Telomeres are composed of highly repetitive nucleotide sequences (5’-(TTAGGG)n-3’ in humans) and telomere-binding proteins at the ends of each chromosome, the latter of which preserve chromosome integrity (Blackburn, 1991). Telomere length shortening is induced naturally over time with each cell division due to end-replication problem; telomeres can also shorten as a result of oxidative stress (von Zglinicki, 2002). Observational studies have revealed that telomere lengths are shorter among older subjects and/or those exposed to more oxidative stress (Aubert et al., 2012; Guan et al., 2020). Telomere length is a reliable biomarker for cellular and biological aging and for quantifying an estimated accumulated exposure to oxidative stress (von Zglinicki, 2002). Observational studies have revealed that telomere lengths are shorter among older subjects and/or those exposed to more oxidative stress (Aubert et al., 2012; Guan et al., 2020). Telomere length is a reliable biomarker for cellular and biological aging and for quantifying an estimated accumulated exposure to oxidative stress (Babizhayev et al., 2011). Previous studies have reported an association between telomere length and a higher risk of being diagnosed with aging-related diseases including dementia, cardiovascular diseases, and diabetes mellitus (Sanders and Newman, 2013), thus popularizing the use of telomere length in the studies of human biology.

Several methods, such as Southern blotting, fluorescence in situ hybridization, or real-time quantitative polymerase chain reaction (qPCR), have been used to measure telomere length. Measurement by qPCR (Cawthon, 2002) has recently garnered significant attention because of its low cost, high throughput, and low DNA quantity requirement. In this method, the ratio of telomere repeat copy number (T) to the single-copy-gene copy number (S) (i.e. the T/S ratio) is used as an indicator of telomere length. The values of T and S are relative quantities calculated by comparison to a standard curve with serial dilutions of a reference DNA sample. The S value reflects DNA concentration (i.e. the number of chromosomes in a particular sample) and is used to normalize the value of T. The amplification primers of telomere sequences have mismatched bases to prevent the production of a primer dimer. Hence, the qPCR of telomere sequences may be sensitive to several factors such as preparation procedures for qPCR and minor errors in the temperature program due to imperfectly matched primers for telomeres. Thus, substantial inter-well or inter-plate variations in telomere length measured by qPCR using 96-well plates have been reported (Martin-Ruiz et al., 2015; Eastwood et al., 2018) possibly due to minor spatial inequalities in temperature within a plate (Eisenberg et al., 2015). Nevertheless, previous studies usually tested inter-well variations only for two or three wells which were close to each other and did not check variability throughout a 96-well plate in detail (Lin et al., 2010).

qPCR instruments can work not only with 96-well plates but also with 8-well tubes; 96-well plates are more commonly used due to the higher throughput they offer. The 8-well tubes have an advantage that stems from the structural char-
acteristics; the PCR solution mixes and collects at the bottom of each well more reliably in 8-well tubes than in 96-well plates, which can potentially affect qPCR results.

In human biology field surveys, dried blood spot (DBS) samples from a finger prick are commonly used instead of blood samples collected from the cubital vein, because the procedure is minimally invasive and samples can be stored at room temperature for a considerable time. However, the reliability of telomere length in DBSs measured by qPCR has not been fully investigated (Zanet et al., 2013; Stout et al., 2017; Goldman et al., 2018).

Here, we report the results of our investigation on the reliability of DBS telomere length measurement by qPCR. The telomere lengths measured in two different containers (96-well plates and 8-well tubes) were compared. Because the amount of DNA extracted from DBSs is low, the reagent needs to be very thoroughly and carefully mixed with template DNA and primers. This is far easier with 8-well tubes than with 96-well plates. Careful selection of primers is also indispensable to producing accurate results. To determine which method is most reliable, we assessed the intra- and inter-assay variations in DBS telomere length measured by qPCR using two different containers (96-well plates and 8-well tubes) and two common primer sets.

Methods

Sampling

DBS samples from a male subject were collected on Whatman 903 Protein Saver Cards (Cytiva, Marlborough, MA, USA) by finger prick and stored at –80°C after drying until DNA extraction for telomere length measurement. The current study was approved by the Ethics Committee of the Faculty of Medicine at the University of Tokyo (No. 12033-3).

Determination of telomere length of DNA extracted from DBSs

Chromosomal DNA was extracted from six punches (3 mm in diameter) of DBS using the QIAamp DNA Investigator Kit (QIAGEN, Hilden, Germany). Following the manufacturer’s protocol, DNA was eluted in 50 μl buffer.

The telomere length of the DNA extracted from the DBS was measured by qPCR methods with minor modifications from previous studies (Cawthon, 2002; Lin et al., 2010). The methods determine a T/S ratio, which is used to approximate the subject’s telomere length. Human genomic DNA from human blood (Merck & Co., Inc., Darmstadt, Germany) was diluted serially and used as the reference DNA for calibration curves to compute relative quantities in qPCR. qPCR with 96-well plates was conducted using FastSYBR Green Master (Thermo Fisher Scientific, Waltham, MA, USA) with the Applied Biosystems StepOne real-time PCR system (Thermo Fisher Scientific), whereas that with 8-well tubes was run on LightCycler Nano (Roche, Penzberg, Germany) with the FastStart Essential DNA Green Master (Roche). Two common primer sets (tel1–tel2 and tel1b–tel2b primer sets) were used for telomer PCR, whereas the 36B4u-36B4d primer set was used for single-copy-gene PCR. Primer sequences and temperature programs are displayed in Table 1. The linearities of all calibration curves were adequate ($r^2 > 0.98$). Wells used for qPCR on 96-well plates were scattered throughout including edges, whereas all wells in the tubes were used for 8-well tube protocols.

Statistical analysis

We calculated an intra-assay coefficient of variation (CV) of relative quantity of telomere and 36B4 amplification as well as the T/S ratio in each protocol. The inter-assay CV of four repeated measurements by the protocol having the lowest intra-assay CV was also assessed. Two-way analysis of variance (ANOVA) was used for comparison between effects of container types and primer sets.

Results

Table 2 shows that the intra-assay CV of relative quantities of telomere PCR differed greatly among the four protocols; the CV was the highest in the 96-well plate/tel1b–tel2b protocol and the lowest in the 8-well tube/tel1–tel2 protocol. As expected, the intra-assay CV of the relative quantities of 36B4 PCR did not vary (2.8–4.9%). Intra-assay CVs of T/S ratios also differed among protocols; the CV was the lowest in the 8-well tube/tel1–tel2 protocol and the highest in the 96-well plate/tel1b–tel2b protocol. The 8-well tube/tel1–tel2 protocol showed sufficiently low inter-assay CV (5.0%, $n = 4$). Two-way ANOVA indicated a statistically significant difference in T/S ratio depending on type of container and primer set; the interaction term between type of container and primer set was also significant ($P < 0.001$).

### Table 1. Primer sequences, concentrations, and temperature programs

| Primer   | Sequences [5′–3′]                          | Concentration (nM) | Temperature program* |
|----------|-------------------------------------------|--------------------|----------------------|
| tel1     | GGTTTTTGAGGTTAGGGTGTTAGGGTTAGGGTTAGGG    | 270                | 95°C 15 s            |
| tel2     | TCCCCGACTATCCCCCATCCCCCATCCCCCATCCCCCAT   | 900                | + 54°C 120 s         |
| tel1b    | CGTTTTGGTTTTGGTTTTGGTTTTGGTTTTGGTTTTGGTT | 100                | 96°C 1 s             |
| tel2b    | GCCCTTGCTTACCCCTTACCCCTTACCCCTTACCCCTT   | 900                | + 54°C 60 s          |
| 36B4a    | CAGCAAGTGGGAAAGGTGTAATCC                 | 300                | 95°C 15 s            |
| 36B4d    | CCCATCTTATCATCAACGGGTACAA                | 500                | + 58°C 60 s          |

* Denaturing + annealing/extension. The number of cycles was 40.
We found that the T/S ratios determined by qPCR with 8-well tubes and the tel1–tel2 primer set had the lowest intra-assay CV, whereas the T/S ratios determined by qPCR with 96-well plates and the tel1b–tel2b primer set, which are commonly used in previous studies, had the highest intra-assay CV. Variation of T/S ratios was also explained by the interaction between container and primer set. A possible explanation for the lower intra-assay variation in T/S ratios determined by qPCR with 8-well tubes is the simplicity of preparation procedures in the 8-well tubes, such as mixing PCR solution. Additionally, compared with 8-well tubes placed around the center of a heating well-block, inter-well variations in temperature on 96-well plates might be slightly larger due to their relatively larger surface; common procedural knowledge suggests that wells at the edges of 96-well plates do not yield accurate qPCR measurements. Even if the inter-well variation in temperature is negligible in 36B4 qPCR experiments, this can nevertheless result in high intra-assay CVs in telomere measurements as primer sequences used in telomere qPCR include mismatched bases (Cawthon, 2002); primers with mismatched bases for the telomere sequence amplification process are most likely sensitive to the minor spatial inequalities in temperature within a plate (Eisenberg et al., 2015). Mean values of T/S ratios also varied substantially among different protocols. It was difficult to judge which ratio was correct, because the true value of a T/S ratio is unknown. The gold standard for measuring telomere length is the mean terminal restriction fragment length, determined by Southern blotting. However, the DNA yield extracted from DBS samples is insufficient for this method and is thus a limitation of this study. However, our findings suggest that T/S ratios vary with the difference in primers used for the amplification process of telomere sequences. Therefore, we strongly recommend that the names of primers should always be reported in studies using qPCR to determine telomere length.

In conclusion, we found that DBS T/S ratios measured by qPCR using common protocols (i.e. 96-well plates) can have large intra-assay CVs. Assessing the intra-assay CV prior to measurements of DBS telomere length with qPCR is crucial.

### Discussion

We declare no actual or potential competing financial interest.

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### Conflicts of interest

We declare no actual or potential competing financial interest.

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