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Telomere length measurement by qPCR – Summary of critical factors and recommendations for assay design

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Abstract

Research in the last decade has explored the length of telomeres, the protective ends of eukaryotic chromosomes, as a biomarker for the cumulative effects of environmental exposures and life experiences as well as a risk factor for major diseases. With a growing interest in telomere biology across biomedical, epidemiological and public health research, it is critical to ensure that the measurement of telomere length is performed with high precision and accuracy. Of the several major methods utilized to determine telomere length, quantitative PCR (qPCR) remains the most cost-effective and suitable method for large-scale epidemiological and population studies. However, inconsistencies in recent reports utilizing the qPCR method highlight the need for a careful methodological analysis of each step of this process. In this review, we summarize each critical step in qPCR telomere length assay, including sample type selection, sample collection, storage, processing issues and assay procedures. We provide guidance and recommendations for each step based on current knowledge. It is clear that a collaborative and rigorous effort is needed to characterize and resolve existing issues related to sample storage, both before and after DNA extraction, as well as the impact of different extraction protocols, reagents and post extraction processing across all tissue types (e.g. blood, saliva, buccal swabs, etc.) to provide the needed data upon which best practices for TL analyses can be agreed upon. Additionally, we suggest that the whole telomere research community be invited to collaborate on the development and implementation of standardized protocols for the assay itself as well as for reporting in scientific journals. The existing evidence provides substantial support for the continuation of telomere research across a range of different exposures and health outcomes. However, as with any technological or methodologic advance in science, reproducibility, reliability and rigor need to be established to ensure the highest quality research.

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Declaration of interest

Jue Lin is a co-founder of Telomere Diagnostics Inc. and serves on its scientific advisory board. The company plays no role in the current manuscript and the research conducted in the author's laboratory. The other authors have no conflict of interest.

Keywords

Telomere length measurement; Aging; Biomarker; Quantitative PCR; Preanalytical factors; Analytical factors

1. Background

Telomeres are the protective complexes at the ends of eukaryotic chromosomes, consisting of short tandem DNA repeats and their associated proteins. Telomere maintenance is not only essential as a fundamental cellular process; it is also etiologically linked to a range of different human diseases (Stanley and Armanios, 2015). More importantly, recent works have explored telomere length as a biomarker for both the cumulative effects of environmental exposures and life experiences as well as future disease susceptibility (Blackburn et al., 2015; De Meyer et al., 2018; Haycock et al., 2017; Ridout et al., 2018; Willis et al., 2018). Longitudinal studies further indicate that the change of telomere length over time is shaped by genetic and non-genetic influences throughout life (Factor-Litvak et al., 2017; Shalev et al., 2013). With a growing interest in telomere biology in biomedical, epidemiological and public health research, telomere length is measured in many different laboratories utilizing different assays with no standardization of cohort size. Further, the different approaches to measurement and reporting make it challenging to combine results from different studies or even to compare them. It is now critically important to ensure that the measurement of telomere length across different studies is performed with high precision and accuracy.

Several major methods are utilized to determine telomere length, each with specific strengths and weaknesses, particularly for population based and large sample size biomedical research. These methods include telomere restriction fragment (TRF) length analysis by Southern blot analysis, quantitative PCR (qPCR) amplification of telomere repeats relative to a single copy gene, and fluorescent *in situ* hybridization (FISH) to quantify telomere repeats in individual cells or chromosomes by flow cytometry (Flow-FISH) or in metaphase cells (Q-FISH). Although there are other methods of telomere measurement, these are not included here given their limited utility in population based studies. Recent reviews have summarized the advantages and disadvantages of each method (Aubert et al., 2012; Barrett et al., 2015; Lai et al., 2018; Montpetit et al., 2014; Nussey et al., 2014); therefore, we will not further discuss these issues in detail. Telomere length measurement as a diagnostic tool for diseases caused by rare mutations in telomere maintenance genes has been validated for Flow-FISH under the regulatory agency of Clinical Laboratory Improvement Amendments (CLIA) (Alter et al., 2007; Stanley and Armanios, 2015), is also not discussed here as while this methodology has a high accuracy, it is not feasible in population based studies.

Specific features of the TRF analysis and the Flow-FISH method limit the use of these methods in epidemiological and population studies. For example, Southern blot analysis requires large quantities of genomic DNA, therefore is not suitable for studies with limited quantities of specimen. Flow-FISH requires fresh blood and is not applicable for solid

tissues or archived samples. Additionally, both TRF analysis and Flow-FISH are labor-intensive and costly. qPCR, on the other hand, is relatively low-cost, and can be performed utilizing small quantities of DNA obtainable from most archived samples. Therefore, qPCR remains most suitable for large epidemiological and population-based studies that typically require hundreds or thousands of samples. However, inconsistencies across multiple components of the qPCR TL assay highlight the need for a careful methodological review of each step of this process. This review begins with the initial selection of the biological sample source (e.g. blood, saliva etc.), but also includes detailed information about collection methodology, storage, extraction of DNA, and post-extraction processing in addition to a precise description of the assay conditions. We highlight components of the assay that are often inadequately described, including: the source of reference sample DNA, quality control tests for DNA integrity and contamination, determination of replicate number, standard practice for cross-plate controls determination of inter and intra assay variability, and methods for data analysis. The goal of this review paper is to summarize critical issues related to the qPCR telomere length assay and to provide guidance and recommendations based on current knowledge. These recommendations are based either on published literature, or personal experience from the authors' labs. We make a distinction between the two levels of evidence throughout the paper. We hope this review will serve as a first step towards developing a set of guidelines endorsed by experts in the field.

2. Brief description of the qPCR method

qPCR telomere length measurement was first described by Richard Cawthon in his 2002 paper (Cawthon, 2002). The principle of this method is that the abundance of telomere signal per genome measured by qPCR represents the average telomere length in a given DNA sample (Fig. 1 A). The amount of input genomic DNA is quantified by measuring the qPCR product of a single copy gene, and is used to normalize the signal from the telomere reaction. The resulting T/S ratio represents the average telomere length per genome. In a follow-up paper, Cawthon described a monochrome multiplex qPCR (mmQPCR) method that simultaneously measures the signals of telomeres and a single copy gene in a one-tube reaction (Cawthon, 2009). This was possible because telomeres are much more abundant than the single copy gene. The telomere signals are collected in early PCR cycles, before single copy gene signals rise above baseline; and the single copy gene signals are collected at a temperature that fully melts the telomere products (Fig. 1B). This monochrome multiplexing PCR method eliminates assay variabilities due to potentially variable amounts of DNA pipetted in the separate T and S reactions, a possible drawback of the protocol described in the 2002 paper. The mmQPCR also has increased throughput and decreased reagent cost.

3. Sample source

Based on tissue type and collection methods, several specimen types have been used for qPCR telomere length measurement (summarized in Table 1). Each specimen type has relative advantages and challenges and, due to cell type differences, may be differentially relevant to a specific outcome or factor being examined in relation to telomere length. We outline these advantages and disadvantages below focusing on samples that can be collected

peripherally and repeatedly in an effort to provide specific guidance to both cross sectional and long-itudinal studies.-

3.1. Blood

Whole blood (and buffy coat, PBMCs and granulocytes that are derived from whole blood) in circulation is one of the most often reported specimens utilized in telomere research. Whole blood is relatively easy to obtain from venous blood draw, and a significant number of past and current epidemiological and population studies have utilized whole blood for telomere length measurement. When cell type specificity is important, cell sorting methods can be used to separate out specific cell types from peripheral blood mononuclear cells (PBMCs). However, the high cost of cell sorting and the low quantity of DNA yield for cell types with low abundance needs to be considered. A number of studies have utilized DNA extracted from finger and heel pricks collected on protein saver cards (Whatman® protein saver cards) (also referred to as dry blood spots, or DBS) and have reported a high correlation with venous blood (Stout et al., 2017; Zanet et al., 2013). However, T/S ratios measured in finger pricks are higher than venous blood from the same person. This is at least partly caused by the action of blotting anti-coagulated WB onto paper, but may also be due to differential cell types obtained from capillaries compared to veins (Zanet et al., 2013). Also, the inter-assay coefficient of variation (CV) is higher in dry blood spots compared to venous blood and this could be due to the low concentration/yield from DBS, which is shown to in-fluence qPCR (Hsieh et al., 2016). Nevertheless, the potential to determine individual telomere lengths from the large numbers of already-archived DBS samples from newborns would represent a significant advance for the field. In the future, microtainer tubes (https://www.bd.com/en-us/offerings/capabilities/specimen-collection/blood-collection/capillary-collection/bd-microtainer-blood-collection-tubes) that can collect a few hundred microliters of blood directly into an anticoagulant might be a good alternative to DBS. It is also important to point out that given the profound changes of the immune system in response to recent infections/illness, including mobilization of hematopoietic stem cells and proliferation of naïve cells (Glatman Zaretsky et al., 2014), it is likely that telomere length measured at the time of acute infection is different from before and after the infection. Studies should avoid collecting blood samples if the participant is showing signs of illness, has recently had a major injury or surgery, or is taking immunosuppressive agents (e.g. steroids). When this is not feasible, attention to measures of immune function as potential covariates is recommended.

3.2. Other peripheral DNA sources: passive drool, saliva swabs and buccal cell swabs

Although blood has the largest body of literature to date, an increasing number of studies have utilized DNA extracted from less invasive sources, most prominently saliva from passive drooling, saliva collected with swabs and buccal cells from cheek swabs. In addition to being less invasive, these alternative collection methods decrease the cost of collection, pose less risk of blood borne pathogen exposure, allow potential home collection with shipping by participants directly to research sites, and provide simpler post-collection immediate storage conditions. A significant number of studies have successfully used salivary DNA obtained from proprietary kits that use a stabilizing solution (e.g. Oragene) (https://www.dnagenotek.com/US/index.html). These kits offer several significant

advantages including streamlined collection methods, stability at room temperature for long periods of time, high quantity of DNA and appropriately designed collection devices for infants through adults. However, caution is warranted when comparing telomere length measured from saliva obtained through passive drool (as with the kits described above) and saliva obtained using swabs or sponges (e.g. Oragene DNA/OG575 for assisted collection from DNA Genotek and collection devices by Oasis Diagnostics®) as the percentage of buccal cells when swabs and sponges are utilized for saliva collection is significantly higher than that found in saliva collected though passive drool (Theda et al., 2018).

Swabs designed to specifically collect buccal cells (as opposed to a mixture of saliva and buccal cells collected in the Oragene DNA/OG575 kit or collection devices by Oasis Diagnostics®) offer several advantages over saliva collection kits including significantly decreased cost and more uniform cell type. Buccal swabs collect predominantly buccal epithelial cells with rare white blood cells. For studies examining phenotypes associated with neural tissue and pathways, particularly psychiatric disorders, the use of buccal cells may be preferred given that both are derived from the ectoderm and the immune cells found in blood and saliva are mesodermal in origin. The presence of buccal cells in saliva likely account for the greater epigenetic overlap with the brain compared to blood (Smith et al., 2015).

3.3. Cross tissue comparisons

Several studies have compared telomere length in different tissues from the same individual, and concluded that telomere lengths from these different tissues are significantly correlated (Daniali et al., 2013; Friedrich et al., 2000; Stout et al., 2017). Correlations between TL measured from venous blood and saliva from the same individual are reported to be modest, but statistically significant (Goldman et al., 2018; Stout et al., 2017). However, the difference between different tissues are still considerably larger than the group differences found using a single tissue source in most studies. Thus, combining data from different tissues is not advisable.

An important factor to consider in interpreting telomere length data measured in blood, saliva, or buccal cells is that each contain both a variable number of different cell types as well as different ratios of cell types. In addition, the ratio of different cell types can even vary within a single tissue source at different time points of collection, indicating that careful consideration of the relative length of telomeres by cell type is needed when designing studies. Although telomere lengths of these cell types from the same individual are correlated (Lin et al., 2010), there is evidence that different immune cells can have consistently different telomere lengths. For example, B cells have longer telomere length compared to T cells; naïve cells have longer telomere length compared to memory cells. Therefore, interpretations of telomere length difference, either in cross-sectional or longitudinal studies should always consider the possibility that apparent difference/change maybe due to changes in cell compositions. Ideally, telomere length of specific cell types should be measured in sorted cells, but this is often impractical for archived samples and studies with large sample size and/or limited quantities of specimen. Statistically adjusting for cell compositions, measured by differential cell complete blood count (CBC) or other

immune phenotyping methods, is an alternative in this case and, for the most part, does not appear to significantly change the conclusions.

4. Sample storage

As mentioned earlier, saliva samples in a stabilizing solution can potentially be stored at room temperature for several years. Although studies definitely confirming stability of telomeres in saliva stored at room temperature for extended periods of time have not been systematically evaluated, in our experience, DNA extracted after saliva has been collected and stored in Oragene kits (OG-500) at room temperature for up to 3 years appear intact when run on agarose gels. Buccal swabs stored with a desiccator pellet are also stable for several months at room temperature before DNA is extracted. If DNA extraction is expected to be delayed by more than six months, we recommend that buccal swabs be stored at −20 °C based on our lab's experience. Venous blood samples, however, need to be kept immediately at −80 °C for long term storage. Although blood from finger pricks collected on FTA cards (Whatman® FTA® card) or protein saver cards (Whatman® protein saver cards) stored at room temperature is considered suitable for DNA stability in general, we recommend that these cards be kept at −80 °C for long term storage until studies comparing DNA stored under different conditions can be conducted. At the very least, when examining telomere length in archived DBS samples, studies should include a clear description of how these samples were stored and for how long. Most epidemiological and population studies involve biospecimens collected and stored over a long period of time. It is not uncommon that telomere length measurement is conducted several years after the completion of specimen collection, especially for studies where telomere length or change of telomere length over time is the independent variable used for prospective correlations. To the best of our knowledge, no study has investigated the effect of long-term storage of whole blood on qPCR telomere length measurement. A systematic examination of this important pre-analytical factor is required to fully understand how sample storage impacts qPCR telomere length assay and what is the best approach to handle this. We do know that freezing-thaw cycles of blood had minor effects on qPCR telomere length measurement (Tolios et al., 2015; Zanet et al., 2013). A recent paper by Dagnall et al found that T/S ratios of low concentration genomic DNA (1 ng/ul) stored at 4 °C or −30 °C for 6 months correlated poorly with their original values, however samples at higher concentrations (25 ng/ul) demonstrated strong correlations (Dagnall et al., 2017). Given the high sensitivity of DNA samples to storage conditions and extraction methods, we suggest that samples should be stored as frozen blood at −80 °C and DNA extracted right before the assay. We further recommend that length of time stored prior to extraction be recorded and evaluated as a potential covariate until larger, systematic studies are conducted. For studies utilizing buccal samples, we recommend an alternative approach. Specifically, we recommend that DNA is extracted within six months and stored at −80 °C in aliquots, in order to minimize the number of future freeze/thaw cycles of the DNA. While bacterial contamination has been raised as a concern for both saliva and buccal analyses, experiments in our laboratories that varied the amount of bacterial DNA failed to impact telomere length measurement from saliva, buccal, or whole blood samples.

5. DNA extraction and DNA storage

By far, DNA extraction methods appear to be the most impactful factor for qPCR telomere length assay, particularly for blood. Several studies published in the last few years showed that telomere length measured by qPCR using DNA samples extracted with different extraction kits are not only systematically different, but poorly correlated (Boardman et al., 2014; Cunningham et al., 2013; Denham et al., 2014; Hofmann et al., 2014; Raschenberger et al., 2016; Tolios et al., 2015). Currently, we do not fully understand how these different extraction methods impact qPCR telomere length assay. These kits use different technologies to remove other cellular components and contaminants. For example, the salting out method (Miller et al., 1988) employed by commonly used kits such as Puregene from QIAGEN removes protein by precipitation in a high salt concentration solution. On the other hand, the spin-column method, such as the QIAamp mini columns, removes impurities by passing the cell lysate through silica membrane columns that only bind to DNA under certain salt and pH conditions (Boom et al., 1990). These different ways of purification may contribute to potential variations. The sizes of the genomic DNA are reported to be different, with 100–200kbp for the salting out method and up to 50 kb for the mini columns based on the specifications for the kits. The size difference can also potentially influence the assay. Ostensibly, these methods all yield DNA samples that pass basic quality control criteria, namely, above certain concentration threshold and with OD260/OD280 between 1.7–2.0 (Raschenberger et al., 2016). It is possible that residual impurities that are not detectable by spectrometry and/or residual chemicals used in the DNA extractions affect qPCR reactions. To further add complications, post-extraction DNA purification methods to remove potential inhibitors introduce another level of variability in qPCR telomere length assay (Dagnall et al., 2017). Importantly while a significant number of studies have evaluated the impact of different extraction methods on telomere length measured from blood (Boardman et al., 2014; Cunningham et al., 2013; Dagnall et al., 2017; Denham et al., 2014; Hofmann et al., 2014; Raschenberger et al., 2016), to our knowledge, no data exists examining different extraction and purification techniques for salivary and buccal samples.

The reports described above did not compare TL from different DNA-extraction methods to another TL-detection method, such as Southern blot or Flow-FISH; therefore, it is difficult to conclude which of the several DNA extraction kits used in these papers yields the most accurate and precise qPCR TL data. However, we are aware of several studies that compared the qPCR measurement with the Southern blot analysis or Flow-FISH using DNA extracted with the QIAamp® DNA Kit (Aviv et al., 2011; Imam et al., 2012; Martin-Ruiz et al., 2015). Aviv et al reported a study of blind telomere length measurements on the same samples from 50 donors performed in with Southern Blot analysis and qPCR on two different occasions. Both the qPCR and Southern blots displayed highly reproducible results as shown by $r > 0.9$ for the correlations between results obtained by either method on both occasions. The correlation between these two methods is also $r > 0.9$ (Aviv et al., 2011). Imam et al reported that TL measured by qPCR and Flow-FISH in 26 cord blood DNA samples showed that the two methods yielded highly correlated TL measures ($r = 0.91$; $P < 0.0001$) (Imam et al., 2012). Finally, a comparison of telomere length assessment by 10 different laboratories, employing 3 different techniques [2 Southern blot labs, 1 single telomere length analysis

(STELA) lab and 7 real-time quantitative PCR (qPCR) labs] used the QIAamp® DNA Midi kit and showed that TL measured by these three methods are highly correlated (Martin-Ruiz et al., 2015). Hsieh et al also described optimization of the mmQPCR on the LightCycler using samples purified with QIAamp Mini DNA kit and showed that the correlations with Southern Blot and flow FISH to be r = 0.938 and 0.9 respectively (Hsieh et al., 2016). Based on the results described above, we recommend the QIAamp® kits for DNA extraction for qPCR telomere length measurement. Other DNA extraction methods may also be appropriate and direct comparisons across different methods is warranted. Comprehensive studies that compare telomere length obtained from DNA extracted utilizing different extraction kits and methods that further test the relative correlation of TL estimates across different measurement assays (e.g. Southern blot, qPCR and FLOW-FISH) are necessary to further determine the best DNA extraction methods for telomere length determination that is relevant for both clinical trials and studies in smaller cohorts and methods amenable to larger population cohorts such as qPCR.

As mentioned before, DNA storage conditions are known to impact quantitative PCR reactions, although with potentially less impact than on the Southern blot (TRF) method. The Southern blot technique requires intact high molecular weight DNA, while qPCR amplification can potentially tolerate partially degraded DNA, as the amplicons are typically short (< 100bp). As described earlier, Dagnall *et al* showed that samples stored at 25 ng/ul maintained strong correlations to the original results after 6 month at both 4 °C (r = 0.927) and −30 °C (r = 0.916). Samples at 1 ng/ul were very weakly correlated to their original results when stored at 4 °C (r = 0.33) and only moderately correlated when stored at −30 °C (r = 0.7). It appears that both low concentration and storage of DNA at refrigeration temperature, rather than a preferable −80 °C, introduce considerable variability. Since DNA sample degradation impacts telomere sequence significantly (Tolios et al., 2015), assessing DNA integrity by visualization on agarose gels or other methods such as the measurement of double stranded DNA content using Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA) or Quanti-iT Picogreen dsDNA assay (Thermo-Fisher) is required.

6. qPCR assay conditions

The first publication of the qPCR TL method by Cawthon (Cawthon, 2002) has been cited 839 times based on Pubmed records as of August 27, 2018. The more recent 2009 paper that described the novel monochrome multiplex quantitative PCR method (mmQPCR) has also been cited 280 times. Adaption of the original methods by many different groups means that modifications have been made for many aspects of the assay. These variations may contribute to the relatively large inter-lab variations reported by the recent international 10 lab collaboration (Martin-Ruiz et al., 2015). As noted by the authors of this paper, telomere primer sequences, primer concentration, choice of single copy gene, master mixes made by different vendors or homemade master mixes, and their associated reaction chemical components, PCR program conditions, inclusion of quality control (QC) samples, number and types of QC samples, PCR instruments, data analysis method, mmQPCR vs. monoplex, and QC criteria all have the potential to impact the assay (Martin-Ruiz et al., 2015). These factors, together with the preanalytical factors mentioned above, may independently contribute to assay variability, limiting the ability to adjust for these differences in a

systematic way. Below we discuss some of these factors in more detail by organizing them as three classes of considerations.

6.1. Assay reagent

Both homemade and commercial master mixes are used for qPCR telomere length measurement. Commercial master mixes are available from many vendors. Due to the propriety nature of these commercial products, we do not know the individual components of each master mix and how they might impact the assay (Hsieh et al., 2016). However, commercial master mix has the benefit of being readily available. A recent report by Jiménez and Forero examined 8 commercial SYBR Green and High-Resolution Melting (HRM) mixes on the measurement of TL by the mmQPCR method master and discovered that master mix influences the measurement of TL, affecting specificity and consistency of the results (Jimenez and Forero, 2018). Whether using homemade or commercial reagents, it is important to validate the assay when first setting it up and to qualify each new lot of reagents.

6.2. Reference standard

By design, the T/S ratios of the qPCR telomere length method are relative values compared to a reference standard, typically a genomic DNA sample from a commercial source or pooled genomic DNA generated within the lab. Attention to the source of the DNA for the standard is also important. For example, the use of a pooled DNA standard from blood as a reference for salivary telomere length measurement introduces an additional source of variance and should be avoided. Studies should report the source of all standards and confirm that the same reference DNA was utilized for all telomere measurements in the specific cohort.

Comparison of T/S ratios across different labs is also hindered by the fact that reference standards differ between labs. One approach to address this is the absolute telomere length method, initially developed by Michael Fenech's group (O'Callaghan and Fenech, 2011). This method uses an oligonucleotide with telomere sequence and an oligo-nucleotide with a single copy gene sequence to serve as the reference standard for telomere reaction and single copy gene reaction respectively. Since the absolute quantities of the telomere and single copy gene oligonucleotides are known, the average telomere length per genome, expressed in base pairs, can be calculated. One challenge using these oligonucleotides is that very low amounts are used in the reactions, making it difficult to obtain accurate and reproducible dilutions. Procedures to accurately quantify the amount of oligonucleotides and calibrate them against DNA samples with known telomere length, measured by other methods, should be implemented before adopting the telomere and single copy gene oligonucleotides as universal cross laboratory references standards.

6.3. Data analysis methods

Quantification of telomere length by qPCR adopted methods originally used in quantitative gene expression analysis. The $2^{-\Delta\Delta CT}$ algorithm uses the difference of cycle thresholds (CT) of the target gene and a caliber gene to calculate the relative gene expression levels (Livak and Schmittgen, 2001). This has been adopted for telomere length measurement, with

the difference between the CTs of the telomere reaction (T) and single copy gene (S) representing the relative abundance of telomere sequence normalized by the single copy gene. For the ΔΔCT calculation to be valid, the amplification efficiencies of the telomere and single copy gene must be approximately equal. This needs to be validated before applying this method for a specific study. The absolute quantification method calculates the abundance of telomere and single copy gene relative to a standard curve, which is created by serial dilutions of a reference standard DNA. This absolute quantification approach assumes that the PCR efficiencies of the reference standard DNA and the test samples to be equal, which often is not validated. In order to address the possibility that the PCR efficiency of each sample might be different, a custom-made data analysis method called LinReg was developed (http://www.hartfaalcentrum.nl/index.php?main=files&fileName=LinRegPCR.zip&description=LinRegPCR:%20qPCR %20data %20analysis&sub=LinRegPCR) (Pfaffl, 2001). The LinReg program determines the PCR efficiency of each sample by a Window-of-Linearity and then determines the CT value (Ramakers et al., 2003; Ruijter et al., 2009). However, it appears that groups adopting this analysis method have routinely calculated the average PCR efficiency of all samples from each assay plate and applied this average efficiency to individual samples (Raschenberger et al., 2016). At the very least, studies should be required to report the quality metrics utilized prior to analysis for PCR efficiencies of the standard curves if the absolute quantification method is used.

Also, qPCR telomere length assays are typically run in triplicate wells, with some labs running duplicate runs of triplicate wells. Steps to assess data quality should be reported. These should include methods of excluding outliners within the triplicate wells or duplicate wells, samples that fall outside of the standard curve range and criteria for repeat runs. As studies increasingly use longitudinal samples, it is important to have all samples from a particular individual on the same plate to ensure that trajectory analyses are not impacted by plate to plate variability.

7. Special consideration for longitudinal studies

The impacts of sample storage and variable qPCR assay conditions pose a special challenge on longitudinal telomere length studies, especially those with a long follow-up time. By the time follow-up samples are collected, the baseline samples would have been stored for years. We are faced with several choices: A) Assay baseline and follow-up samples right after they are collected and DNA extracted from them. In this case, the DNA extraction and assay reagents from different time points are most likely from different lots for longitudinal studies that span multiple years. B) Extract DNA from baseline samples right after they are collected, store the DNA and assay these early samples together with the follow-up samples. In this case, the DNA extraction kits used in the baseline and follow-up visits are most likely from different lots. Furthermore, there is a possibility of DNA degradation over time for the baseline samples. C) Extract DNA from baseline and follow-up samples after the follow-up samples are collected. In this case, DNA extraction and assay reagents are from the same lot. However, the baseline samples would have been stored for years, and the impact of long-term storage effects, especially for whole blood samples, have not be examined carefully. Nevertheless, weighing these options and taking into consideration of the importance of

DNA extraction, we recommend option C as we think extracting all samples with the same lot of DNA extraction kit is the most critical aspect. When immediate DNA extraction before the assay is not practical or feasible, for example, in longitudinal studies with long follow-ups, where only DNA has been archived, we recommend using consistent storage conditions and DNA extraction kits with careful attention to the purity of DNA samples, through measurement of the OD260/OD230 and OD260/280 ratios and double stranded DNA integrity. When this is not feasible, analyses should include extraction conditions and storage time as initial covariates in order to determine if batch effects are present. Studies should work with the vendors of the extraction kits to ensure the same lot of kits is used for all studies. In cases when this is not feasible, a comparison study using different lots should be considered to adjust for any systematic difference between lots. Systematic examination of the effects of long-term storage of whole blood, saliva and buccal samples will be required in the future to further optimize longitudinal study designs of telomere length assay using qPCR. Current studies should report which of the above approaches was taken. Beyond these considerations, samples from the same individual should be run on the same plate to prevent additional sources of variance due to plate to plate variability.

8. Validation and report of assay performance

Key assay parameters need to be validated when a lab first sets up the qPCR TL assay. Minimally, assay specificity, linearity, precision and accuracy, reproducibility with intra and inter-assay coefficient of variations, lower and upper limits of quantification (LLOQ and ULOQ), and the performance of at least three quality control samples that representing short, medium and long telomeres should be assessed when establishing the assay. For full validation, the effects of freeze-thaw cycles, short-term and long-term sample stability as well as reagent stability should also be determined. This information should be described in the method section in publications.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines are recommendations that target the reliability of real-time PCR results. They have been in place for nearly a decade and help ensure the integrity of scientific literature, the transparency of experimental procedures and consistency between different labs (Bustin et al., 2009). These guidelines help reviewers and readers assess the reliability and validity of the protocol used. With the wide-use of the qPCR TL method, implementing a similar list of guidelines will help move the field towards adopting a more rigorous standard for qPCR TL. We propose a checklist of critical information that should be included in publications (Table 2).

9. Conclusions

Quantitative PCR remains the most commonly used method for telomere length measurement due to its low cost, ease of adaptation, low DNA quantity requirement, and obvious advantages for high throughput analyses. Our review of publications that addressed the preanalytical and analytical factors that influence qPCR for telomere length measurement revealed that preanalytical factors are likely the most important sources of experiment-induced variation. Critically, studies clearly indicate that different DNA

extraction kits resulted in significant differences in telomere length measurement using the qPCR assay. The field urgently needs to understand how these preanalytical factors contribute to assay variability and to resolve these methodologic issues for each tissue type in order to develop standardized procedural recommendations that can be implemented across different laboratories and peer reviewed journals.

In addition to a set of reference standards distributed to labs that perform qPCR TL, as suggested by the 10-lab study (Martin-Ruiz et al., 2015), we also recommend a set of guidelines that describes the minimum information necessary for evaluating qPCR TL for publication so that reviewers and readers can assess the validity of the protocol used (Table 2). Comparison of a set of common quality control samples across existing labs currently performing qPCR telomere length assays that would be available for new labs seeking to develop the technology would be highly beneficial. It is clear that a collaborative and rigorous effort is needed to resolve existing issues related to sample storage and processing across all tissue types. Additionally, we suggest that the whole telomere research community be invited to collaborate on the development and implementation of standardized protocols for the assay itself as well as for reporting in scientific journals. The existing evidence provides substantial support for the continuation of telomere research across a range of different exposures and health outcomes. However, as with any technological or methodologic advance in science, reproducibility, reliability and rigor need to be established to ensure the highest quality research.

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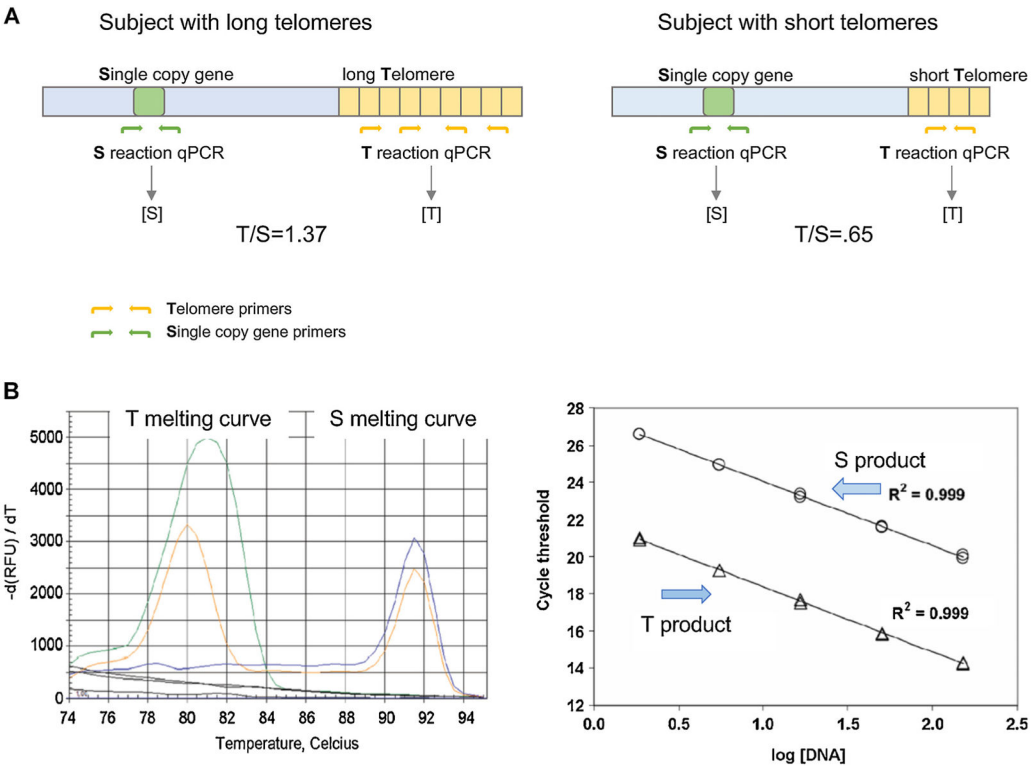

Fig. 1.

A. Schematic diagram of qPCR telomere length measurement as described in Cawthon 2002 (Cawthon, 2002). Examples of two subjects, with high and low T/S ratios, representing long and short average telomere length, are presented. These numbers are arbitrary, but are in the typical range of values for normal, healthy adults when a pooled human genomic DNA is used as the reference standard. B. Schematic diagram of monochrome multiplex quantitative PCR method (mmQPCR) for telomere length measurement, adapted from the original publication (Cawthon, 2009).

Table 1

Comparisons of Commonly Used Specimens for Telomere Length Assay.

Specimen type	Pros	Cons
Whole blood	Low biovariability	Need a phlebotomist
	Post blood draw processing is minimal	Need to process specimen on site
	Excellent DNA yield and quality	Need to have a − 80 °C freezer for storage
		Mixed immune cell type
Buffy coat	Low biovariability	Same as whole blood except longer processing time (~l/2h)
	Excellent DNA yield and quality	Mixed immune cells
	Higher DNA yield compared to whole blood	
PBMCs	Low biovariability	Need a phlebotomist
	Excellent DNA yield and quality	Need to process specimen on site within hours of collection
	Can also assay telomerase activity and other assays that require live cells if stored in liquid nitrogen	Sample processing takes 2–3 hours, is complex and needs a fully equipped lab
		Need to have a − 80 °C freezer for storage
		Mixed immune cell type
Granulocytes	Excellent DNA yield and quality	Same as PBMC + extra ~ 1 hour processing time
	Pure cell type	
Finger pricks or heel pricks (dry blood spot)	Less invasive sample collection compared to venous blood draw	DNA yield much lower. Concentrations may be low and require several punches or extensive processing to ensure appropriate DNA amount
	Can be collected by the participant (adult)	Assay CV higher (~ 6% vs ~ 3%) in Jue Lin's lab using a modified method based on Cawthon, 2002
	Simple to ship and stable with desiccant	
Salivary collection kits (Oragene) *	Easy sample collection (can be collected at home)	Higher biovariability
	Can be stored at room temperature for years	Mixed cell type (leukocytes + buccal cells)
	Large DNA amount and simple extraction that can be	Inter individual variability in ratio of cell types
	automated	Differences in stability dependent upon kit
Buccal swabs	Easy sample collection	Relatively short storage time
	Can be stored with desiccator pellet for months	May not reflect systemic exposure
	Lower biovariability	
	Majority of cells are buccal	
Cord blood	Excellent DNA yield and quality	Potential contamination from maternal source

Specimen type	Pros	Cons
	Higher DNA yield compared to whole blood	

*Note: infant Oragene kits collect a mixed ratio of cells that are more likely to contain a higher percentage of buccal cells. Comparison between infant swabs and passive drool collection methods should be done with caution.

Table 2

Suggested Publication Guideline for qPCR TL.

Experiment Design	Importance
Experimental and control groups (numbers): race, age, sex	E
Laboratory where the assays carried out	E
Author contribution	D
Sample	
Sample type	E
Sample processing procedure (time frame over which samples extracted, storage time before DNA extraction	D
Storage condition and duration including buffer and number of freeze thaw cycles	E
DNA extraction	
Kit or instrumentation	E
DNA quantification method	D
DNA quality control metrics (260/280 ratio, integrity, dsDNA content (OD260/OD280, yield)	E
qPCR validation	
PCR efficiencies	E
Linear dynamic range	D
NTC Ct	D
Assay Conditions	
Assay setup condition (manual vs. instrumentation, what instrument)	D
PCR Master mix (home-made vs. commercial master mix, vendor name)	E
Primer sequence	E
Complete assay condition (dNTP, Mg + +, primer and polymerase concentrations, DNA amount, other components, reaction volume, thermo-cycling conditions)	E
Monoplex vs. multiplex	E
Number of replicates and +/− duplicate plates and change in well position	E
qPCR instrument	E
Data analysis	
Analysis program	E
Method of data analysis	E
Reference standard (source and concentrations used to create standard curve)	E
Normalization and adjustment	E
Acceptance and rejection criteria	E
Intra and inter-assay cv	E

E: Essential information, must be submitted with the manuscript.

D: Desirable information, should be submitted if available.