A quantitative real-time PCR method for absolute telomere length

Nathan J. O'Callaghan, Varinderpal S. Dhillon, Philip Thomas, and Michael Fenech
Commonwealth Scientific and Industrial Research Organization (CSIRO)-Human Nutrition, Adelaide, Australia

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Telomere shortening is an important risk factor for cancer and accelerated aging. Here we describe the development of a simple and reproducible method to measure absolute telomere length. Based on Cawthon's quantitative real-time PCR (qRT-PCR) assay, our method uses an oligomer standard that can be used to generate absolute telomere length values rather than relative quantification. We demonstrate a strong correlation between this improved method and the "gold standard" of telomere length measurement—terminal restriction fragment analysis (TRF) by Southern hybridization. The capability to generate absolute telomere length values should allow a more direct comparison of results between experiments within and between laboratories.

Telomeres are composed of long hexamer (TTAGGG) repeats that protect against spontaneous DNA damage (1–4). An important risk factor for cancer and accelerated aging (1–4) is telomere shortening. We have developed a simple and reproducible method to measure absolute telomere length. This method is based on Cawthon's quantitative real-time PCR (qRT-PCR) assay, which, in its original format, produces a relative measure of telomere length (5).

Genomic DNA was isolated using the silica-gel-membrane–based DNeasy Tissue Kit (Qiagen, Melbourne, Australia) as described by Lu et al. (7). All buffers were purged with nitrogen and supplemented with 50 µM phenyl-tert-butyl nitrone (Sigma, Sydney, Australia) to minimize oxidative damage to DNA, which may alter the efficiency of the PCR if abasic sites are generated (6). The initial high temperature lysis and proteinase K protein digestion (10 min at 56°C) was replaced by an extended incubation (6 h) at 37°C to minimize abasic site generation (7). Following elution of purified DNA, 1 mM DTT (dithiothreitol) was added and the DNA solution stored at -80°C until required (7). DNA was quantified in triplicate using a NanoDrop spectrophotometer (Biolabs, Melbourne, Australia).

Quantitative real-time amplification of the telomere sequence was performed as described by Cawthon (5) with the following modifications. A standard curve was established by dilution of known quantities of a synthesized 84mer oligonucleotide containing only TTAGGG repeats (Geneworks, Adelaide, Australia). The number of repeats in each standard was calculated using standard techniques as follows:

- The oligomer standard is 84 bp in length (TTAGGG repeated 14 times), with a MW of 26667.2.
- The weight of one molecule is MW/Avogadro's number. Therefore, weight of telomere standard is: $2.6667 \times 10^4/6.02 \times 10^{23} = 0.44 \times 10^{-19}$ g.
- The highest concentration standard (standard A) had 60 pg of telomere oligomer (60×10^{-12}g) per reaction.
- Therefore there are $60 \times 10^{-12}/0.44 \times 10^{-19} = 1.36 \times 10^9$ molecules of oligomer in standard A.
- The amount of telomere sequence in standard A is calculated as: $1.36 \times 10^9 \times 84$ (oligomer length) $= 1.18 \times 10^8$ kb of telomere sequence in standard A.

A standard curve was generated by performing serial dilutions of standard A (10^{-1} through to 10^{-6} dilution). Plasmid DNA (*pBR3222*) was added to each standard to maintain a constant 20 ng of total DNA per reaction tube. The standard curve was used to measure content of telomeric sequence per sample in kb (Figure 1). The amount of test sample DNA per reaction was adjusted so that the cycle threshold (C_T) values were within the linear range of the standard curve.

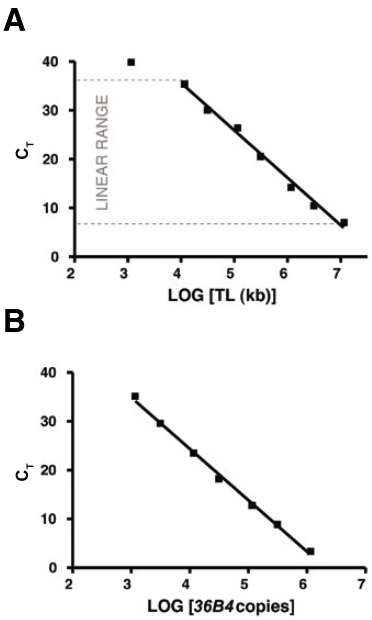

Figure 1. Standard curves used to calculate absolute telomere length. C_T (cycle threshold) is the number of PCR cycles for which enough SYBR Green fluorescence was detected above background. (A) Graph shows standard curve for calculating length of telomere sequence (TL) per reaction tube. x-axis represents amount of telomere sequence in kb per reaction. Correlation coefficient within the linear range was 0.98. The graph shown here represents the linear range of the PCR. The DNA amount was optimized so that experimental samples are detected within the linear range. The value generated from the experimental samples using this standard curve was equal to kb of telomere sequence per sample. (B) Graph shows standard curve for calculating genome copies using *36B4* copy number. Correlation coefficient was 0.99. Standard curves were generated using an AB 7300 Sequence Detection System with the SDS Ver. 1.9 software.

All samples were run on an ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software (Applied Biosystems [AB] Foster City, CA, USA). Each sample was analyzed in duplicate. A single copy gene, *36B4*, which encodes the acidic ribosomal phosphoprotein P0, was used as a control for amplification for every sample performed, as described in Cawthon (5). The latter data was used for the relative measure, but was also

essential for the absolute method to quantify the amount of DNA or number of genomes in each well. Each 20 µl reaction was performed as follows: 20 ng DNA, 1 × SYBR Green master mix (AB), 100 nM telomere forward primer (CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT), 100 nM telomere reverse primer (GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT) (5,8). Cycling conditions (for both telomere and *36B4* products) were: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. DNA from the 1301 lymphoblastic cell line was used as a long telomere control (telomere length of 70 kb) in each plate run. The inter- and intra-experimental coefficient of variation of the 1301 telomere length measurement by absolute qRT-PCR was 7% and 1.1%, respectively.

After amplification was complete, the AB software produced a value for each reaction that was equivalent to kb/reaction based on the telomere standard curve values. This kb/reaction value was then exported into MS Excel where the final calculations were done to determine total telomere length per diploid genome. The kb/reaction value was then used to calculate total telomere length in kb per human diploid genome. The number of diploid genomes was calculated using the *36B4* product. Similar to the oligomer used for standardization of absolute telomere length, a 79mer oligomer was synthesized containing the *36B4* product (79 bp). The telomere kb per reaction value was divided by diploid genome copy number (calculated from the *36B4* C_T and standard curve) to give a total telomeric length in kb per human diploid genome. This value can be further used to give a length per telomere (by dividing by 92, which is the total number of telomeres on 23 pairs of chromosomes).

Genome copy number per reaction was calculated as follows:

• The synthesized *36B4* oligomer standard is 79 bp in length with a MW of 23268.1.

• The weight of one molecule is MW/Avogadro's number. Therefore, weight of the synthesized *36B4* oligomer standard is: $2.32681 \times 10^4/6.02 \times 10^{23} = 0.38 \times 10^{-19}$ g.

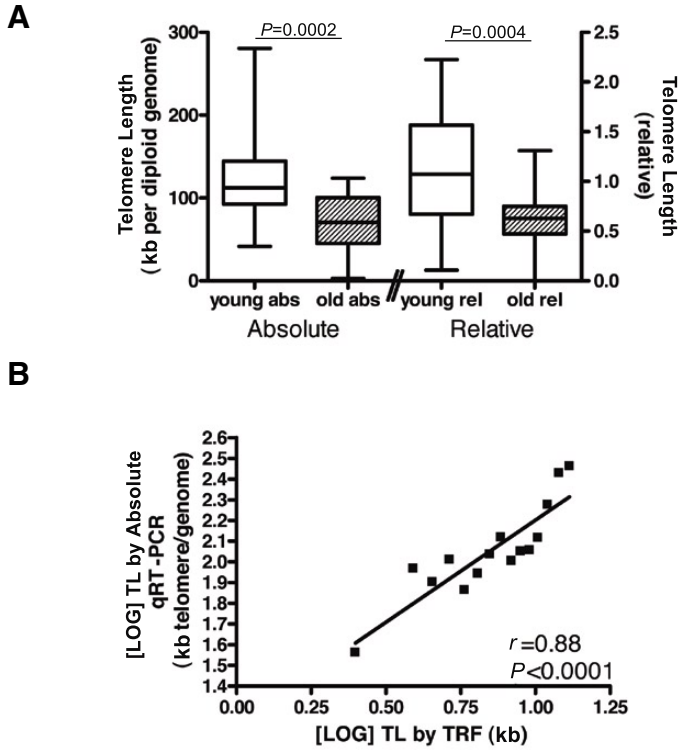

Figure 2. Comparison of methods for telomere length measurement. (A) Absolute and relative telomere length in whole-blood DNA of young controls (mean age ± SEM 22.5 ± 2.2, $N = 26$) and old controls (mean age ± SEM 68.7 ± 2.6, $N = 25$). Telomere length determined using the Cawthon method (Relative) and the same dataset analyzed using a 84mer TTAGGG repeat as a standard curve (Absolute). Using the relative method, the old controls have significantly shorter telomeres ($P < 0.001$). Similarly, using the absolute method, the old controls have significantly ($P < 0.0001$) shorter telomeres. Data are shown as box plots, which represent five-number summary of the data (the minimum, lower quartile, median, upper quartile, and maximum). (B) Graph represents correlation between terminal restriction fragment analysis (TRF) and absolute qRT-PCR methods. Telomeres were measured in whole-blood, lymphocytes, mid-rectal biopsies, and low and high controls (Telo Telomere length assay, Roche) ($r = 0.88$, $P < 0.0001$). Data was LOG transformed. TL, telomere length.

• The highest concentration standard had 200 pg of *36B4* oligomer (20×10^{-12} g) per reaction.

• Therefore, there are $200 \times 10^{-12}/0.38 \times 10^{-19} = 5.26 \times 10^9$ copies of *36B4* product in standard A.

• Therefore, standard A is equivalent to 2.63×10^9 diploid genome copies because there are two copies of *36B4* per diploid genome.

We compared the reproducibility of the relative method for analysis of telomere content, as described by Cawthon (5), with the reproducibility of the absolute method described here. We used a dataset from whole-blood samples of healthy young (mean age 22.5 ± 2.2, $N = 26$) and old (mean age 68.7 ± 2.6, $N = 25$) adults of similar gender ratios (15/15 and 11/15, respectively) (Figure 2A). While the expected differences between young and old subjects were consistent between methods, inter-individual variation for measured telomere content in the young and old group were lower using the absolute method [(mean ± SD) 126.1 ± 59 and 69.5 ± 37] as compared with using the relative method (1.12 ± 0.56 and 0.61 ± 0.34, respectively). Data from 320 samples (including samples from the above dataset) showed that the coefficient of variation for intra-experimental variation (i.e., variation between duplicate measures for each sample) was 40% for the relative method and 12% for the absolute method, which suggest better reproducibility for the absolute qRT-PCR method. Furthermore, using these datasets, a correlation between age and telomere length can be obtained. For

the relative method the r value was -0.46 ($P = 0.001$) and for the absolute the r value was -0.56 ($P < 0.0001$). This was similar to previously reported decline of telomere length with age, using terminal restriction fragment analysis (TRF) (9).

We also compared the absolute qRT-PCR telomere method against TRF, the gold standard of telomere length measurements (10). Telomere lengths were determined by a TRF diagnostic kit (Roche Diagnostics, Sydney, Australia). We demonstrate a strong correlation between results for TRF and the absolute qRT-PCR methods ($r = 0.88$, $P < 0.0001$) (Figure 2B). However, there was a consistent discrepancy between the two values, with the TRF value being somewhat greater than that observed with the absolute qRT-PCR method (an approximate 7 kb difference). It is recognized that the TRF method tends to overestimate telomere length because there is a considerable nontelomeric component of inter-individual variation within TRFs (11). In addition to TTAGGG, terminal restriction fragments in human DNA contain variable amounts of repeat sequences, which are detected as telomere sequence in the TRF assay. These include telomere repeat variants proximal to the telomere and the telomere adjacent sequences (reviewed in Reference 11). Additionally, as TRF is based on hybridization, the shorter the telomere the lower the hybridization signal—which indicates there is a telomere length threshold below which TRF analysis will not detect. Interestingly, two of the test samples used in this comparison were not detected by TRF but had very short telomere length as measured by absolute qRT-PCR. Although TRF analysis biases toward longer telomere length, this can be corrected to a certain degree by dividing the signal intensity by length in bp (4), although this is not always done.

Cawthon first described the use of qRT-PCR to measure telomere length in 2002 (5). Since then there have been numerous reports that have effectively used this method (12–17). In conclusion we have developed a modification to the Cawthon method introducing an oligomer standard to generate absolute telomere length values rather than relative quantification. We also show that such a method might be more reproducible than the relative method, possibly because only results within the linear C_T range are used for both the telomere and genome copy measures. The capability to generate absolute telomere length values using qRT-PCR technology should allow a more direct comparison of results between experiments within and between laboratories and provides a more practical quantitative method compared with the TRF assay.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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Address correspondence to Nathan O'Callaghan, Commonwealth Scientific and Industrial Research Organization (CSIRO)-Human Nutrition, Genome Health, Nutrigenomics and Nutrigenetics, P.O. Box 10041, Adelaide, BC 5000, South Australia, Australia. e-mail: nathan.o'callaghan@csiro.au; or Michael Fenech (CSIRO). e-mail: michael.fenech@csiro.au