WALAILAK JOURNAL
http://wjst.wu.ac.th Health Sciences

Preliminary Study of Alpha-Globin mRNA as a Molecular Tool for Age Estimation of Human Bloodstains

Unyarat TUCKPRAKHON[1], Khomsorn LOMTHAISONG[2], Sakda DADUANG[2] and Khemika LOMTHAISONG[1,*]

[1]Forensic Science Program, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand
[2]Department of Biochemistry, Faculty of Science, Khon Kaen University, Khon Kaen 40002, Thailand

([*]Corresponding author's e-mail: khemlo@kku.ac.th)

Received: 7 April 2015, Revised: 16 September 2015, Accepted: 24 October 2015

Abstract

This research aims to investigate the possibility of using α-globin mRNA for both human blood identification and age estimation of bloodstains. Primers specific to human α-globin mRNA were designed. These primers were then used to amplify cDNA prepared from human and animal blood samples. A 168 bp DNA band was only detected in the reaction containing the human blood sample. This result suggesting that the designed primers could be able to be used for PCR amplification of human blood evidence. For age estimation of human bloodstains, bloodstains on cotton clothes (3 males and 3 females) were prepared and then separately kept at 30 ºC for 0, 30, 60, 90, 120, and 150 days, respectively. The quantity of α-globin cDNA in each sample was then examined in 3 replicates by real-time PCR. Interestingly, the quantity of α-globin cDNA tended to decrease with the age of the bloodstain. The coefficients of determination (R^2) of male and female bloodstains were 0.90 and 0.88, respectively. Our observed results suggested that age estimation of human bloodstains could be performed by comparing the quantity of α-globin mRNA presented in the samples.

Keywords: Human alpha-globin mRNA, human blood identification, age estimation, forensic science

Introduction

The identification and age estimation of human bloodstains is a valuable tool for forensic investigation in order to reduce the number of samples used for DNA analysis, to exclude a suspect as innocent, and/or disprove a suspect's statement in which they insist that their bloodstains originated before the crime has occurred. Previously, the analyses of bloodstain age were mostly based on the changes of hemoglobin to its derivatives which could be observed by the color change [1], which had led to the study of imaging analysis for the age estimation of blood stains [2,3]. Other techniques, e.g., the activities of enzyme and proteins [4], or the racemization rate of aspartic acid [5] found in blood, had been reported for age estimation of human bloodstains. However, none of these methods could discriminate human blood from that of animals, and their results also depended on the quantity of samples. Therefore, if any method can be found to identify human bloodstains, estimate the age of bloodstains, and analyze samples in small quantities, it will be very useful.

Many studies have shown that DNA is a potent target for species identification, and that results can be obtained even with a small amount of sample present [6,7]. However, DNA lacks information on age estimation due to its stability. Previous study elucidated that DNA could be successfully extracted from ancient human bones of ages greater than 10,000 years [8]. Although the telomere shortening at the end of chromosomes had been examined for human age estimation [9,10], limitations such as the high error of prediction and dependent factors affected the results. Therefore, RNA has been focused on. The

degradation rate of rRNA and mRNA in different ages of bloodstains has been previously compared [11]. Interestingly, mRNA significantly decreased over time, as it did not combine with protein like rRNA. Hence, for age estimation of bloodstains, mRNA is a better choice. Although the method of bloodstain age estimation by quantifying β-actin mRNA in the samples had been successfully reported in previous study, such a method was not specific to blood samples, as β-actin are housekeeping genes which can be found in all cell types.

This study, therefore, aimed to estimate the age of bloodstains by using the molecular target specific to a blood sample. In this case, hemoglobin was chosen. Hemoglobin is a major component of red blood cells. They are composed of 2 α- and 2 β-globin chains. In 1992, the protein patterns of bloodstains at different ages were examined using high performance liquid chromatography [12]. The results showed that α-globin protein in the bloodstains had decreased over time. Therefore, it could be possible to use α-globin mRNA as a target of this study. So, the objectives of this study were to identify human blood samples and to estimate the age of bloodstains by using α-globin mRNA.

Materials and methods

Samples

Human blood samples were obtained from the blood bank, Faculty of Medicine, Khon Kaen University, with permission from the Khon Kaen University Ethics Committee in Human Research. Animal blood samples: *Bos taurus* (cow), *Sus scrofa* (pig), *Canis lupus familiaris* (dog), and *Gallus gallus* (chicken) were taken from Ubon Ratchathani College of Agriculture and Technology. All blood samples were collected in sample tubes with the addition of 50 µl 200 mM EDTA to prevent the blood clotting process.

Human bloodstains (from 3 males and 3 females) were prepared by spotting 50 µl of fresh blood samples onto 5×10 cm sterilized white cotton cloths. Five separate spots were applied to each fabric piece. The cloths were kept in an incubator at 30 °C for 0, 30, 60, 90, 120, or 150 days. Each experiment was performed in 3 replicates. For 0 days, the stains were left to dry at 30 °C and then directly used to extract RNA.

RNA extraction

RNA extraction was performed using a GF-1 Blood total RNA kit (Vivantis technologies, Malaysia) according to the manufacturer's instructions. Briefly, 400 µl blood samples were mixed with 300 µl Buffer BR, and then 180 µl RNase-free water. Subsequently, 20 µl Proteinase K was added. The solution was mixed and incubated at 65 °C for 10 min. The mixture was centrifuged. The supernatant was transferred to a homogenization column and then centrifuged. The flow through liquid was kept, and then 80 % ethanol was added at 0.5 % volume. The liquid was mixed and loaded onto an RNA Binding column. The column was centrifuged and then washed with 500 µl Wash buffer. DNase I digestion Mix was subsequently added to the column and incubated at room temperature for 15 min before being added by use of 500 µl Inhibitor Removal buffer. The liquid was removed from the column by centrifugation and the column was then washed with 500 µl Wash buffer twice. Total RNA was then eluted from the column by adding 60 µl of RNase-free water. The eluted RNA was then quantified by using a UV-VIS spectrophotometer (Shimadzu, Japan). For RNA extraction from bloodstain samples, the blood spots were cut into small pieces with sterilized scissors. The small fabric pieces were then transferred into microcentrifuged tubes before adding Buffer BR, RNase-free water and Proteinase K. The following steps were then conducted as described earlier.

cDNA synthesis

A RevoScript™ Reverse Transcriptase PreMix (Oligo dT_{15}primer) kit was used for cDNA synthesis. Briefly, 20 ng of RNA was added into the RevoScript™ RT PreMix tubes. RNase/DNase-free water was then added to make the total volume up to 20 µl. The tubes were incubated at 50 °C for 60 min

to allow the cDNA synthesis. The reaction was then ceased by incubation at 95 °C for 5 min to inactivate the RTase enzyme. The cDNA solutions were then stored at −20 °C until use.

Primer design

The nucleotide sequences of human and animal (cow, pig, dog, and chicken) α-globin mRNAs were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank). The human α-globin mRNA sequence (Accession Number NM_000558) was used as a template DNA for primer design by the Primer3 program. The forward and reverse primers were then compared with animal α-globin mRNA using the Primer-blast program. The α-globin mRNA sequences were retrieved from the GenBank database. The accession numbers of cow, pig, dog, and chicken α-globin mRNAs were NM_001077422, XM_003481084, NM_001270885, and NM_001004376, respectively. Primers showing no complementary DNA sequence with those of animals were chosen for PCR amplification.

PCR amplification

The total volume of PCR reaction was 20 µl, containing 5 ng cDNA templates, 0.5 µM of each primer, 2XPCR Master Mix, and deionized water. Amplification was performed in a Thermal Cycler with the following cycling conditions: initial denaturation step at 94 °C 3 min, followed by 35 cycles of 94 °C 30 s, 65 °C 45 s, 72 °C 45 s. and a final extension at 72 °C 3 min. The PCR product was subsequently analyzed on 1.5 % agarose gel.

Real-time PCR

This experiment was conducted to quantify α-globin cDNA presented in bloodstains aged 0, 30, 60, 90, 120, and 150 days. Tests were performed using a LightCycler®480 SYBR Green I Master, and the results were then analyzed by a LightCycler®480 (Roche, Germany). A housekeeping gene, GAPDH, was also quantified, in order to normalize the expression of α-globin gene in the samples. The GAPDH primers used in this study were described by Fang *et al.* [13]. A reaction of real-time PCR, comprising of 1xLightCycler®480 SYBR Green I Master, 0.25 µM of each forward and reverse primer, and 5 ng cDNA templates was prepared in a multiwell plate 96. Water was then added to make the total volume of reaction up to 20 µl. The reaction was thoroughly mixed and then briefly centrifuged. The plate was placed in a LightCycler®480. The appropriate program was subsequently set. For α-globin cDNA, 45 cycles of 95 °C 20 s, 65 °C 20 s, and 72 °C 30 s was carried out, while 45 cycles of 95 °C 35 s, 54 °C 45 s, and 72 °C 35 s was performed for GAPDH cDNA. All experiments were performed in 3 replicates. The fluorescence signal above the background or threshold cycle (Ct) of each sample was then examined.

Relative RNA quantification

The α-globin mRNAs in different ages of bloodstains were measured by relative quantification as shown by the equation;

$$R = 2^{-\Delta\Delta Ct} \quad (1)$$

Ct is the threshold cycle
ΔΔCt is the ΔCt sample – ΔCt control
ΔCt sample is Ct of α-globin cDNA – Ct of GAPDH cDNA
ΔCt control is Ct of α-globin cDNA – Ct of GAPDH cDNA (at 0 days)

Melting temperature analysis

As the SYBR Green I Dye used in the reaction of real-time PCR is not specific to the amplified product of α-globin or GAPDH cDNA, the fluorescent signal may have resulted from the amplification of primer-dimer or other non-specific DNA regions. To ensure that the amplified products are the targets of interest, the melting temperature (Tm) of PCR products must be analyzed. For Tm analysis of α-globin and GAPDH PCR products, the temperatures of the LightCycler® 480 were set as follows; 95 °C for 5 s,

77 °C for 10 s, and 95 °C continuously. The melting temperature of PCR product for each age of bloodstain sample was then determined.

Results and discussion

Primer design

The nucleotide sequence of human α-globin mRNA obtained from GenBank was used as a template DNA for primer design by the Primer3 program. The nucleotide sequences of forward and reversed primers designed by Primer3 are 5'-GGTCCCCACAGACTCAGAGA-3' and 5'-AGTGCGGGAAGTAGGTCTTG-3', respectively. The expected size of the amplified PCR product was 168 bp. These primers were then blasted with the α-globin mRNA of 4 animal species, including cow, pig, dog, and chicken, using the Primer-BLAST program. No homology sequence was found (data not shown). In addition, primers were also compared with human α-globin DNA, to check whether or not these primers could differentiate the PCR products amplified from contaminated DNA templates. The binding sites of forward and reverse primers were situated on exon1 and exon2 of α-globin DNA, respectively (data not shown). Therefore, if there was any possible contaminated human DNA presented in the cDNA sample, it was identifiable by the bigger size of the PCR product (~290 bp).

PCR assay of designed primer on human and animal blood samples

The designed primers were analyzed by PCR technique, using the cDNA synthesized from human and animal fresh blood samples as the template. A DNA fragment of 168 bp was amplified in the PCR reaction, which contained a human cDNA template. As predicted, such PCR product was not observed with animal samples (**Figure 1**). These results confirmed that the designed primers were human specific. Therefore, they can be used to identify human blood samples. In addition, no PCR product with a size greater than 168 bp was shown. Therefore, the cDNA was of good quality, and the designed primers were effective enough to specifically identify human blood from the cDNA template.

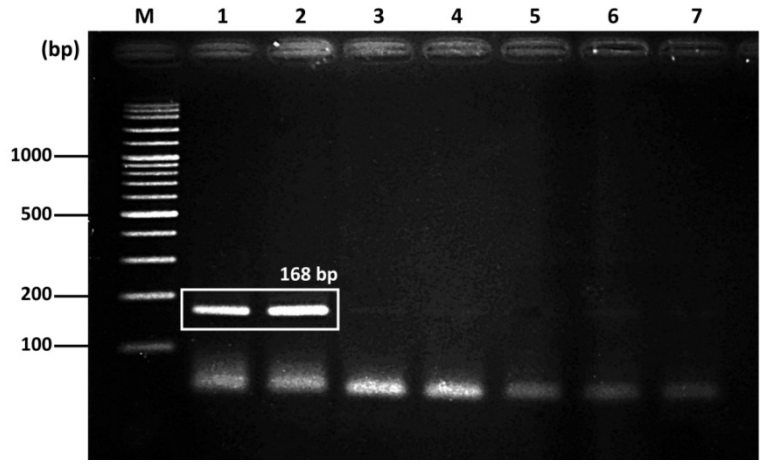

Figure 1 PCR amplification of human α-globin cDNA on different cDNA templates prepared from blood samples of man (1), woman (2), cow (3), pig (4), dog (5), and chicken (6), respectively. Experiments were compared with negative control (7). M represents the 100 bp DNA ladder.

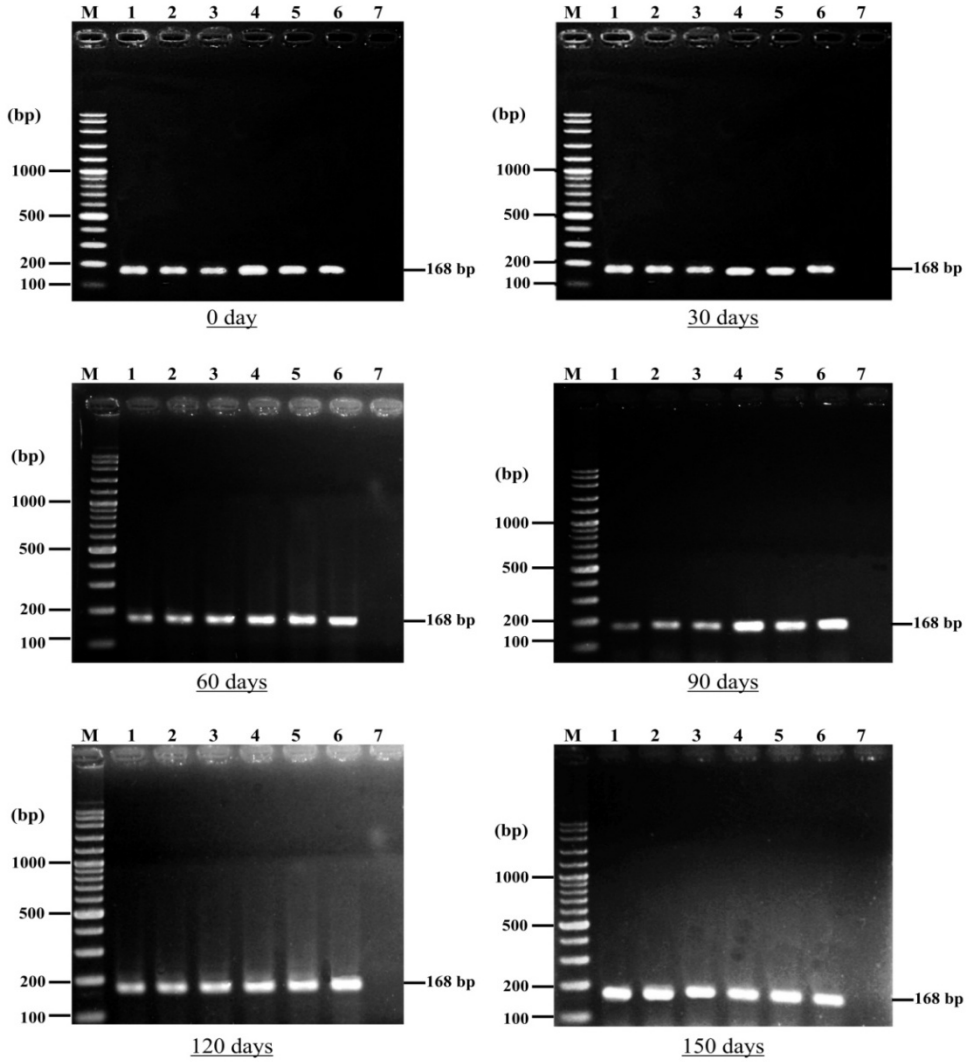

Figure 2 PCR amplification of cDNA prepared from male (lane 1 - 3) and female (lane 4 - 6) bloodstains aged 0, 30, 60, 90, 120, and 150 days using human α-globin primers. Negative control (lane 7) was run together in all reactions. The size of PCR product was compared with 100 bp ladder (M).

PCR analysis of α-globin cDNA on different ages of human bloodstains

This experiment was set up to establish that the human α-globin mRNA at different ages of bloodstains could be amplified by the designed primers. The cDNA was prepared from male and female bloodstains aged 0, 30, 60, 90, 120, and 150 days. The PCR reaction was then performed. PCR amplification was examined on 1.5 % agarose gel. The results demonstrated that human α-globin cDNA could be amplified from human bloodstains up to an age of 150 days (**Figure 2**). These results confirmed that the designed primers could be used to examine α-globin mRNA in every age of human bloodstains. Therefore, it would be possible to use α-globin mRNA as a molecular marker for the age estimation of bloodstains. For this reason, the quantity of α-globin mRNA in the bloodstains was subsequently examined by real-time PCR technique. The number of amplified PCR product was monitored by

fluorescent dye, SYBR Green I. This technique can detect the amplified PCR product during the exponential stage of PCR reaction, which enables comparison of the quantity of starting mRNA and subsequently the cDNA template. However, the accuracy of cDNA quantification by real-time PCR is dependent on nucleic acid integrity, the efficiency of reverse transcription, and the amount of sample loaded [14]. Consequently, a reference for normalizing sample needs to be performed. Housekeeping genes have been used as references in many studies, because of their unchanged expression [15]. There are many housekeeping genes e.g. β-actin (BACT), TATA box-binding protein (TBP), 18S ribosomal RNA (18S RNA), and phosphoglycerate kinase (PGK1). In this study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was chosen as a reference gene. For age estimation of human bloodstain by mRNA quantification, this was the first report using GAPDH as a reference gene. In previous studies, quantities of 18S rRNA [11,16] and cyclophilin [17] had been used as reference genes for the relative quantification of β-actin mRNA presented in the different ages of bloodstains.

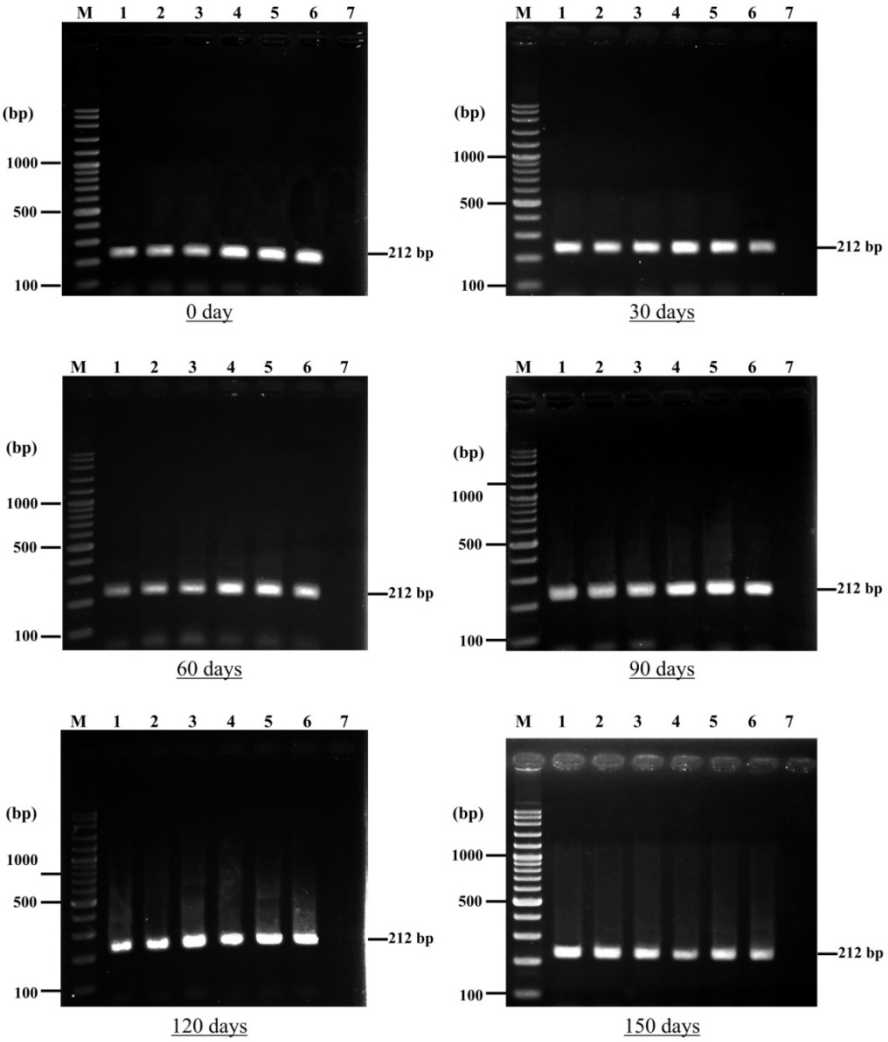

Figure 3 PCR amplification of GAPDH cDNA prepared from male (lane 1 - 3) and female (lane 4 - 6) bloodstains aged 0, 30, 60, 90, 120, and 150 days. Negative control (lane 7) was run in together in all reactions. The size of PCR product was compared with 100 bp ladder (M).

PCR analysis of GAPDH cDNA on different ages of human bloodstains

The quantity of GAPDH mRNA was used to normalize the quantity of α-globin mRNA in real-time PCR. However, before performing the GAPDH real-time reaction, amplification of GAPDH cDNA had to be checked in all bloodstain samples. The cDNAs synthesized from bloodstains aged 0 - 150 days were amplified by GAPDH primers. The results are presented in **Figure 3**. It can be seen that the size of the amplified product was 212 bp. Such PCR products were also observed in all bloodstains ages, indicating that the primers used for GAPDH amplification had worked properly.

Relative quantification of α-globin mRNA in bloodstains

The quantity of α-globin mRNA for each age of human bloodstain was determined by real-time analysis. The results showed that the Ct values tend to increase with the age of bloodstains (data not shown), indicating that α-globin mRNA in the bloodstains had reduced. These results suggest that the reduction of α-globin mRNA was proportional to bloodstain age. Therefore, it is possible to use α-globin mRNA as a marker to estimate bloodstain age. However, in order to compare the quantity of α-globin mRNA at each age, the quantity of α-globin mRNA must be normalized by a quantity of a housekeeping gene, GAPDH. The quantity of α-globin mRNA in differently aged of bloodstain samples was compared by relative quantification (R) using the formula $2^{-\Delta\Delta Ct}$. The results are shown in **Table 1**. Trend analysis was then performed to examine the coefficient of determination (R^2). It can be seen that the graphs were dramatically reduced with the age of bloodstains, in both the male and female groups (**Figure 4**). Our observations were similar to the study reported earlier by Anderson *et al*. [11]. They reported that the number of β-actin mRNA in the bloodstains had decreased with time, but their reported graphs differ from our report. Their graphs showed gradual increase because the values on the y axis were calculated from the Ct value of β-actin mRNA divided by the Ct value of 18S rRNA. However, Alrowaithi (2014) recently discussed Anderson's report and stated that the determination of bloodstain age should be the ratio of β-actin mRNA over 18S rRNA [18]. This is the reason why our study used a relative ratio of α-globin mRNA over GAPDH.

Considering the values of R^2, the values in both male and female bloodstains were 0.90 and 0.88, respectively. These results suggested that 90 and 88 % of male and female bloodstain ages could be predicted. The effects of gender on the relationship between time and RNA ratio in the bloodstains had been reported earlier [19]. They found that subject's gender affected the ratio of 18S rRNA:β-actin mRNA. However, the RNA ratio was quantified in bloodstains under uncontrolled room conditions, unlike our study, in which bloodstains samples were kept at a certain temperature (30 °C). Nevertheless, in real-life cases, bloodstains are found in different conditions. Previous studies had reported the effects of conditions on age estimation [20]. Therefore, further studies on the effects of environment conditions, e.g., temperature, moisture, and deposited substrate on α-globin cDNA quantification need to be examined. In addition, for further study, multivariate analysis for the age estimation of bloodstains by measurement of the number of α-globin mRNA should be performed, because expression of the housekeeping gene is independently regulated [16]. Thus, the ratio between mRNA and reference gene may not be stable. To overcome this problem, an experiment could be conducted either by semi-quantitative duplex RT-PCR or competitive RT-PCR, as described by [17]. At present, this powerful tool for the age estimation of bloodstains is still to be investigated. However, in the past few years, the alteration of red blood cells is a favorable target for this study [21,22]. Despite this, the RNA expression method is still used as a tool for the age prediction of hair evidence [23].

Table 1 Relative quantification of α-globin mRNA in 3 male and 3 female bloodstains aged 0 - 150 days. Each sample was done in triplicate.

Bloodstain age (day)	Relative quantification (R)							
	Males				Females			
	1	2	3	Mean±SD	1	2	3	Mean±SD
0	1.000	1.000	1.000	1.000±0.000	1.000	1.000	1.000	1.000±0.000
30	0.974	0.972	0.978	0.975±0.003	0.977	0.976	0.982	0.978±0.003
60	0.952	0.967	0.953	0.957±0.008	0.963	0.971	0.971	0.968±0.005
90	0.915	0.927	0.921	0.921±0.006	0.924	0.931	0.932	0.929±0.004
120	0.887	0.891	0.895	0.891±0.004	0.932	0.928	0.936	0.932±0.004
150	0.915	0.898	0.911	0.908±0.008	0.936	0.918	0.930	0.928±0.009

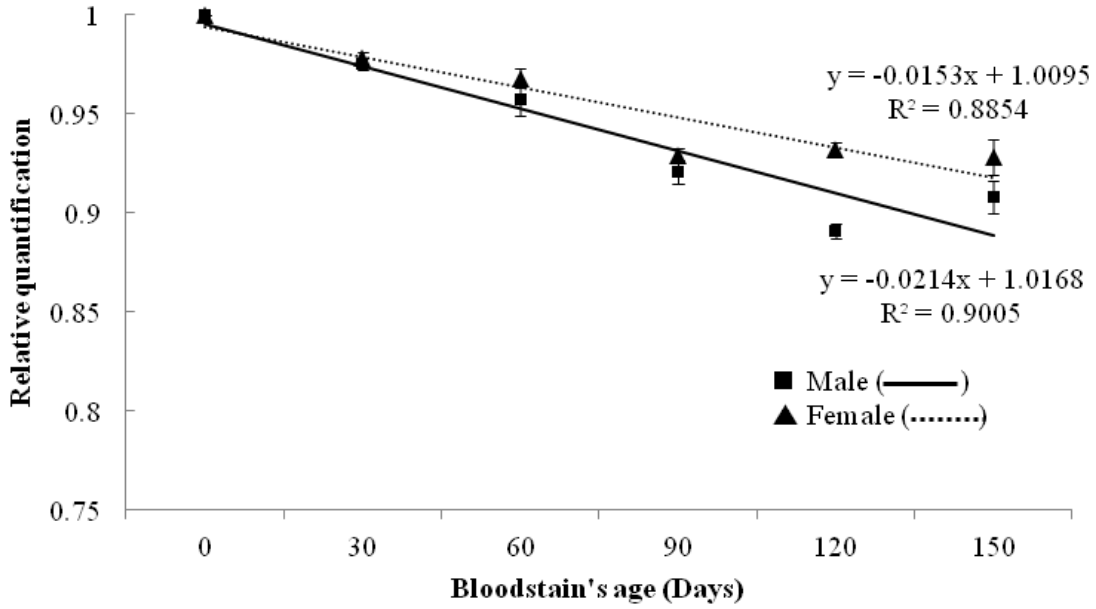

Figure 4 Plotted graphs of α-globin mRNA relative quantification between male and female bloodstains aged 0 - 150 days.

Tm analysis of PCR product

The melting temperatures of PCR products in the real-time PCR reaction of α-globin and GAPDH cDNA on human's bloodstains aged 0 - 150 days showed that the melting temperatures of PCR products amplified in the real-time reaction of α-globin and GAPDH cDNA were approximately 88 and 82 °C, respectively. These melting temperatures were as expected. Therefore, these results also confirmed that no primer-dimer or non-specific amplification had occurred in the reaction of real-time PCR [24].

Conclusions

In this study, human blood has been identified by reverse-transcription PCR, using a pair of primers specific to human α-globin mRNA producing a 168 bp PCR product. Our study has proven that it is possible to estimate the age of a bloodstain by relative quantification of α-globin cDNA. The coefficients of determination (R^2) of male and female bloodstains were 0.90 and 0.88, respectively. However, as blood evidence may be found in different conditions, α-globin cDNA quantification of bloodstain samples in various conditions needs to be further studied.

Acknowledgements

This research was financially supported by Khon Kaen University (Grant No. 563801).

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