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Establishment of in-house telomere length measurement using qPCR

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KEYWORDS
Telomere length; Chronological age; Quantitative polymerase chain reaction (qPCR).

ABSTRACT

Telomere is a nucleoprotein complex at the ends of chromosomes which can be used as a biomarker for aging and health condition. The gold standard method for telomere length measurement is quite a complication. Recently, quantitative polymerase chain reaction (qPCR) is a widely used method for molecular study with high throughput and cost-effectiveness. This study aimed to develop the in-house qPCR for estimating telomere length in kilobase in healthy samples. The in-house qPCR was established for the telomere gene and 36B4 gene. The analytical performance was verified prior to applying to 190 healthy participants, 139 females and 51 males. The telomere length (kilobase) was calculated and compared to the reference value. The results for telomere length by in-house qPCR method were 7.48 ± 1.78 kb for males and 7.53 ± 1.45 kb for females with the range of 4.66 – 10.69 kb and 4.03 – 11.50 kb, respectively. A total of 190 participants showed moderate correlation with a reference value at R^2 = 0.5672. The Bland-Altman analysis from two different methods showed only 5.26% (10 out of 190) were out of ± 25% bias in females. The in-house qPCR was successfully demonstrated for telomere length measurement with an acceptable performance compared to the reference values. However, the validation in more clinical samples should be performed in further study.

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Introduction

Telomeres are nucleoprotein structures composed of short tandem repeats, TTAGGG, at the ends of chromosomes. The average telomere length is about 10 – 15 kb in humans[1]. Telomeres reduce during each cell division, due to an end-replication problem that leads to cell death. The shorter telomeres imply a higher biological age. Therefore, telomere length has been proposed as a biomarker of aging and general health condition. Terminal restriction fragment (TRF) is a gold standard for telomere length measurement by southern blot[2]. However, the TRF method requires a large amount of DNA and expertise, and uses more time-consuming. Nowadays, several methods have been developed for telomere length measurement including quantitative fluorescence in situ hybridization (q-FISH), flow cytometry fluorescent in-situ hybridization (flow-FISH) and quantitative polymerase chain reaction (qPCR). Q-FISH and flow-FISH can determine the average telomere length using a fluorescent peptide nucleic acid (PNA) probe. The labor-intensive, requiring high skill level, expensive and technically demanding system are the limitations of these techniques[3].

Quantitative polymerase chain reaction (qPCR) is a widely used technique for population-based comparisons. The qPCR measurement for telomere length was first reported by Cawthon et al[4]. The principle is based on the abundance of telomere (T) signals per genome representing the average telomere length in a DNA sample. The single copy gene (S) is used to normalize the signal from the telomere reaction. Therefore, the T/S ratio shows the average telomere length per genome. The advantage of this method is the small amount of DNA requirement and high throughput performance[5]. Previous studies reported the T/S ratio for relative telomere length in biological age prediction and many diseases[6]. In this study, we aimed to establish in-house qPCR to estimate the telomere length (kilobase) using convert calculation from T/S ratio in a healthy population.

Materials and methods

Participants and DNA extraction

Blood samples were drawn from 190 healthy volunteers under written informed consents approved by the Ethics Committee of Khon Kaen University, Thailand with the approval no. HE622269. The inclusion criteria of volunteers were 18-80 years old with no history of high blood pressure or any chronic diseases such as diabetes, cancers, cardiovascular disease, kidney disease and liver disease. Moreover, participants who are taking any drugs for chronic diseases, having waist circumference more than 41 inches and having body mass index (BMI) more than 30 kg/m^2 were excluded. DNA was extracted using Qiagen® DNA Blood Kit (Qiagen® GmbH, Hilden, Germany) according to the manufacturer's protocol and kept frozen at -80 °C until use.

Telomere length analysis by quantitative polymerase chain reaction (qPCR)

The qPCR for telomere length measurement was modified from the Cawthon et al[4]. Primers and standard oligomers sequences of Telomere (T) and a single copy gene, 36B4, as an internal control synthesized by *Integrated DNA Technologies* (IA, USA) are listed in table 1. Two separate reactions for T and 36B4 gene were prepared to amplify each of an individual sample. A total of 6.25 ng of DNA template was added into the 25 µL of PCR reaction mixture, which was composed of 10 µmol of each primer and 12.5 µL of Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific, MA, USA). The reactions were amplified under thermocycler (*Rotor-Gene Q, QIAGEN*, Germany) for telomere and single copy gene with initial denaturation at 93 °C for 10 min followed by 40 cycles of 93 °C for 15 sec and 54 °C for 1 min.

Telomere length calculations in Kilobases

The cycle threshold of telomere in each sample was relatively normalized with single copy gene (36B4) as telomere gene/single copy gene ratio (T/S) regarding the formula:

ΔCt telomere = [Ct (telomere of DNA sample) - Ct (telomere of DNA control)]

ΔCt single copy gene
= [Ct (single copy gene of DNA sample)
- Ct (single copy gene of DNA control)]
ΔΔCt sample = [ΔCt telomere - ΔCt single copy gene]
Relative telomere (T/S ratio) = $2^{-\Delta\Delta Ct\ sample}$

To control the precision between assays, a large pooled genomic DNA of one donor was performed within the assay as the internal control throughout this study.

The absolute telomere length in Kilobase (Kb) was further estimated from the T/S ratio of the calibrator obtained from the Absolute Human Telomere Length Quantification qPCR Assay Kit (ScienCell™ Research Laboratories, CA, USA).

Evaluation of assay performance of telomere length measurement

The performance in precision and accuracy of in-house telomere length qPCR assay was conducted in this study. A pooled DNA control was used to determine within-run (n = 18) and between-run (n = 20 days) imprecision of Telomere and 36B4 gene and indicated as the coefficient of variation (CV). The acceptable performance of qPCR should be less than 15%[8,9]. For the accuracy of in-house telomere length by qPCR, the telomere length of 190 samples was compared with the qPCR results obtained from Teloage® analysis (Mediage, South Korea) as reference values. The Bland-Altman analysis was used to compare the differences between the two methods with 95% limits of agreement. The mean difference less than 25% was used as an acceptable inaccuracy.

Table 1 Oligonucleotide sequences for telomere length measurement using qPCR

Primer Name	Oligomer sequences* (5' - 3')	Amplicon size
Telomere (Forward)	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT	76
Telomere (Reverse)	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT	
36B4 (Forward)	CAGCAAGTGGGAAGGTGTAATCC	75
36B4 (Reverse)	CCCATTCTATCATCAACGGGTACAA	

Note: *Oligomer sequences were followed from the previous study of Callaghan et al[7].

Results

Characteristic data of participants

A total of 190 healthy participants with 139 females and 51 males were included in this study. The overall age ranged from 20 to 76 years old with an average at 40.41 ± 13.53 years. The physical and blood parameters are listed in table 2. The upper and lower range of biochemical parameters were followed the criteria of the previous study which covered the biochemical values in older normal adult[10].

Table 2 Characteristic data of participants

Parameters	Mean (SD)	Median (min - max)
Age (years)	40.41 (13.53)	41 (20-76)
Weight (kg)	56.17 (8.77)	55 (40.8-92.6)
Height (cm)	161.95 (8.15)	160 (145-189)
Body mass index; BMI (kg/m^2)	21.31 (2.15)	21.2 (17-29)
Systolic blood pressure; SBP (mmHg)	115.69 (13.00)	115 (75-149)
Diastolic blood pressure; DBP (mmHg)	71.10 (8.15)	70 (50-89)
Fasting glucose (mg/dL)	83.92 (7.09)	83 (63-107)
Blood urea nitrogen; BUN (mg/dL)	12.58 (2.84)	12 (6-19)
Creatinine (mg/dL)	0.84 (0.18)	0.8 (0.4-1.4)
Cholesterol (mg/dL)	190.67 (25.37)	189 (119-253)
Triglycerides; TG (mg/dL)	77.17 (38.25)	67 (17-274)
High-density lipoproteins; HDL (mg/dL)	57.98 (11.61)	57 (32-97)
Low-density lipoproteins; LDL (mg/dL)	117.22 (24.09)	118 (50-172)
Aspartate aminotransferase; AST (U/L)	26.60 (5.19)	27 (16-47)
Alanine aminotransferase; ALT (U/L)	19.57 (8.01)	18 (9-68)
Alkaline phosphatase; ALP (U/L)	50.96 (15.32)	50 (16-92)
Albumin (g/dL)	4.68 (0.37)	4.68 (2.9-6.1)

The imprecision of the In-house telomere length qPCR method

The coefficient of variation (CV) for within-run and between-run are summarized in table 3. For the within-run assay, the %CV of cycle threshold of telomere gene and 36B4 gene was found with 3.75% and 1.16%, respectively, whereas the %CV of the between-run assay showed slightly higher than within-run assay as 3.38% of telomere gene, and 3.19% for 36B4 gene.

Table 3 Analytical imprecision of cycle threshold (Ct) of telomere gene and 36B4 gene using qPCR

Precision	Ct value of Telomere gene Mean ± SD (%CV)	Ct values of 36B4 gene (S) Mean ± SD (%CV)
Within-run (n = 18)	11.78 ± 0.44 (3.75)	18.28 ± 0.21 (1.16)
Between-run (n = 20)	12.07 ± 0.40 (3.38)	18.26 ± 0.58 (3.19)

The regression analysis of telomere length between the in-house qPCR method and the reference values

To evaluate the better correlation of telomere length between in-house qPCR method and the reference values, the gender separation was analyzed compared to all participants. The result showed that the correlation in male samples (R^2 = 0.6846) was better than that in females (R^2 = 0.5687) and all 190 participants (R^2 = 0.5672), respectively (Figure 1). The average converted telomere length measured by the in-house qPCR method was 7.48 Kb for males and 7.53 Kb for females, whereas the telomere length for males and females measured by the reference method was 7.44 Kb and 8.30 Kb, respectively (Table 4). In this study, the telomere length was calculated from the linear regression formula. To estimate the lower limit and upper limit or detectable range of this calculation, we have verified with the data from the values of 190 samples. Therefore, the lower detection of telomere length with our established method was obtained from the elderly group over 60 years with 4.66 kb in male and 4.03 kb in female as shown in table 4. The detectable range of telomere length in our in-house method was obtained at 4.66 – 10.69 Kb in males and 4.03 – 11.50 Kb in females compared to the reference method at 4.28 – 10.57 Kb in males and 4.79 – 11.94 Kb in females.

Table 4 The correlation analysis of telomere length between in-house qPCR and the reference method

	Telomere length (Kilobase)	
	In-house qPCR method	Reference method
Male (n = 51)		
Mean (SD)	7.48 (1.78)	7.44 (1.52)
Range (min - max)	4.66 – 10.69	4.28 – 10.57
Female (n = 139)		
Mean (SD)	7.53 (1.45)	8.30 (1.48)
Range (min - max)	4.03 – 11.50	4.79 – 11.94

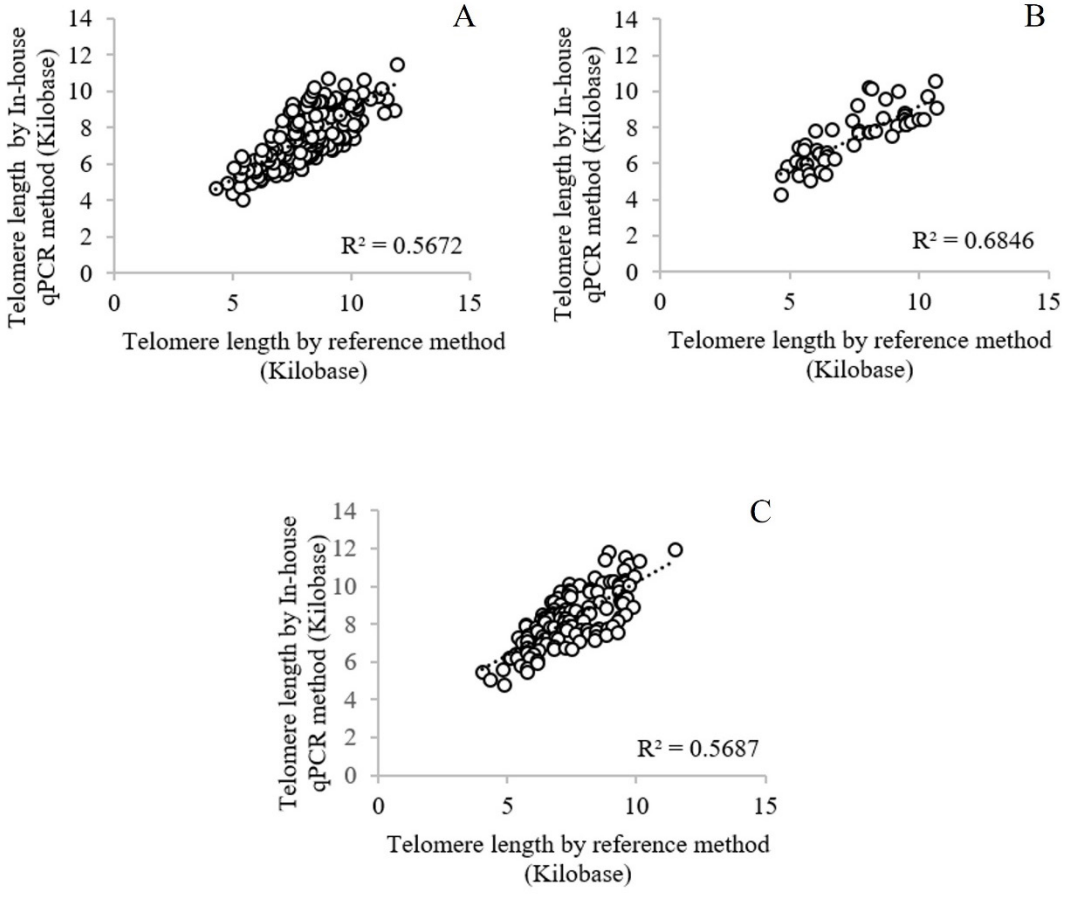

Figure 1 Linear regression plot of telomere length measured between in-house qPCR method and the reference method among A) total participants (n = 190), B) males (n = 51), and C) females (n = 139).

The Bland-Altman analysis of telomere length

To evaluate the difference of telomere length between two methods, Bland-Altman plot was performed as shown in figure 2. For total 190 samples, the mean of difference percentage of telomere length was -6.37 with 95% confidence interval at -31.7% to 18.9%. Moreover, the mean of difference percentage of more than ± 25% was set as an unacceptable inaccuracy. Only 5.26% (10 out of 190) were out of ± 25% which was obtained from females whereas no data from males was out of this range indicating approximately 95% of the study samples with acceptable accuracy.

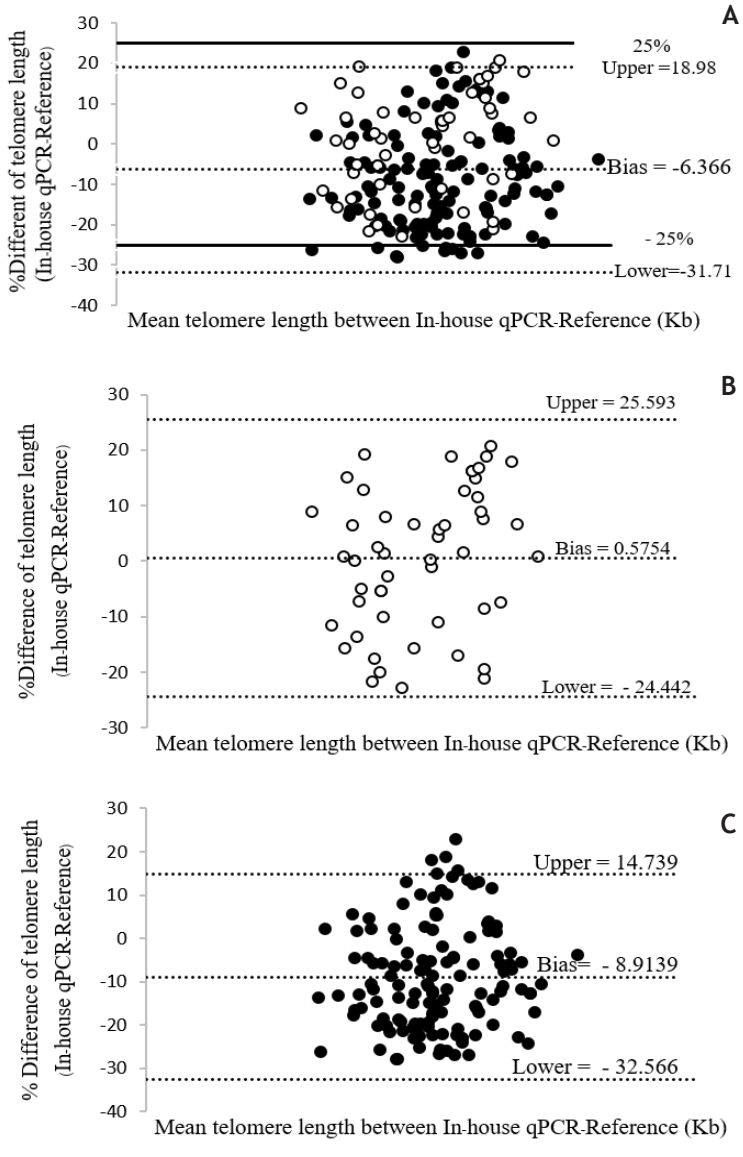

Figure 2 Bland-Altman plot for mean difference and 95% confidence upper and lower interval of telomere length measurement between in-house qPCR and reference method among A) total participants, B) males and C) females. A white circle indicates males and a black circle indicates females.

Discussion

Telomere length can be measured in many types of cells which have nucleus such as white blood cells (WBCs), buccal cells and tissues. However, the best types of cells are controversial among these reports. Thomas et al[14] investigated the telomere length using extracted DNA from WBCs, buccal cells, and brain tissues. Their results showed that absolute telomere length which was extracted from buccal cells was significantly shorter than that from WBCs even in control group or Alzheimer's group. On the other hand, Gadalla

et al[15] reported that the telomere length from blood cells was shorter than that of buccal cells and fibroblast from patient with dyskeratosis congenita and inherited bone marrow failure syndrome. In this study, WBCs were used according to their advantages in terms of high DNA yields and purity[13].

Nowadays, there is a commercial assay kit for human telomere length using qPCR available in the market; however, its high cost renders its limited uses. This study showed the successful establishment of in-house qPCR to estimate the telomere length using convert calculation from the T/S gene copy ratio.

The analytical performance of the developed method showed an excellent precision for both within-run and between-run CV of < 5% regarding the recommended imprecision for qPCR of less than 15%[8,9]. The minimal imprecision was achieved by including well internal control correction in every assay.

Regarding the reference value of comparison study, the telomere length measured from Teloage® analysis (Mediage, South Korea) has already been published[10]. In accordance with gender-related telomere length in table 3, longer telomere length was also observed in females which can be explained by the estrogen enhancement of telomerase activity[11]. Moreover, telomere shortening is correlated with many other factors, such as smoking, alcohol consumption, obesity, and lack of exercise exerting as the main role in increasing oxidative stress and inflammatory[12].

The comparison study between in-house qPCR and reference values using the Bland-Altman plot showed a significant and higher correlation in males than in females with 0% and 5.26% inaccuracy. The result illustrated that male group showed the lowest difference of telomere length between two methods compared to females and total participants; hence the estimation for males is more accurate than for other groups. This may be caused by an unequal number of males compared to females. Therefore, more sample sizes with equal number should be obtained for accurate estimation among gender difference in further study.

Conclusion

The development method of in-house qPCR for telomere length measurement was achieved with an acceptable performance of precision and accuracy compared to the reference values. However, this study was performed in a small sample size. Therefore, to apply the in-house qPCR, more sample size and aging status prediction from telomere length should be examined in a further study.

Take home messages

According to spontaneous cellular division, telomere length was known as aging biomarker in human. Shortening of telomere can be used to estimate the cellular aging of individual. To date, qPCR is concerned as one of the most convenient methods. The established in-house qPCR for telomere length provided acceptable performance compared to the reference values.

Conflicts of interest

The authors declare no conflict of interest.

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