µl primer mix (f+r, diluted to 5 µM), 1 µl cDNA, and 3 µl H_2O. The recommended cycling conditions were as follows: 50 °C for 2 min, 95 °C for 2 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a continuous melt curve ramp increment. C_q values were determined manually using a threshold of 0.1 and an automatic baseline. One qPCR negative control per target was included in each run.

The melting curve analysis was performed to check the specificity of the qPCR assay since it is an easy and sensitive method that may be used to identify artifact amplification [28,29]. It is more sensitive than size fractionation by gel electrophoresis, for the melting temperature of a DNA fragment is given by its size, nucleotide sequence, composition, and reaction conditions [30]. DNA-binding dye such as SYBR Green will be released upon DNA denaturation resulting in a decrease in fluorescence. The negative first derivative of the melting curve depicts the temperature at which the examined DNA fragment denatures fastest. The temperature at which the negative first derivative peaks approaches the melting temperature (T_m), which describes the temperature at which 50% of the DNA exists as double-stranded and 50% as single-stranded. Therefore, a single amplification product will generate one peak only at the product's melting temperature. Multiple peaks in a melt curve denote the presence of non-specific amplification products and/or primer dimers [31].

2.4. Statistical analysis

Statistical analyses were performed on the indoor samples from both laboratories. In the first part of the analysis, we did a simple regression analysis to explore the association between TsD and Delta C_q (ΔC_q). This was carried out for each marker using training data from each laboratory and also combined. Statistical testing of the association was conducted by calculating p-values and R^2 values from the regression model.

In the second part of the analysis, machine learning models for TsD predictions were created using the elastic net (EN) model implemented in the glmnet R-package (v4.1–6) with the argument "alpha=0.5" [32], using the marker-specific Delta C_q (ΔC_q) measurements as explanatory variables. We built models using the training data within each laboratory separately or combined. The regularized regression method elastic net estimates linear models using both lasso and ridge regression penalties, allowing for variable selection and utilizing dependency between the explanatory variables. The model penalty was estimated using two different measures of errors: Mean Square Error (MSE) or Mean absolute error (MAE) employing the in-built cv.glmnet function where leave-one-out cross-validation was performed. Subsequently, a fixed value of the penalty was used to fit the model: Either the "min" penalty or the "1se" penalty was used. Hence each model is assigned as a combination of selected error measurement and penalty type. The importance of each marker was quantified based on the estimated coefficients where the explanatory variables were standardized before doing the model fit. We required the samples to have at most one missing marker, and these values were imputed as "column means" using the makeX function from the glmnet library. We investigated the performance of the EN model with independent test sets not used in the training of the model. A negative prediction of TsD was restricted to zero days (TSD=0).

3. Results

3.1. qPCR results

In Laboratory 1 only one extraction negative control obtained C_q values, all were higher than for ordinary samples (range 33–38). All RT minus reactions and all qPCR negative controls were negative (C_q >40). C_q values were detected for all markers in all samples except for the LGLAS2 3′ in two samples at day 42 and one sample at 310 days. The C_q values increased for the samples collected up to 42 days, while a small decrease was detected in the samples at 210 days.

In Laboratory 2 no extraction negative controls were available from the long-term time series experiment conducted by Salzmann et al. [7]. RT minus controls were only prepared for the independent test sample set. Mainly for the markers CLC and B2M, C_q values were sporadically detected, either in the 3′ or the 5′ target (all C_q values above 35, except for B2M 3′ in B5 (28.3)). Not all of the qPCR negative controls were negative, but they displayed high C_q values (>32.5). C_q values were detected for almost all markers in indoor and outdoor samples, however, the detection was less reliable for outdoor samples (Fig. 1). Fig. 1 shows in how many samples the C_q values could be measured for each target.

Table 1
Targets included in the qPCR analysis and the sequences of the corresponding primers.

| Marker Name	Reference Sequence	Primer forward (5′ to 3′) Primer reverse (5′ to 3′)	Size (bp)	Reference
S100A12 5′	NM_005621.1	GGGGTTAACATTAGGCTGGGA TGTCAAAATGCCCCTTCCGA	102	[15]
S100A12 3′		TCCAAGGCCTGGATGCTAATC TGTGGTAATGGGCAGCCTTC	93	[15]
LGLAS 5′	NM_006498.2	CGGGGGAACTTGAGGTTAAGA TTACAAAGCCATCAGTGCCATC	93	[15]
LGLAS 3′		ATGGGCACGAGCTGACTTTT CTTGAAAGAGGACATGTTGAACCC	92	[15]
CLC 5′	NM_001828	GGAGACAACAATGTCCCTGCT AGTGGTCGCCCTTTGATTGTC	90	[15]
CLC 3′		ATGGTGCAAGTGTGGAGAGAT AGGGATTCCTTGGCAACATGA	91	[15]
B2M 5′	NM_004048.2	TGGAGGCTATCCAGCGTACT CCCAGACACATAGCAATTCAGG	95	[15]
B2M 3′		TCTTCAATCTCTTGCACTCAAAGC TCCCCCAAATTCTAAGCAGAGT	91	[15]
ALAS2 5′	NM_000032.5	AGAGGTTGTTTTGTGAGGACTT GGGACAGCGTCCAATACCAA	149	This study
ALAS2 3′		TGATGGAAGATTTTGTGGAGAAGC CCCCATGTTCCCGAAGTAGG	155	This study
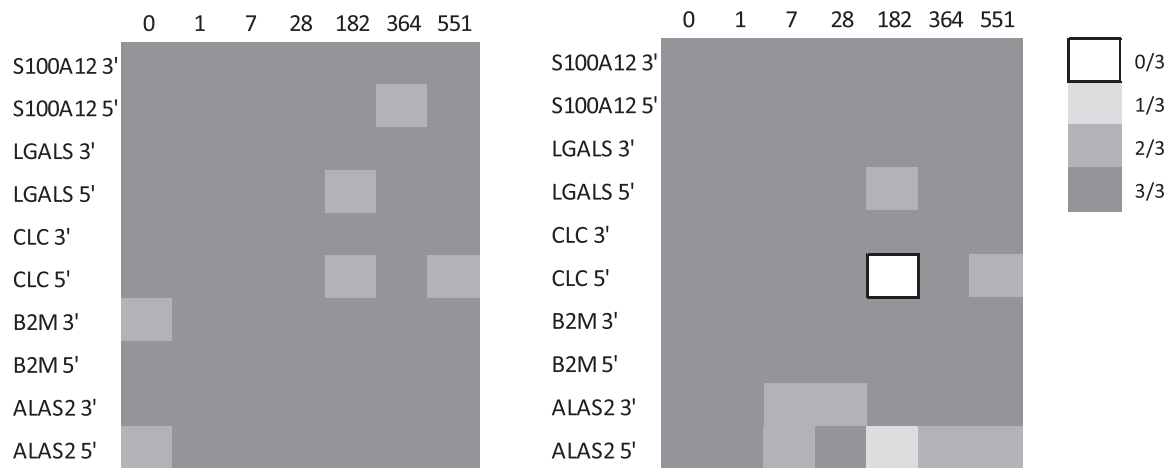
Fig. 1. Successful qPCR analysis for the long-term time series experiment of Laboratory 2. The different shades of grey to white represent the number of samples where C_q values were obtained, displayed by targets. The figure is divided into indoor samples on the left and outdoor samples on the right.

The remaining samples showed C_q values > 40. All C_q values can be found in the Supplementary Material (Supplementary Material, Table S1).

In Fig. 2, C_q values for each target in each transcript are displayed for both laboratories. Measured C_q values for the respective targets are comparable between the two laboratories.

3.2. Melt curve analysis

Laboratory 1 observed single peaks in the melting curves except for the occurrence of an additional peak at a lower temperature for the LGALS 5′ target in 7/38 samples. The occurrence was random in regards to samples and time points. In Laboratory 2, some of the melt curves indicate the presence of either primer dimers or non-specific amplification, since peaks at lower melting temperatures were observed. The melt curves of the targets in B2M (Supplementary Material, Fig. S1), S100A12 and CLC 3′ (data not shown) did not depict any signs of non-specific amplification. However, primer dimers seem to be an issue in the LGALS2 5′ and the ALAS2 5′ targets (Supplementary Material, Fig. S1). Mostly the older samples (time points 364d and 551d) show signs of primer dimers and non-specific amplification, more often in, outdoor than in indoor samples (Supplementary Material, Fig. S1).

3.3. Regression analysis per marker

For both data sets, the ΔC_q values were calculated to investigate the hypothesis of a faster degradation at the 5′ end of the transcript than at the 3′ end, as suggested by Fu and Allen [17]. Calculations of the ΔC_q values were performed by subtracting the C_q value measured for the 3′ target from the C_q value measured from the 5′ target (Supplementary Material, Fig. S2). In addition, the average ΔC_q value was plotted against time to capture the overall trend of the ΔC_q values (Supplementary Material, Fig. S3).

Moreover, a linear regression was conducted to examine the correlation between ΔC_q and time per marker. The regression analysis was performed on the data set of each laboratory separately first (Fig. 3, columns 1 and 2). Significance for the markers analyzed and corresponding R^2-values were recorded (Table 2, significance level: $p < 0.05$). In addition, short-time TsD (up to 54 days) was considered separately (Supplementary Material, Fig. S4). Only CLC displayed a correlation between ΔC_q and time ($p = 0.04$) for Laboratory 1, while no correlations ($p < 0.05$) were observed in the Laboratory 2 indoor data, Table 2. To increase the amount of data in the short-term setting the data from both laboratories were combined up to 54 days (0, 1, 2, 7, 16, 28,

42, and 54 days) and subjected to regression analysis. The results of the short-term regression analysis on the combined data set are shown in Fig. 3 (column 3) and Table 2.

A significant correlation ($p < 0.05$) between ΔC_q and time was observed for all markers except ALAS2 for Laboratory 1 and all markers except ALAS2 and LAGLS2 for Laboratory 2. The trend of differential degradation between the 3′ and 5′ ends appears less obvious in the 551d samples from Laboratory 2 (Fig. 3). On the short time scale, there was a significant correlation with time for S100A12 and CLC when the data was combined.

As for the outdoor time series from Laboratory 2, ΔC_q values that could be obtained per time point showed even more variation than in indoor conditions. A regression analysis was performed on the qPCR data of the full time series that revealed a significant correlation for CLC only in outdoor conditions (Supplementary Material, Fig. S4). Regarding the short-time setting, a significant correlation between the degradation pattern of the markers CLC and LGALS2 was observed (Supplementary Material, Fig. S5). Since considerable inter-individual variation occurred in the outdoor data, in addition to the melt curves showing signs of non-specific amplification, we decided to not further analyze the outdoor data. Moreover, C_q values could not be obtained for all the targets examined.

3.4. Analysis using machine learning model

For the analysis based on the machine learning model, the data from the outdoor samples from Laboratory 2 was excluded. The ALAS2 result for sample B2 for Laboratory 1 was removed, due to being a clear outlying data point. The importance of each marker for the elastic net model can be seen in Table 3. Overall, the MSE and MAE model penalties gave quite similar importances, and the two models selected the same markers. For Laboratory 1, we found that mostly all markers are important, but to various degrees, whereas this was not the case for Laboratory 2. The importance of CLC was overall higher than for the other markers. LGALS2 was the additional marker included for a less strict model ("Min"), with only a small importance (5–6%). For Laboratory 2, the marker CLC was the only marker of importance when a stricter model penalty was applied ("1se"), whereas LGALS2 and S100A12 were also included for the less strict model ("Min"), however with small importance ($<15\%$). When combining the two datasets we obtained an "in-between dataset model", including the markers S100A12 and B2M as less important compared to CLC.

Table 4 summarizes the estimated TsD for the independent test samples of the models using different penalties. The top row of Table 4

Fig. 2. C_q values measured for indoor samples for each target in all five transcripts for Laboratory 1 (red) and Laboratory 2 (blue). *(For interpretation of the references to color in this figure, the reader is referred to the online version of this article).*

shows the training data used for each model (penalty combination). An overview of the Mean absolute deviation (MAD) scores is given in the Supplementary Material (Tables S2 and S3).

The model based on Laboratory 1 training data performed very well for Laboratory 1 test samples, the lowest overall MAD observed was 2 days (model penalty MAE/1se, all markers being important except LGALS2, Table 4, Supplementary Material Table S2, and S3). Interestingly, we observed that a model which also included LGALS2 obtained better prediction for test samples with larger TsD (beyond 100 days, based on model penalty "Min"). For the prediction of both test datasets together, the best-performing model gave an MAD of 11.5 days based on Laboratory 1 training data only (model penalty MSE/Min, Tables S2 and S3).

When including all time points in the Laboratory 2 training data, the predictions for the Laboratory 2 test samples were poor with an MAD of 137.8 days. The Laboratory 2 test samples were best predicted with the Laboratory 1 training data with an MAD of 39.9 days (Supplementary Material, Table S2). A restriction of including samples only with TsD up to 320 drastically improved the performance of the models for Laboratory 2. The restriction only affected Laboratory 2 and combined predictions, as Laboratory 1 did not include any training samples with TsD above 320 days. The best prediction performance for Laboratory 2 was an MAD of 15 days where the markers CLC, LGALS2, and S100A12 were included (using model penalty MSE/Min, Supplementary Material, Table S3).

4. Discussion

In this study, we investigated the reproducibility of the TsD estimation method suggested by Fu and Allen [17] using the four markers B2M, LGALS2, S100A12, and CLC in blood. In addition, we tested the blood-specific marker ALAS2 for differential transcript degradation at the 5′ and 3′ ends. To do so, we examined the degradation pattern in aging bloodstains analyzed in two different laboratories. Correlations with time were assessed for each marker using linear regression. Statistical models based on machine learning techniques incorporating
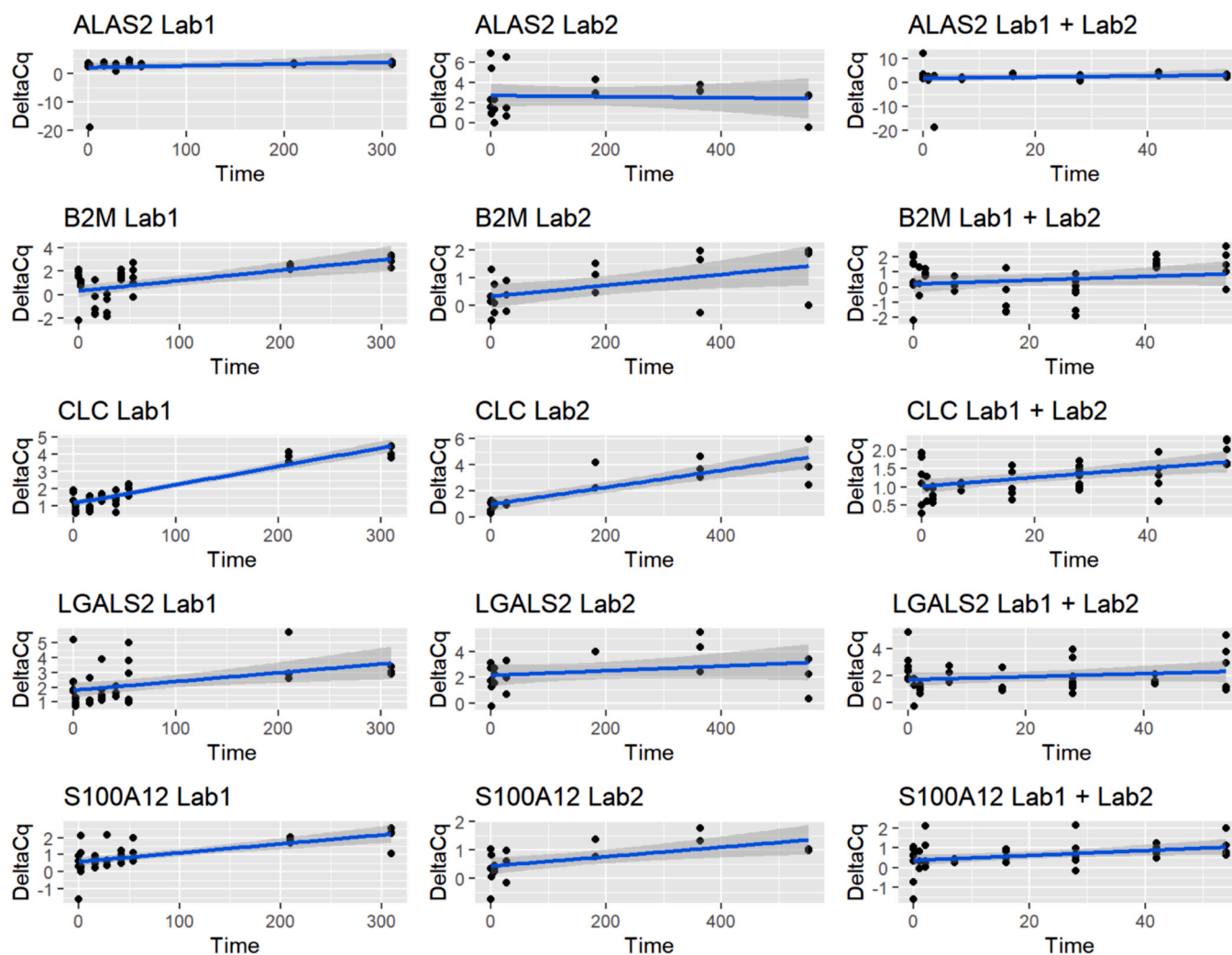
Fig. 3. Scatterplot of the ΔC_q values (natural logarithm) over time in Laboratories 1 and 2 (indoor setting only) and the combined data sets in a short-term setting (up to 54 days), including regression lines.

Table 2
p-values and R^2-values of the long-term regression analysis for indoor data of each laboratory and the short-term regression analysis (data combined up to 54 days).

Marker	Lab1	Lab1	Lab2	Lab2	Lab1 + Lab2	Lab1 + Lab2
	p-values	R^2-values	p values	R^2 values	p-values	R^2 values
ALAS 2	0.3	0.03	0.28	0.02	0.44	0.024
B2M	0.0001	0.34	0.028	0.22	0.23	0.041
LGALS2	0.008	0.2	0.17	0.06	0.25	0.03
S100A12	$7.8e^{-6}$	0.43	0.01	0.30	0.02	0.14
CLC	$3.94e^{-16}$	0.84	$2.03e^{-06}$	0.72	0	0.22

multiple markers were built using different datasets. Data from separate laboratories or combined were used for 1) building the model (train) or 2) investigating the performance of the model (test).

While Fu and Allen [17] analyzed bloodstains over regular TsD intervals (every two weeks for the first 24 weeks of storage, then every four weeks up to week 52), the current dataset from Laboratory 1 contained frequent measurements for up to 54 days followed by additional measurements at 210 and 310 days. The first period was chosen since it showed a rather strong increase in ΔC_q values for some markers in the study of Fu and Allen [17], who reported that "for B2M the ΔC_q value rises rapidly up to about 8 weeks of storage and then levels off rising only slightly over the course of one year of storage". In the current study, additional samples with larger TsD were included later on, since the

preliminary results showed fewer changes in ΔC_q than expected. Laboratory 2 focused on a longer time frame, and analyzed seven time points during 1.5 years, expecting to see considerable changes between the more spaced-out time points.

The melt curve analysis showed some nonspecific amplification and primer dimers for some of the targets for Laboratory 2 and LAGALS2 5' for 7 samples from Laboratory 1. Amplification artifacts in qPCR experiments are a frequently discussed topic in the literature, nevertheless, why they arise is not entirely clear. It is evident though that the absence of template DNA or low-template input may lead to low melting artifacts, most likely primer dimers [33]. Very low target concentration increases the probability of nonspecific product formation [33,34]. Moreover, the occurrence of PCR artifacts depends on primer

Table 3
Importance of markers given as a percentage (%) tested with different "model penalties" and training data sources (TsD up to 310d).

Training Data	Model	ALAS2	B2M	CLC	LGALS2	S100A12
Lab1	MSE/Min	11.9	1.1	63.4	5.8	17.8
Lab1	MAE/Min	12.1	1.2	63.6	5.3	17.9
Lab1	MSE /1se	10	2.2	70.2	0	17.7
Lab1	MAE/1se	6.1	2.4	74.9	0	16.6
Lab2	MSE/Min	0	0	77.6	14.9	7.5
Lab2	MAE/Min	0	0	80.2	13.5	6.4
Lab2	MSE /1se	0	0	100	0	0
Lab2	MAE/1se	0	0	100	0	0
Lab1 + Lab2	MSE/Min	0	7.5	77.8	0	14.7
Lab1 + Lab2	MAE/Min	0	6.9	77.6	0	15.5
Lab1 + Lab2	MSE /1se	0	6	78.4	0	15.5
Lab1 + Lab2	MAE/1se	0	4	80.9	0	15.1

concentrations and the time for setting up the qPCR experiment. C_q values of reaction wells that produced specific amplicons are affected by the concentration of non-template cDNA, in that a minimal amount of carrier DNA is essential for a successful quantification [33]. Although both laboratories experienced issues regarding the qPCR amplification process, they were more prevalent in Laboratory 2. For one, it might be expected that lower amounts of RNA can be recovered from older samples, as Laboratory 2 sampled over a longer period. Additionally, a storage time of approximately 2 years at -80 °C of the RNA extracts might have affected the RNA integrity and quality of the time series samples as well. Generally, artifact amplification should be circumvented by assay design and optimization [31], and validation and optimization of novel primer designs are necessary to examine specificity and sensitivity [34]. Nonetheless, since Laboratory 1 did not observe the same issues as Laboratory 2, and it was not systematically prevalent for all samples or all targets, we propose the matter is more likely connected to the samples analyzed than the primer design or the experimental setup. However, the assay might benefit from a future re-design of the LGALS2 5′ target, since Laboratory 1 also observed occasional non-specific amplification.

A rather large inter-individual variability in ΔC_q values was observed by both laboratories and also the course of the ΔC_q over time curves was not as conclusive. For freshly extracted bloodstains (0d), one would expect that ΔC_q values are close to zero since no preferential degradation of the mRNA should have taken place. Nevertheless, a rather large variation in ΔC_q values at timepoint 0 was observed by both laboratories. The timepoint 0 samples in the study by Fu and Allen were extracted at 16 h while the current study extracted samples 0–2 hours after deposition (thus not completely dry). For some of the markers, we observed a drop in ΔC_q values in the 1d/2d samples indicating a change in the dried samples. After 6 months and up to one year, a larger change in ΔC_q values can be observed in the results from Laboratory 2 before a drop in the ΔC_q values can be noticed after one year in indoor and outdoor samples. Salzmann et al. [7], who provided the RNA extracts of the long-term time series experiment, described difficulties with the identification of putative markers displaying long-term stability or a steady degradation. Besides, no transcript with a significant resilience to degradation beyond the 1-year mark was found.

The results of Laboratory 1 revealed a correlation ($p < 0.05$) between ΔC_q values and TsD for all markers except ALAS2 in a long-term setting (up to 310d). In Laboratory 2, correlations ($p < 0.05$) could be detected for the markers CLC, B2M, and S100A12 in indoor conditions in up to 1.5 years old samples. The blood-specific marker ALAS2 showed no correlation with TsD. The primers for the ALAS2 markers amplified longer sequences than for the other markers. In addition, ALAS2 is a longer transcript and the 3′ and 5′ targets are located further apart (127–256 and 1523–1658) than in the other markers. ΔC_q values of B2M increased over time for both laboratories, however, not as much as for some of the other transcripts and some time points displayed large variations between samples. Thus, this marker was not strongly correlated with time. Likewise, Fu and Allen [17] observed differences in the degradation kinetics of the inspected markers. Nonetheless, it was the transcript B2M that differed from the rest in that it displayed signs of degradation mostly over the first 6–8 weeks, but then the curve flattened (up to week 52). The authors proposed a balancing of the degradation of the 5′ and 3′ ends after 8 weeks, thus the ΔC_q values became stable.

In the outdoor samples, C_q values could not be obtained for all markers, probably due to stronger degradation in the targets in outdoor conditions and the uncontrolled environment the samples were exposed to. Moreover, inter-individual variation was even bigger than for indoor samples (Supplementary Material, Fig. S1), hence emphasizing the unpredictable effect on transcript stability and degradation of

Table 4
Predicted age in days for test samples of Laboratory 1 (A1 to A11) and Laboratory 2 (B1 to B11) based on all different model penalties (model). Predictions closest to the true TsD are bold and underlined.

ID	True TsD	Lab1 MSE/Min	Lab1 MAE/Min	Lab1 MSE/1se	Lab1 MAE/1se	Lab2 MSE/Min	Lab2 MAE/Min	Lab2 MSE/1se	Lab2 MAE/1se	Lab 1 + 2 MSE/Min	Lab 1 + 2 MAE/Min	Lab 1 + 2 MSE/1se	Lab 1 + 2 MAE/1se
A1	1	0	0	0	0	18.86	19.76	25.59	28.75	0	**1.1**	6.09	12.62
A2	1	**0**	**0**	**0**	**0**	4.51	6.89	20.61	25.72	27.99	27.72	24.51	17.21
A3	1	**12.53**	12.63	29.43	38.9	26.85	26.61	28.12	30.3	91.42	84.66	76.39	61.41
A4	21	14.47	14.35	17.9	**22.23**	25.61	26.61	31.91	32.61	13.74	19.6	23.57	29.09
A5	21	15.36	14.83	17.34	23.91	23.5	24.42	30.45	31.72	**21.18**	26.58	29.77	33.79
A6	74	76.14	**75.76**	70.99	69.94	48.36	47.56	42.72	39.21	72.47	71.14	70.17	68.73
A7	74	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A8	277	316.61	**303.59**	160.25	144.22	0	0.86	54.73	46.54	151.83	141.6	134.13	123.22
A9	357	371.28	**369.92**	320.73	289.77	151.27	140.5	84.21	64.54	316.07	288.75	269.71	242.71
A10	1100	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
A11	1200	0	0	0	0	0	0	0	**9.39**	0	0	0	0
B1	1	**0**	**0**	**0**	7.58	15.81	18.18	30.33	31.65	5.85	12.27	16.55	22.46
B2	1	**0**	**0**	**0**	**0**	7.85	9.77	21.32	26.15	**0**	**0**	**0**	**0**
B3	3	NA	NA	54.45	56.22	NA	NA	37.9	**36.27**	55.6	57.08	57.81	58.58
B4	14	0	0	0	0	0	0.53	**13.45**	0	0	0	0	0
B5	21	4.09	4.08	6.38	9	17.18	**18.84**	27.88	30.15	0	1.52	7.07	14.77
B6	24	89.23	88.89	90.83	94.79	66.75	64.14	49.83	**43.55**	112.78	107.1	103.14	97.52
B7	25	160.94	160.22	143.76	135.52	80.8	77.19	57.09	**47.98**	146.99	137.45	131.05	122.18
B8	32	197.58	196.99	174.25	159.61	89.94	84.72	57.79	**48.41**	170.1	158.66	150.4	138.45
B9	32	249.81	249.27	211.77	184.47	98.94	93.71	64.52	**52.52**	183.71	169.72	160.37	147.49
B10	85	129.84	129.6	118.89	111.53	**70**	67.13	51.19	44.38	118.55	111.98	107.44	101.04
B11	2310	NA	NA	139.45	135.57	NA	NA	56.53	47.64	**155.66**	145.34	138.04	127.59

environmental conditions. Additionally, the qPCR data is less reliable since the melt curves showed non-specific amplification for certain targets while the reason for this is not entirely clear. Hence, the results of the regression analysis are less reliable than for indoor conditions. In outdoor conditions, CLC was the only transcript whose degradation pattern showed a correlation ($p < 0.05$) with TsD in a long-term setting. However, up to 28d, both CLC and LGALS2 correlated with TsD, but the preferential degradation of the $5'$ end over the $3'$ end became less pronounced over time in the marker LGALS2.

Since the ΔC_q values of both laboratories were comparable, the indoor data of both laboratories was combined in a short-term regression analysis (0, 1, 2, 7, 16, 28, 42, and 54 days). The markers CLC and S100A12 showed the strongest association with TsD, also when the data sets were analyzed individually using the entire time series. In the statistical analysis, we calculated the marker importance using an elastic net model trained on different data sets and penalty combinations. CLC had the highest importance in all analyses regardless of training data and penalty combination. S100A12 was also influential in models especially using qPCR data from Laboratory 1 or all data as training data. LGALS2, B2M, and ALAS2 had less influence in the models. Therefore, C_q values of CLC were most strongly correlated with time. Fu and Allen [17] used a linear regression curve to estimate the age of additional blood stains to test the limitations of their method. TsD was estimated based on each marker individually. The authors did not comment on which marker performed best, however, B2M was not informative above a TsD of 8–10 weeks due to its specific degradation pattern.

We estimated the TsD of independent test samples, from both laboratories. Predictions were made for all test samples using models trained on the qPCR data from Laboratory 1, Laboratory 2, and the combined data. The model that provided the overall lowest MAD was always based on the training data from the respective laboratory. However, predictions for the Laboratory 1 test samples with TsD< 100 days provided a MAD of 4.6 days using the model based on both datasets. Predictions of TsD for test samples from Laboratory 1 provided an overall lower MAD than the Laboratory 2 test samples. The training data from Laboratory 1 contains more data points, 5 individuals (4 for 210 and 310 days) at 8 time points, while Laboratory 2 included data from 3 individuals and only 5 time points. Two of the time points for Laboratory 2 (364 and 551 days) showed a stagnation in the ΔC_q values. Including these data points in the training data for any of the prediction models increased the MAD, hence these time points were excluded from the training data for the final model. Moreover, some of the test samples in the independent test set of Laboratory 2 were extracted with a different extraction method (B6 – B9). The C_q values measured for the targets in these samples were overall slightly lower compared to samples with similar age, though the ΔC_q values were comparable. Nonetheless, compared to test samples of similar age extracted with the same method as the training data, the C_q values per marker were quite different. Consequently, the greatest prediction errors for samples with TsD of < 100 days were observed for samples B6 – B9. An important observation is that the older stains seem to have ΔC_q values closer to fresh ones, which will lead to misclassifications. Three of the test samples included in the test set had ages outside the range of the training data. The predicted ages of these were either non-conclusive or estimated considerably lower than the true TsD (B11). This supports the finding that after a period of more than 1.5 years a shift in ΔC_q can be observed and this can lead to incorrect predictions of old stains. Older stains may experience more similar degradation at both ends of the transcript.

5. Conclusion

Although the change in ΔC_q values in the current study differs to some degree in the time-wise change from the data Fu and Allen [17] reported, we have observed a correlation between ΔC_q and TsD for some of the markers and demonstrated how this can be used in a statistical model. Two laboratories performed the analysis on bloodstains stored under indoor conditions with a similar methodology, although a certain degree of inter-laboratory variation was observed. We found that the samples with similar TsD (up to 54 days) gave comparable ΔC_q values and that there is a significant correlation between ΔC_q and time when the data was combined. In the elastic net model, predictions could also be made based on the combined datasets as training data, especially for Laboratory 1 where the lowest MAD was 4.3 days. Nevertheless, the prediction performance using test data from Laboratory 1 was good and resulted in an MAD of 2.0 days. As seen for the outdoor data, assessing samples that were highly exposed to environmental effects might be very challenging. Therefore, further test samples exposed to different conditions or on different carrier materials are needed to fully explore the predictive potential of this method. The qPCR assay requires a large amount of sample (1 μl for each marker) and many pipetting steps, thus making it an error-prone and time-consuming process. Combining all TsD markers in one analysis could help to accelerate and to streamline the process, also sample consumption could be minimized. Considering the results of this study, it is obvious that the training data is a crucial factor for the performance of the prediction model. The prediction performance of a future model could be improved by homogenizing the training set in that more sampling points at regular time intervals could be included. Further, biological replicates from the same individual could increase the robustness of the model even more.

CRediT authorship contribution statement

Nadescha Viviane Hänggi: Investigation, Roles/Writing − original draft, Writing − review & editing, Visualization. **Øyvind Bleka**: Formal analysis, Writing − review & editing, Visualization. **Cordula Haas**: Conceptualization, Methodology, Writing − review & editing. **Ane Elida Fonneløp**: Conceptualization, Methodology, Investigation, Writing − review & editing. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.forsciint.2023.111785.

References

[1] G. Zadora, A. Menżyk, In the pursuit of the holy grail of forensic science–Spectroscopic studies on the estimation of time since deposition of bloodstains, TrAC Trends Anal. Chem. 105 (2018) 137–165.

[2] J. Wang, X. Cheng, J. Zhang, Z. Liu, F. Cheng, J. Yan, G. Zhang, Estimating the time since deposition (TsD) in saliva stains using temporal changes in microbial markers, Forensic Sci. Int. Genet. 60 (2022), 102747, https://doi.org/10.1016/j.fsigen.2022.102747.

[3] C. Díez López, M. Kayser, A. Vidaki, Estimating the time since deposition of saliva stains with a targeted bacterial DNA approach: a proof-of-principle study, Front. Microbiol. 12 (2021), 647933, https://doi.org/10.3389/fmicb.2021.647933.

[4] A.P. Salzmann, N. Arora, G. Russo, S. Kreutzer, L. Snipen, C. Haas, Assessing time dependent changes in microbial composition of biological crime scene traces using microbial RNA markers, Forensic Sci. Int. Genet. 53 (2021), 102537, https://doi.org/10.1016/j.fsigen.2021.102537.

[5] S. Anderson, B. Howard, G.R. Hobbs, C.P. Bishop, A method for determining the age of a bloodstain, Forensic Sci. Int. 148 (1) (2005) 37–45, https://doi.org/10.1016/j.forsciint.2004.04.071.

[6] S. Alshehhi, N.A. McCallum, P.R. Haddrill, Quantification of RNA degradation of blood-specific markers to indicate the age of bloodstains, Forensic Sci. Int.: Genet. Suppl. Ser. 6 (2017) e453–e455.

[7] A.P. Salzmann, G. Russo, S. Kreutzer, C. Haas, Degradation of human mRNA transcripts over time as an indicator of the time since deposition (TsD) in biological crime scene traces, Forensic Sci. Int. Genet. 53 (2021), 102524, https://doi.org/10.1016/j.fsigen.2021.102524.

[8] S.L. Fordyce, M.-L. Kampmann, N.L. van Doorn, M.T.P. Gilbert, Long-term RNA persistence in postmortem contexts, Invest. Genet. 4 (1) (2013) 1–7.

[9] F. Kohlmeier, P.M. Schneider, Successful mRNA profiling of 23 years old blood stains, Forensic Sci. Int.: Genet. 6 (2) (2012) 274–276.

[10] M. Bauer, D. Patzelt, Evaluation of mRNA markers for the identification of menstrual blood, J. Forensic Sci. 47 (6) (2002) 1278–1282.

[11] J. Juusola, J. Ballantyne, Messenger RNA profiling: a prototype method to supplant conventional methods for body fluid identification, Forensic Sci. Int. 135 (2) (2003) 85–96, https://doi.org/10.1016/s0379-0738(03)00197-x.

[12] C. Haas, C. Muheim, A. Kratzer, W. Bär, C. Maake, mRNA profiling for the identification of sperm and seminal plasma, Forensic Sci. Int.: Genet. Suppl. Ser. 2 (1) (2009) 534–535.

[13] A. Lindenbergh, M. Pagter, G. de, Ramdayal, M. Visser, D. Zubakov, M. Kayser, T. Sijen, A multiplex (m)RNA-profiling system for the forensic identification of body fluids and contact traces, Forensic Sci. Int. Genet. 6 (5) (2012) 565–577, https://doi.org/10.1016/j.fsigen.2012.01.009.

[14] A.P. Salzmann, M. Bamberg, C. Courts, G. Dørum, A. Gosch, T. Hadrys, G. Hadzic, M. Neis, P.M. Schneider, T. Sijen, mRNA profiling of mock casework samples: results of a FoRNAP collaborative exercise, Forensic Sci. Int.: Genet. 50 (2021), 102409.

[15] M. Bauer, S. Polzin, D. Patzelt, Quantification of RNA degradation by semi-quantitative duplex and competitive RT-PCR: a possible indicator of the age of bloodstains, Forensic Sci. Int. 138 (1–3) (2003) 94–103, https://doi.org/10.1016/j.forsciint.2003.09.008.

[16] S.E. Anderson, G.R. Hobbs, C.P. Bishop, Multivariate analysis for estimating the age of a bloodstain, J. Forensic Sci. 56 (1) (2011) 186–193, https://doi.org/10.1111/j.1556-4029.2010.01551.x.

[17] J. Fu, R.W. Allen, A method to estimate the age of bloodstains using quantitative PCR, Forensic Sci. Int. Genet. 39 (2019) 103–108, https://doi.org/10.1016/j.fsigen.2018.12.004.

[18] Lohr, C.: Detection of RNA Methylation Patterns in Forensically Relevant Transcripts in Dried Bloodstains, Oklahoma State University (2022).

[19] N. Heneghan, J. Fu, J. Pritchard, M. Payton, R.W. Allen, The effect of environmental conditions on the rate of RNA degradation in dried blood stains, Forensic Sci. Int. Genet. 51 (2021), 102456, https://doi.org/10.1016/j.fsigen.2020.102456.

[20] S. Alshehhi, P.R. Haddrill, Estimating time since deposition using quantification of RNA degradation in body fluid-specific markers, Forensic Sci. Int. 298 (2019) 58–63, https://doi.org/10.1016/j.forsciint.2019.02.046.

[21] C. Hampson, J. Louhelainen, S. McColl, An RNA expression method for aging forensic hair samples, J. Forensic Sci. 56 (2) (2011) 359–365, https://doi.org/10.1111/j.1556-4029.2010.01641.x.

[22] A.-M. Simard, L. DesGroseillers, V. Sarafian, Assessment of RNA stability for age determination of body fluid stains, Can. Soc. Forensic Sci. J. 45 (4) (2012) 179–194.

[23] S. Alshehhi, P.R. Haddrill, Evaluating the effect of body fluid mixture on the relative expression ratio of blood-specific RNA markers, Forensic Sci. Int. 307 (2020), 110116, https://doi.org/10.1016/j.forsciint.2019.110116.

[24] C. Zhao, M. Zhao, Y. Zhu, L. Zhang, Z. Zheng, Q. Wang, Y. Li, P. Zhang, S. Zhu, S. Ding, J. Li, The persistence and stability of miRNA in bloodstained samples under different environmental conditions, Forensic Sci. Int. 318 (2021), 110594, https://doi.org/10.1016/j.forsciint.2020.110594.

[25] Y. Wei, J. Wang, Q. Wang, B. Cong, S. Li, The estimation of bloodstain age utilizing circRNAs and mRNAs biomarkers, Forensic Sci. Int. 338 (2022), 111408, https://doi.org/10.1016/j.forsciint.2022.111408.

[26] K.D. Weinbrecht, J. Fu, M.E. Payton, R.W. Allen, Time-dependent loss of mRNA transcripts from forensic stains, Res. Rep. Forensic Med. Sci. (2017) 1–12.

[27] M.-H. Lin, D.F. Jones, R. Fleming, Transcriptomic analysis of degraded forensic body fluids, Forensic Sci. Int.: Genet. 17 (2015) 35–42.

[28] S.A. Bustin, V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Clin. Chem. 55 (4) (2009) 611–622, https://doi.org/10.1373/clinchem.2008.112797.

[29] J.S. Farrar, C.T. Wittwer, High-resolution melting curve analysis for molecular diagnostics, in: G.P. Patrinos (Ed.), Molecular Diagnostics (Third Edition), Academic Press, 2017, pp. 79–102.

[30] K.M. Ririe, R.P. Rasmussen, C.T. Wittwer, Product differentiation by analysis of DNA melting curves during the polymerase chain reaction, Anal. Biochem. 245 (2) (1997) 154–160.

[31] J.M. Ruijter, A. Ruiz-Villalba, A.J.J. van den Hoff, Q.D. Gunst, C.T. Wittwer, M.J.B. van den Hoff, Removal of artifact bias from qPCR results using DNA melting curve analysis, FASEB J. 33 (12) (2019) 14542–14555, https://doi.org/10.1096/fj.201901604R.

[32] J. Friedman, T. Hastie, R. Tibshirani, Regularization paths for generalized linear models via coordinate descent, J. Stat. Softw. 33 (1) (2010) 1.

[33] A. Ruiz-Villalba, E. van Pelt-Verkuil, Q.D. Gunst, J.M. Ruijter, M.J.B. van den Hoff, Amplification of nonspecific products in quantitative polymerase chain reactions (qPCR, Biomol. Detect. Quantif. 14 (2017) 7–18, https://doi.org/10.1016/j.bdq.2017.10.001.

[34] T. Nolan, R.E. Hands, S.A. Bustin, Quantification of mRNA using real-time RT-PCR, Nat. Protoc. 1 (3) (2006) 1559–1582, https://doi.org/10.1038/nprot.2006.236.