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Original research article

Quantification of Signal-Joint T-Cell Receptor Excision Circles (sjTRECs) for Age Estimation in the Thai Population

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ABSTRACT

The age of an individual is beneficial in predicting human appearance, which can facilitate personal identification. Several studies have shown that a decrease of signal-joint T-cell receptor excision circle (sjTREC) levels in peripheral blood correlates with an increase in age. The potential application of using age-related sjTRECs has been validated in various ethnic groups, yielding a model of age estimation specific to each population. To specifically validate the use of sjTRECs as a marker for age estimation in Thais, the present study quantified sjTRECs in peripheral blood from 198 healthy individuals (20 to 91 years old) using quantitative real-time polymerase chain reaction (qRT-PCR). The results showed that sjTREC levels were negatively correlated with age ($r^2 = 0.60$, r = -0.77). The equation for age estimation was Age = -7.776 (dCt (Ct_{TBP}-Ct_{sjTREC}))-49.39 with a standard error (SE) for age estimation of ±9.97 years. The determination of sjTREC levels in dried blood stored at a temperature between 4°C and 65°C revealed that sjTREC levels did not change in those samples stored at 65°C for 60 days, when compared to the fresh blood. This suggests that sjTRECs are relatively stable at ambient temperature in Thailand and that this age estimation model could also be applied to bloodstain samples found at temperatures up to 65°C.

Keywords: Age estimation; Bloodstain; Peripheral blood; Signal-joint T-cell receptor excision circle (sjTREC); Thai

1. Introduction

Personal identification is one of the critical processes in forensic investigation used to link individuals with crimes or illegal events. DNA profiling is the ideal method for personal identification when known references or DNA databases are available for comparison to the unknown sample;

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however, in the absence of such references or databases, phenotypic traits could be useful for personal identification since it could give clues about an individual's appearance. Age is an important piece of information that helps to describe how a person looks, assisting an investigator in searching for that individual. To date, age can be estimated using various methods ranging from morphological analyses to sophisticated molecular methods. Depending on the types of biological evidence recovered at a scene, teeth and bones can be used for age estimation through morphological, chemical, and physical principles; however, these analyses need the availability of the sample [1-3]. Several molecular markers have been demonstrated to be associated with age that allows for the flexibility of age estimation using a variety of biological samples such as blood and tissue.

Among age-associated molecular markers, signal-joint T-cell receptor excision circles (sjTRECs) have been shown to be reliable and accurate for use in age estimation. sjTRECs are episomal DNA circles in T-cells that are generated during excisional rearrangement of T-cell receptor (TCR) genes. This process happens in the thymus where T-cells undergo maturation and development. It is known that the thymus undergoes changes during the process of aging; it has been reported that the thymus begins to atrophy around the first year of birth and its reduction in size continues progressively throughout life [4]. This attenuates the function of the thymus in T-cell maturation and development, subsequently decreasing the levels of sjTRECs in T-cells. Furthermore, sjTRECs are not duplicated during mitosis and thus are diluted out with cell divisions [5], resulting in decreasing levels of sjTRECs with increasing age.

Previous studies in several different populations have shown that sjTREC quantity in the blood was strongly correlated to age, posing sjTRECs as a promising marker for age estimation [6-13]. These studies have produced equations for age estimation; however, the equations were derived from particular populations and cannot be applied to the Thai population, possibly due to differences in genetic background. The present study aimed to determine the correlation between sjTRECs and age in the Thai population, and subsequently, an equation for age estimation was generated. To examine whether temperature affects the stability of sjTRECs, quantification of sjTRECs was carried out in dried blood stored at various temperatures between 4°C and 65°C in a time-course experiment. Results from this investigation would provide an insight into the potential use of sjTRECs in practical casework in which blood may be deposited within the range of analyzed temperatures.

2. Materials and Methods

2.1 Sample collection and DNA extraction

The present study divided subjects into 10 age ranges with 5-year intervals. The sample size calculation of each age range was carried out using the formula $n = (Z\alpha * S)^2/E^2$ where $Z\alpha = 1.96$, S = SD of dCt(Ct_{TBP}-Ct_{sjTREC}) and E = 40% of SD. Based on the dCt values from a previous study [8], the result of sample size calculation was approximately 24 subjects. Thus, the present study set out 20 subjects for each age range, resulting in a total of 200 subjects. Following the exclusion of unqualified subjects, peripheral blood was collected from 198 healthy Thai volunteers (20-91 years old) in BD Vacutainer K2 EDTA tubes (Becton, Dickinson and Company, USA). DNA extraction was immediately performed using QIAamp DNA Blood mini kit (QIAGEN, Germany) according to manufacturer's instructions. DNA was stored at -20°C until use.

For the preparation of lyophilized blood, to mimic dried blood and bloodstains, 100 µl of blood was placed into a 1.5 ml tube and the liquid component of the blood was

evaporated at 30°C for 2 hours using a Vacufuge Vacuum Centrifuge (Eppendorf, Germany). The lyophilized blood was stored in dark conditions, at 4°C, room temperature (25°C), 50°C, and 65°C for 15, 30, 45, and 60 days. After a specific amount of time, DNA extraction was performed using a Genomic DNA Isolation Kit (Bio-Helix, Taiwan). Initially, 600 µl Phosphate Buffer Saline (PBS) was added into the 1.5 ml tube containing the lyophilized blood. The tube was kept at 4°C, overnight.

The next day, the tube was incubated at 37°C for 1 hour and then spun at 4,000 revolutions per minute (rpm), room temperature for 5 minutes. The supernatant was decanted and the cell pellet was resuspended with 200 µl PBS. DNA extraction was then performed following the manufacturer's protocol with a modified cell lysis procedure by which 20 µl Proteinase K (10 mg/ml) was added into buffer CC and cells were incubated at 60°C for 1 hour.

The study was approved by the Ethics Review Sub-Committee for Research Involving Human Research Subjects of Thammasat University, No. 3 in accordance with the Declaration of Helsinki (COA No. 041/ 2561). Written informed consent was obtained from the subjects prior to the venipuncture.

2.2 Assessment of DNA quantity and purity

DNA was quantified and its purity was assessed using the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). The maximal absorbance for nucleic acid and protein is 260 and 280 nm, respectively. A 260/280 ratio of 1.8-2.0 is generally accepted to indicate DNA with minimal protein contamination.

2.3 sjTREC quantification

For the analysis of sjTREC levels, quantitative real-time polymerase chain reaction (qRT-PCR) was conducted using the Thunderbird SYBR qPCR Mix (TOYOBO, Japan). The forward and reverse primer sequences of sjTREC (Accession AE000521) were 5′-CCATGCTGACACCTCTGGTT-3′ and 5′-TCGTGAGAACGGTGAATGAAG-3′ respectively, as described in previous studies [7, 10]. Primers for human TATA binding protein (TBP) (Accession NG_008165.1) were designed using the open-source Primer 3 program (http://frodo.wi.mit.edu). The sequence of forward primer was 5′-TGTTTGTTTCTTGCGAGTGC-3′ and the reverse primer was 5′-CCCGGGAGGGTTCTTAGTAG-3′. The reactions were carried out in 96-well plates. The total reaction volume of 10 µl contained 5 µl of 2x SYBR mix, 0.5 µl of forward primer (5 pmol/µl), 0.5 µl of reverse primer (5 pmol/µl), 0.1 µl of 50x ROX reference dye, DNase and RNase-free water, and DNA template. The amount of DNA in each reaction was 30-60 ng, from individuals aged 20-60 years and 90 ng from individuals aged 61-91 years. The plate was run on a Bio-Rad CFX96 (Bio-Rad, USA). The cycling conditions consisted of the initial denaturation at 95°C for 5 minutes, amplification for 45 cycles at 95°C for 15 seconds, and 60°C for 1 minute. All reactions were carried out in duplicate and the average of the Ct values from each of the duplicates was used for subsequent data analysis. In each sample, sjTREC was normalized to TBP and the data were presented as dCt (Ct_{TBP}-Ct_{sjTREC}).

2.4 Statistical analysis

Correlation between sjTREC levels and age was assessed using the Pearson correlation coefficient, and a linear regression equation was obtained for age estimation. Differences in dCt values between the lyophilized blood, stored at various temperatures and for various time periods, and the fresh blood were assessed using two-way ANOVA.

3. Results and Discussion

3. 1 Amplification of sjTREC and TBP using the SYBR qRT-PCR

Quantification of sjTREC was carried out using SYBR qRT-PCR. The typical illustrations of amplification curves and dissociation curves from a young individual (29 years old) and an old individual (54 years old) showed that sjTREC and TBP were successfully amplified without non-specific amplification (Fig. 1). Based on the dCt value, it was demonstrated that the sjTREC level of a 29 year-old individual was higher when compared to a 54 year-old individual. This was consistent with previous studies showing that sjTREC decreases with increasing age [6]. Taken together, these results indicated that the SYBR qRT-PCR assay can be used for sjTREC quantification.

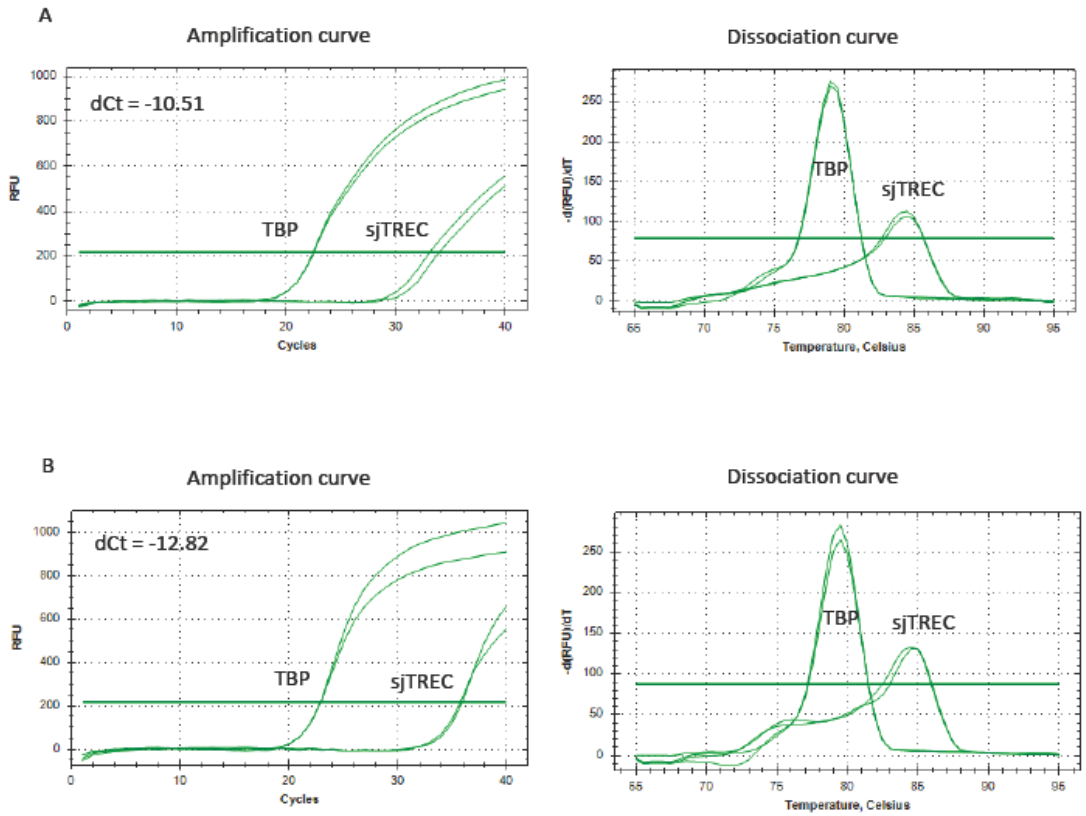

Fig. 1. Amplification of sjTRECs and TBP. Amplification curves and dissociation curves of sjTRECs and TBP from (A) a young individual at the age of 29 and (B) an old individual at the age of 54. sjTRECs were quantified using SYBR qRT-PCR and normalized to TBP. The qRT-PCR reaction of each sample was carried out in duplicate.

3.2 Quantification of sjTREC in Thai population

In an effort to generate a specific model for age estimation in the Thai population, sjTRECs in fresh peripheral blood were quantified by SYBR qRT-PCR in 198 healthy Thai individuals ranging from 20 to 91 years old. The result of 10 age ranges with 5-year intervals revealed that sjTREC quantity (dCt) progressively decreased with increasing age (Table 1). The dCt values were plotted against chronological age and linear regression was applied to determine the correlation between sjTREC quantification and age (Fig. 2). The analysis showed that sjTREC levels were relatively

correlated with age (r^2 = 0.60, r = -0.77) and the linear regression equation for age estimation was Age = -7.776(dCt) -49.39. Standard error of the estimation (SE) was ±9.97 years. In accordance with the present study, previous studies in many populations have shown an equivalent degree of correlation between sjTREC levels and age: Dutch with r^2 = 0.835, SE±8.9 years [6], Chinese with r^2 = 0.668, SE±10.47 years [7] and r^2 =0.758, SE±9.42 years [8], Korean with r^2=0.807, SE±8.49 years [9] and r^2 =0.536, SE±10.55 years [11], Egyptian with r^2=0.870, SE±7.35 years [10], and Japanese with r^2=0.617, SE±8.0 years [12]. This indicates that the prediction performance of age estimation obtained from the present study is comparable to previous reports and the equation of age estimation developed here could be effectively applied to the Thai population.

Table 1. The dCt values of 10 age ranges divided into 5-year intervals.

Age range (Year)	n	Male	Female	dCt (Ct_{TBP}-Ct_{sjTREC})		
				Mean±SD	Min	Max
20-25	20	6	14	-10.22±0.66	-11.03	-8.92
26-30	20	8	12	-10.31±0.7	-11.53	-8.86
31-35	20	9	11	-11.58±0.9	-12.92	-9.25
36-40	20	6	14	-12.05±0.85	-13.48	-10.73
41-45	20	8	12	-12.26±0.66	-13.65	-11.3
46-50	20	4	16	-12.3±0.87	-13.96	-11.19
51-55	20	2	18	-12.91±0.69	-13.85	-11.77
56-60	20	2	18	-13.19±0.9	-14.76	-11.45
61-65	18	0	18	-13.39±1.45	-17.77	-11.8
From 66	20	2	18	-14.16±1.55	-18.18	-12.29
Total	198	47	151			

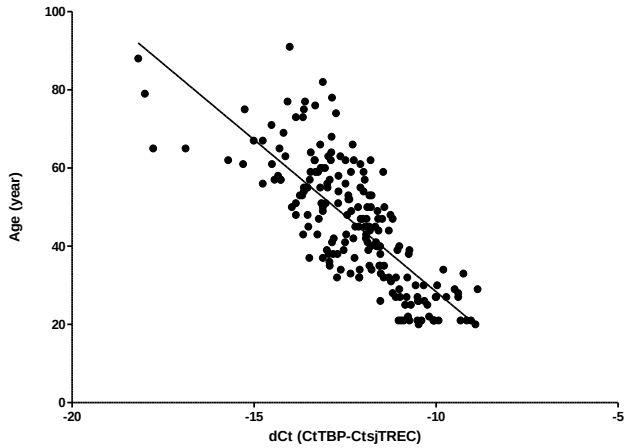

Fig. 2. Correlation between sjTREC quantification and chronological age of Thais. sjTRECs in the blood of 198 Thai individuals aged 20-91 years old were quantified by SYBR qRT-PCR and normalized to TBP. The mean of dCt from 2 technical replicates of each qRT-PCR reaction was plotted against chronological age. The correlation between sjTREC quantification and individual age was presented as r^2 = 0.60, r = -0.77.

It is still unclear whether gender has an effect on sjTREC levels. Zubakov D. *et al.* reported a significant difference in sjTREC quantification between males and females but there was no significant alteration of the age estimation when gender was included as an additional factor in the estimation model [6]. Likewise, Yamanoi E. *et al.* found an association between sjTREC levels and gender in the overall mean of the samples,

however no difference was observed within each age group [12]. Unlike the abovementioned findings, studies in Chinese [7, 8] and Egyptian [10] populations did not observe an effect of gender on sjTREC levels. Altogether, this suggests that gender has a slight effect on sjTREC levels and is unlikely to be a key factor for data analysis and interpretation. Here, sjTREC quantification was compared between age-matched males and females in all age ranges, except the age range of 61-65 years, which consisted of only female participants. In line with previous studies, no differences were found in all age ranges, (Data not shown). Thus, the equation for age estimation derived from male and female study participants reported in the present study is applicable to predict unknown samples regardless of gender.

Although it has been evident that sjTRECs could be a promising marker for age estimation, many factors should be taken into consideration when this marker is used for estimating the age of unknown individuals to which the blood belongs. Due to the fact that sjTREC resides in T-lymphocytes, it is logical to speculate that pathological conditions involving the immune system, especially leukemia and HIV/AIDS, could substantially alter the level of sjTRECs. Indeed, studies of sjTREC levels in peripheral blood mononuclear cells (PBMCs) from B-cell chronic lymphocytic leukemia patients [14] and chronic myeloid leukemia (CML) patients [15] showed a significantly lower level of sjTREC when compared to healthy controls. In untreated HIV-infected patients, sjTREC levels in PBMCs substantially drop, while patients who were on antiretroviral drugs had a level of sjTRECs similar to age-matched controls [16-18]. After antiretroviral therapy, patients showed an elevation of sjTREC levels in CD4+/CD8+ T-cells [18] and PBMCs [19] compared to the baseline. Hence, other immune-related conditions may also complicate sjTREC based age estimation; further studies on such conditions are required to improve sjTREC data interpretation for the purpose of age estimation.

The age estimation strategy using sjTREC has been extensively studied in the last decade and its efficiency is varied among independent studies, with r^2 in the range of 0.5-0.9 and SE ±7-11 years [6-13]. More recently, DNA methylation has emerged as a powerful tool for age estimation and several lines of evidence have shown that it is strongly correlated with age (r^2 approximately 0.8-0.9 and SE±4-7 years) [13, 20-25]. Interestingly, a combination of sjTRECs and 5 DNA methylation markers located in the *ELOVL2, C1orf132, TRIM59,* and *FHL2* genes has been shown to improve prediction accuracy, particularly in the elderly (70-74 years old) [11]. This model could overcome the limitations of sjTRECs in old samples in which sjTREC levels may have declined to the margin of detection, leading to a reduced estimation accuracy. It should be noted, however, that methods of DNA methylation analysis are more complicated and the cost is higher when compared to the quantification of sjTRECs using qRT-PCR. Thus, it appears that sjTREC quantification is still a good model for age estimation in terms of technical challenges and cost-effectiveness, highlighting the value of this model for age estimation in practical forensic casework.

3.3 The effects of temperature on the stability of sjTREC in bloodstains

In the context of forensic investigation, blood samples are usually found in the form of bloodstains, which are inevitably exposed to the environment. Previous studies have measured sjTREC levels in bloodstains stored at room temperature and revealed that sjTREC levels in bloodstains stored for 1 month were not significantly different compared to fresh bloodstains [8, 12], whereas bloodstains stored for 1 [12] and 1.5 years [8] showed a

significant reduction in sjTREC levels compared to fresh sample.

Based on a report by the Thai Meteorological Department, the maximum temperature during summer 2016 in Thailand reached ~45°C in some regions of the country [26]. To apply the age estimation equation in blood recovered in Thailand, the present study determined whether sjTRECs could be resistant to degradation at temperatures up to 65°C. To evaluate the stability of sjTRECs at such heat, the fresh whole blood from 4 samples was evaporated to mimic bloodstains and stored at 4°C, room temperature (~25°C), 50°C, and 65°C for 15, 30, 45, and 60 days (Fig. 3A). Total DNA was extracted at the indicated time points to examine levels of sjTRECs. Four selected samples consisting of 2 young and 2 old individuals were subjected for sjTREC quantification: 018-WS and 019-PM aged 21 years old, 002-KO aged 49 years old, and 001-SP aged 53 years old. Among the 4 samples, only 018-WS showed a significant increase in sjTREC levels in the lyophilized blood kept at room temperature for 30 days and at 50°C for 45 days compared to the fresh whole blood (Fig. 3B-E). Intriguingly, the blood from all 4 samples stored at 65°C for 60 days did not show any significant change in sjTREC levels compared to the fresh whole blood. Thus, it is likely that sjTREC is stable up to 65°C for 60 days.

To further validate the age estimation model, dCt values from the fresh whole blood of 4 independent experiments were analyzed using the equation Age = −7.776 (dCt) −49.39 , SE±9.97 years. As shown in Table 2, means of estimated age from 2 samples: 018-WS and 002-KO were 24 and 45 years old which were close to the actual age of 21 and 49 years old, respectively, while estimated ages of the remaining 2 samples (019-PM and 001-SP) were out of the range of SE. This suggests that further studies may focus on the validation of the age estimation equation in a larger sample size.

Table 2. The estimated age in the fresh whole blood of 4 samples.

Subject identification	Actual age (year)	Estimated Age (Mean±SD) (year)
018-WS	21	24±2.49
019-PM	21	37±2.92*
002-KO	49	45±3.67
001-SP	53	39±1.87*

Note: The results were from 4 technical replications from independent experiments

* Outside the SE±9.97 years

4. Conclusion

In the present study, sjTREC quantification was shown to correlate with age in the Thai population which is consistent with previous reports in other populations. An equation of age estimation was generated though it should be validated using a larger sample size prior to its application for estimating the age of unknown blood donors/samples. To improve the accuracy of the equation, an additional age-related marker e.g. DNA methylation might be combined with sjTREC data. Since sjTRECs reside in T-cells, diseases affecting the immune system could compromise the accuracy of sjTREC markers for age estimation. Quantification of sjTRECs in those with immune-related diseases could provide an insight into how such diseases affect sjTREC levels, which would be useful for result interpretation. Based on the qRT-PCR data from this study, it should be however noted, that the quantification of sjTRECs in old individuals (above 61 years old) requires a relatively large amount of a total DNA (90 ng) due to an age-associated decrease in sjTREC levels. Thus, this method may be not suitable for cases of low DNA yield. The determination of sjTREC stability in relation to temperature revealed that sjTRECs are stable up to 65°C for 60 days, suggesting the possibility to estimate age from bloodstains having endured high temperatures. Prior to applying this age estimation model in forensic casework, further investigation may involve examinations of other environmental factors e.g. UV exposure and humidity, on sjTREC stability.

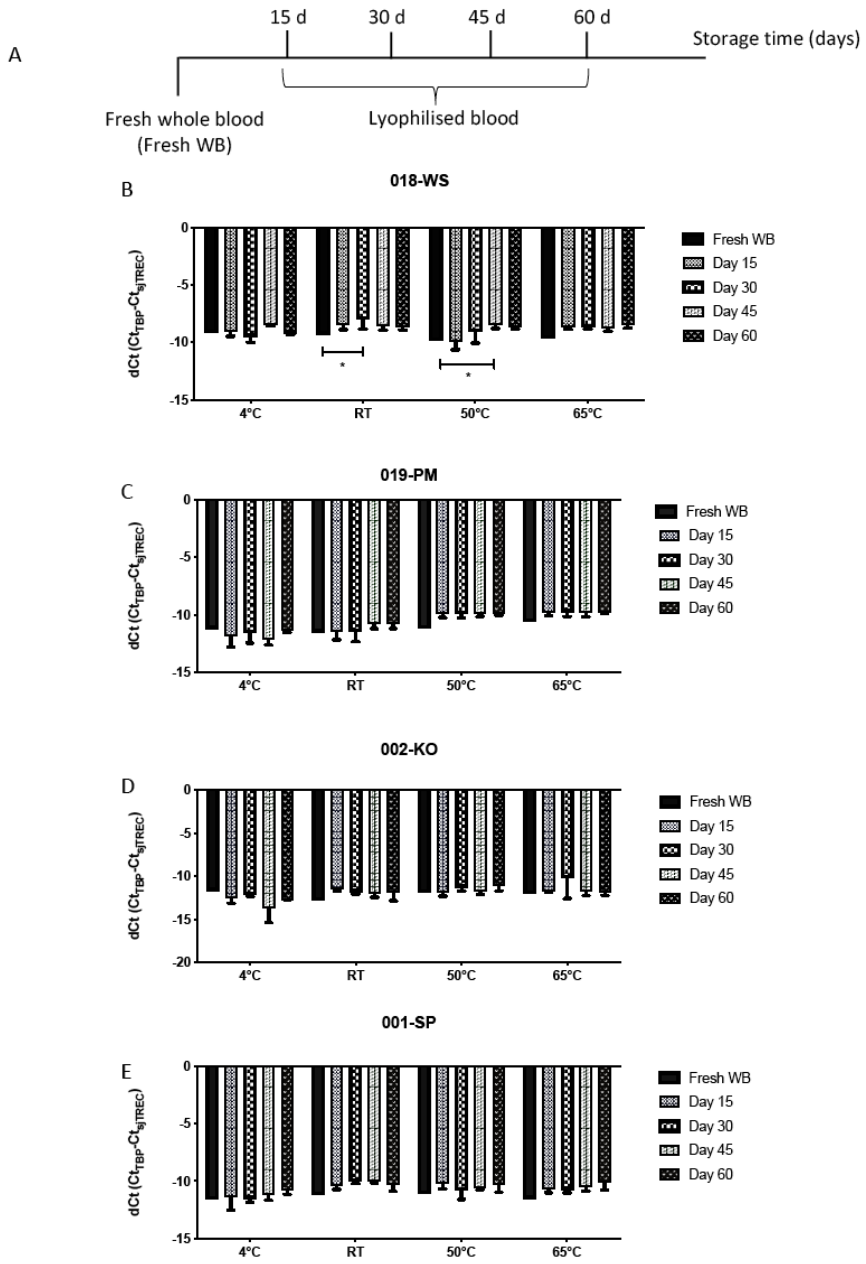

Fig. 3. sjTREC quantification in dried blood under various temperatures and storage times. (A) Experimental design to determine the stability of sjTREC in dried blood. Lyophilized blood was stored at 4°C, room temperature (~25°C), 50°C, and 65°C for 15, 30, 45, and 60 days. At the predetermined temperature and storage duration, total DNA was extracted for sjTREC quantification. (B-E) sjTRECs from 4 samples were quantified using qRT-PCR and normalized to TBP. Data represent means of dCt (±SD) of 3 technical replicates from one experiment. Statistical significance of sjTREC quantification in each temperature compared to fresh whole blood was tested using two-way ANOVA, Dunnett's multiple comparisons test (*p<0.05).

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