Measurement of Telomere Length: A New Assay Using QuantiGene Chemistry on a Luminex Platform

Muhammad G. Kibriya[1], Farzana Jasmine[1], Shantanu Roy[1], Habibul Ahsan[1,2,3,4], and Brandon Pierce[1,4]

Abstract

Background: Telomeres are tandem repeats of sequences present at the end of the chromosomes that maintain chromosomal integrity. After repeated cell division, telomeres shorten to a critical level, triggering replicative senescence or apoptosis, which is a key determinant of cellular aging. Short telomeres also contribute to genome instability and are a hallmark of many cancers. There are several methods for estimating telomere length (TL) from extracted DNA samples. Southern blot is accurate but requires a large quantity of DNA and is expensive. qPCR is cost-effective and requires a small quantity of DNA and is therefore widely used for large-scale epidemiologic studies; however, it typically requires triplicates. We describe a novel multiplexed probe-based non-PCR method for TL measurement.

Methods: A small amount of DNA (~50 ng) is hybridized to telomere repeat sequence–specific probes (T) and a reference single gene probes (R). T and R signals are detected from a single reaction well containing the same input DNA. Branching DNA technology is used to amplify the signal, which is detected by Luminex technology.

Results: The intra- and interassay CV (~3% and ~5%, respectively) shows the precision of the new assay and the measurements from single well correlated well with traditional single-plex qPCR run in triplicate ($r = 0.7$ to 0.8). The assay was also validated in an independent set of samples using Southern blot ($r = 0.74$).

Conclusion: We describe a novel assay for TL assessment using the Luminex platform.

Impact: This may offer an alternative cost-efficient way to study TL in extracted DNA samples.

See all the articles in this *CEBP Focus* section, "Biomarkers, Biospecimens, and New Technologies in Molecular Epidemiology."

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Introduction

Telomeres are tandem repeat sequences (TTAGGG) present at the end of chromosomes and are responsible for protecting chromosomal integrity (1). After repeated cell divisions, telomeres shorten to a critical level, triggering replicative senescence or apoptosis, which is key determinant of cellular aging (2). Cancer cells maintain short and stable telomeres, often by activating telomerase, enabling uncontrolled proliferation (3). Critically short telomere length (TL) contributes to genome instability and carcinogenesis (4–6). TL is shorter in leukocytes of patients with chronic heart failure, atherosclerotic disease (7), and myocardial infarction (8), and nonHodgkin lymphoma (9). A meta-analysis also suggests association of TL and risk for various cancer types (10). Currently available methods for TL measurement are discussed by Aubert and colleagues (11). In 1990, Harley and colleagues measured fibroblast telomere by Southern blot (2) and subsequently Kimura and colleagues established a standard protocol (12). Other methods used are quantitative fluorescence *in situ* hybridization (Q-FISH), developed by Lansdorp and colleagues in 1996 (13). Cawthon and colleagues, in 2002 developed qPCR method for simple, rapid, and high-throughput measurement of TL (14). In 2009, Richard Cawthon improved the assay by multiplexing to reduce variation (15). In 2011, O'Collaghan and colleagues developed another modification of the qPCR method to measure absolute TL (16). Mainly because of cost efficiency and low DNA requirements, most large-scale studies use the qPCR method for telomere measurement. In this article, we describe a novel probe-based non-PCR assay for TL assessment using QuantiGene plex (QGP) chemistry on the Luminex platform.

Materials and Methods

Genomic DNA was extracted from whole blood of 70 adults and 6 children using the FlexiGene DNA Kit (Qiagen).

[1]Department of Health Studies, Biological Sciences Division, the University of Chicago, Chicago, Illinois. [2]Department of Human Genetics, the University of Chicago, Chicago, Illinois. [3]Department of Medicine, the University of Chicago, Chicago, Illinois. [4]Comprehensive Cancer Center, the University of Chicago, Chicago, Illinois.

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Corresponding Author: Muhammad G. Kibriya, Department of Health Studies, Biological Sciences Division, The University of Chicago, KCBD Building, Room 6110, 900 East 57th Street, Chicago, IL 60637. Phone: 773-834-9737; Fax: 773-834-9738; E-mail: kibriya@uchicago.edu

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We used Affymetrix-Panomics QuantiGene Plex (QGP) chemistry for a DNA copy-number assay using custom-designed probes to measure abundance of the telomere repeat sequence on the Luminex platform. The principle of the assay is described elsewhere (http://www.panomics.com/products/dna-copy-number/dna-multiplex-assay/how-it-works). In summary, fluorescent Luminex microbeads with capture probes (CP) are used to capture DNA molecules. For the each DNA target, two target-specific probe sets are designed (see Fig. 1): (i) Capture extenders (CE) and (ii) Label extenders (LE) and blocker (BL) probes. CE has two parts—one part is complementary to the CP sequence on the bead and the other part is complementary to the target DNA sequence that is interrogated. LE has two parts—one is complementary to the target DNA sequence and the other is complimentary to the "pre-amplifier." The target-specific regions of CE, LE, and BL hybridize to contiguous sequences of the target DNA. The preamplifier binds with multiple biotinylated amplifiers. Each amplifier provides multiple hybridization sites for biotinylated label probes that bind Streptavidin R-Phycoerythrin (SAPE) producing fluorescent signals. The signal intensities from the luminex bead and the conjugated SAPE are read on a Luminex 200 instrument. The signal is reported as median fluorescent intensity (MFI) and is proportional to the number of target sequences in the sample. This assay allows multiplexing up to 33 DNA targets in a single well. The probes (both CE and LE) for the telomeric region were designed to target the repeats "TTAGGG." The 24-mer probe was targeted against four repeats—"TTAGGGTTAGGGTTAGGGTTAGGG." For reference single gene, we used *ALK*, which showed very stable copy number (CN = 2) in all the DNA samples detected by oligonucleotide-based microarray SNP chips for our previous study (data not shown).

Assay optimization

As per the manufacturer's protocol for the QGP DNAplex assay, the samples need to be sonicated to get the fragment size up to 500 bp to improve hybridization efficiency. We faced several technical issues with sonication: (i) with Misonix XL-2000 Sonicator with 1/8 inch probe fragmenting the DNA samples one by one in a single tube is a tedious job for large sample size and requires two to three times more DNA due to evaporation during sonication; (ii) when we tried to fragment DNA in a plate in Episonic Bioprocessor, the fragmentation size varied depending on the location of the sample in the plate; (iii) fragmentation improved the signal intensity for the reference gene but it reduced the signal intensity for telomere perhaps due to preferential fragmentation of telomeric region (data not shown). As telomere is the region of interest for this assay, we modified the assay by omitting DNA fragmentation. To improve the signal from reference gene, we increased the number of target-specific probes (CE and LE) to hybridize over a larger span of genomic regions.

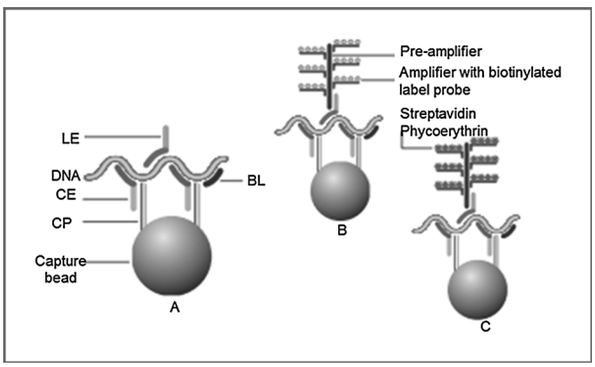

Figure 1. Schematic diagram for the QGP assay. Modified from http://www.panomics.com/products/dna-copy-number/dna-multiplex-assay/how-it-works. A, hybridization step: luminex bead capturing the target DNA sequence with the sequence-specific probes; B, addition of preamplifiers and amplifiers; C, binding of SAPE-producing fluorescent signals.

Protocol

We used a control DNA as a standard (400, 200, 100, 50, 25, 12.5, and 6.25 ng per well) in triplicates with background wells. In Plate-A, we ran 30 unique samples and each DNA was used twice, at 50 and 100 ng per well. In Plate-B, the same 30 samples were run after 2 weeks but with 50 ng/well and 25 ng/well. In Plate-C, we ran an independent set of 23 samples in triplicates at varying input from approximately 50 to 100 ng/well and similarly another set of 23 samples was run in Plate-D.

DNA samples were diluted to 1.25 ng/μL. Then, the 40 μL sample was mixed with 18 μL of Lysis mixture, 5 μL Probe set, and 5 μL of 2.5 mol/L NaOH. The mix was incubated for 30 minutes at room temperature followed by neutralization by 12 μL of buffer provided in the kit. The bead mix was made for 96-well plate (per well Nuclease-free water, 1.8 μL; Lysis mixture, 15 μL; Blocking reagent, 2 μL; Proteinase K, 0.2 μL; and Capture Beads, 1μL) and dispensed in the hybridization plate. The sample mix was added to the hybridization plate and was put on a shaking incubator for 20 hours at 54°C.

The following day, the plate was taken out of the incubator and after a quick spin the whole content was transferred to magnetic separator plate and put on a magnetic washer bed. After 5 minutes, the supernatant was dumped, and the plate was washed three times with the freshly prepared wash buffer. Then, 100 μL of preamplifier (36 μL preamplifier in 12 mL diluent) was added per well and the plate was incubated for 1 hour at 50°C.

The plate was washed on a magnetic washer bed three times with wash buffer. Then, 100 μL of amplifier (36 μL amplifier in 12 mL diluent) was added per well and incubated for 1 hour at 50°C. After similar wash, 100 μL of label probe (36 μL Label probe in 12 mL of diluent) was added, and the plate was incubated again in a similar fashion. The next step was to bind the sample with SAPE. After a similar wash, 100 μL SAPE (36 μL SAPE in 12 mL

diluent) was added to each well. The plate was then incubated in a shaker incubator at 600 rpm for 30 minutes at room temperature. After the binding was done, the plate was washed as before and SAPE buffer was added. After shaking the plate for 3 minutes at room temperature, the plate was read on a Luminex200 reader.

Statistical analysis

The raw intensity data from the Luminex instrument were processed using the Exponent software to generate the MFI data. The MFI values were imported into the Miliplex Analyst software to generate a standard curve using the 5-parametric logistic (5PL) curve (17, 18).

For standard qPCR, we followed the Cawthon method (14). For qPCR index of a given sample, the standard $\Delta\Delta C_T$ method was used and then expressed as percentage of the same control DNA sample run in the same plate. The same control DNA was also used for the QGP assay. For validation with Southern blot in an independent set of DNA samples, we used the measurements from a previously published article by Aviv and colleagues (19) using the standard Southern blot protocol established earlier by the same group (12). Coefficient of variation (CV%) was calculated using standard formula: standard deviation/mean.

Results

The standard curves using the MFI data (Fig. 2) show the linear range for both telomere and reference gene (*ALK*) probe. We expressed the relative TL in two ways: (i) telomere quantity index (TQI)—ratio of the quantity of DNA measured by the telomere probe and by reference gene probe and (ii) telomere intensity index (TII; described later). For each sample, the quantity of DNA in each well is calculated against a standard curve from a "control DNA sample" that is run for each plate. Therefore, the TQI represents TL in a sample relative to the "control DNA" and this measured ratio is supposed to account for plate-to-plate variation in intensity measurement. For the TII, we first make the telomere probe intensity correction for quantity of input DNA (measured by the probe for the reference gene) and then the mathematically corrected intensity of the sample is compared with that of the "control DNA sample" run in each plate. Therefore, this normalization also should account for plate-to-plate variation.

How does the assay work compared to standard qPCR method? Table 1 shows the correlation between the QuantiGene assay (run in single well) and standard qPCR method (mean from triplicates) using the published primer set (14). The assay did not work in only one well in plate A (with ~100 ng input DNA) and one well in plate B (with ~50 ng input DNA) due to technical failure with low bead count and/or low input DNA. Both the measurements (TQI and TII) of the QuantiGene assay from different settings correlated well with the qPCR data (correlation coefficient "*r*" from 0.70 to 0.83) except the TQI from 25 ng input DNA ($r = 0.5697$). Figure 3 shows that the assay correctly detects longer TL in children DNA samples compared with adult DNA.

How does the assay work with different input amount of DNA? We have compared the single measurements from input of approximately 50 ng/well and approximately 100 ng/well in the same plate (plate A) from 29 unique samples. The geometric mean of intraplate CV (between ~50 ng and ~100 ng input DNA) for TQI and TII were 3.03% and 6.52% respectively. (see Table 2).

Interplate variation for the assay. We compared the single measurements from approximately 50 ng input DNA from two different plates (plate A and plate B run more than 2 weeks apart) for 29 unique samples. The geometric mean of interplate CV for TQI and TII were 4.97% and 2.93%, respectively (see Table 2).

Intraplate variation among the triplicates. To assess the precision of the assay, we tested the results from triplicates (in the same plate) of independent set of 46 unique samples. This time the DNA samples were not normalized to achieve similar concentration for all samples to get uniform input quantity. Triplicates of 23 samples were accommodated in Plate-C and triplicates of other 23 samples were run in Plate-D. The geometric mean of intraplate CV of the triplicates for TQI and TII were 5.96% and 3.77%, respectively (see Table 2).

From the calculated DNA input amount in each well using the reference gene probe, we could measure the CV

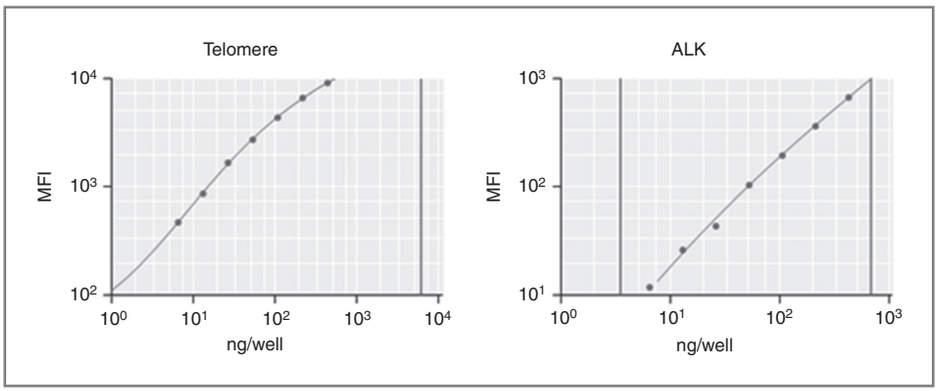

Figure 2. Standard curve for telomere (on the left) and reference gene-*ALK* (on the right). *X*-axis, DNA in ng/well; *y*-axis, MFI.

Table 1. Correlation between QGP assay and qPCR

Plate	Input DNA	QGP measurement	qPCR measurement	Correlation coefficient *r*
Plate-A	50 ng ($n = 30$)	TQI	qPCR Index	0.7086
	100 ng ($n = 29$)	TQI	qPCR Index	0.8068
Plate-B	50 ng ($n = 29$)	TQI	qPCR Index	0.7060
	25 ng ($n = 30$)	TQI	qPCR Index	0.5697
Plate-A	50 ng ($n = 30$)	TII	qPCR Index	0.7461
	100 ng ($n = 29$)	TII	qPCR Index	0.8257
Plate-B	50 ng ($n = 29$)	TII	qPCR Index	0.7378
	25 ng ($n = 30$)	TII	qPCR Index	0.7161

of pipetting accuracy (as the DNA sample is the same, the variation in quantity is supposed to be due to the variation in volume pipetted in the triplicate wells for each sample). It was interesting to note that the geometric mean CV for pipetting accuracy was 6.92% (see Supplementary Fig. S1A). It may be noted that this variation in pipetting volume could potentially affect the traditional single-plex qPCR result as the telomere and reference gene are amplified in different wells.

Is the correlation between QuantiGene assay and qPCR reproducible in different set of samples? From the triplicates, we calculated the mean of TQI for each of this second independent set of 46 unique samples and correlated with qPCR results. An almost similar level of correlation ($r = 0.69$) was seen without plate effect (see Supplementary Fig. S1B), as we found in case of results from single-well measurement and qPCR in the first set of 29 samples.

How does the assay work compared with multiplex qPCR method? The assay showed reasonable correlation ($r = 0.55$; see Supplementary Fig. S2A) to the multiplexed qPCR invented by Cawthon RM (15).

How does the assay work compared with the gold-standard Southern blot method? We carried out the newly developed QGP assay for TL measurement twice on an independent set of 50 DNA samples (shipped from Steven Hunt's laboratory, Cardiovascular Genetics Division of University of Utah School of Medicine) in a blinded way. The same DNA samples were used by Aviv and colleagues in their comparative study published in 2011

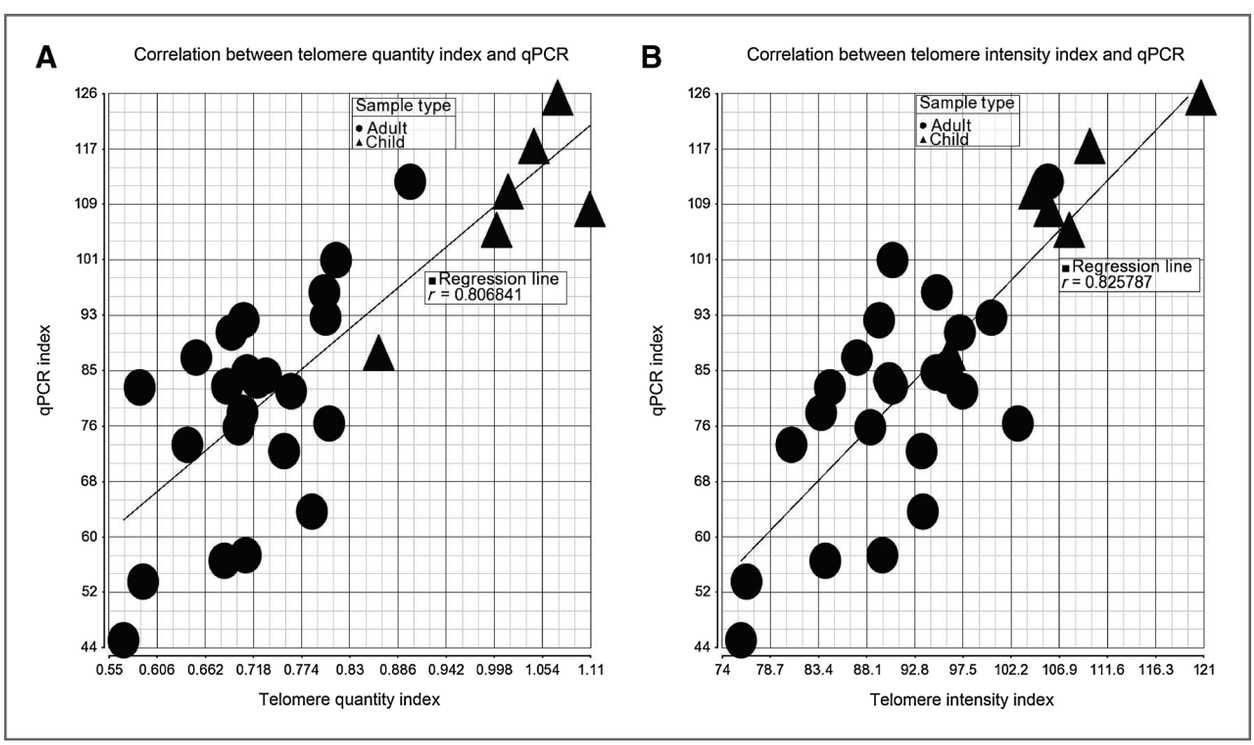

Figure 3. Correlation of the QGP assay measurements (TQI on the left and TII on the right) with conventional single-plex qPCR index.

Table 2. Precision of the QGP assay for telomere length measurement

QGP: comparison between		Geometric mean	Arithmetic mean	Median
50 ng vs.100 ng input	CV% of TQI	3.03	4.63	3.43
	CV% of TII	6.52	8.11	8.45
Interplate 50 ng input	CV% of TQI	4.97	6.40	5.12
	CV% of TII	2.93	4.48	4.19
Intraplate	CV% of TQI	5.96	7.07	6.92
	CV% of TII	3.77	5.02	4.98

(19). Our assay showed strong correlation of telomere intensity index (mean of two measurements) with the Southern blot analysis of the length of the terminal restriction fragments (TRF) of chromosomes (also mean of two measurements; $r = 0.74$, see Supplementary Fig. S2B). The details of the study will be presented in a different article focusing on blinded comparison between the QGP and the Southern blots, in a similar way as described by Aviv and colleagues where they compared qPCR and Southern blots (19).

Discussion

The conventional single-plex qPCR assay (14) for TL measures telomere (T) signals and single copy reference gene (R) signals in separate wells, in comparison with a reference sample, to yield relative T/R ratios that are proportional to average TL. Therefore, variation in the amount of DNA in T and R wells can potentially contribute to inaccurate T/R. This issue was resolved in multiplex qPCR invented by Richard Cawthon (15). We have developed a telomere repeat-specific probe-based, non-PCR multiplexing assay in which the telomere probe (T) and reference gene probe (R) are detected simultaneously by Luminex-based technology from the same well. In this assay, we avoid PCR amplification where the T and R probes are on magnetic beads and the intensity from the probes is amplified by branching DNA technology. The MFI is measured from a minimum of 100 beads. About throughput, a total of 69 DNA samples can be processed in a single 96-well plate (standards and control samples would take the remaining wells) and four such plates (276 samples) can be easily processed in a week. As this is not a PCR-based assay, it is free of PCR amplification biases. Like Q-FISH and Flow-FISH, another major advantage of this assay is the specificity of the CE and LE probes to the telomeric repeats, avoiding measurement of the noncanonical subtelomeric region that confounds the Southern blot method (11, 12). Regarding limitation, the assay may not work for fragmented DNA samples and may be inaccurate with lower than 50 ng of total DNA per sample.

In the present study, our method correlates well with the widely-used qPCR measurement as well as the Southern blot method. Intraplate CV is low and, therefore, unlike qPCR, triplicates are not essential for this assay. The interplate CV% is reasonable for precision. Compared with the Southern blot assay (requiring 3 μg of high-quality DNA), TL can be assayed from just 50 ng of DNA using our method. Reagent cost per sample is significantly lower than Southern blot and comparable with qPCR. Luminex technology has been used for quite some time for various applications, such as quantification of RNA levels; however, to our knowledge, we are the first to use QGP chemistry for TL measurement using this Luminex platform. This novel assay for TL assessment offers an alternative cost-efficient strategy to study TL. In the future, integrating measurements of synthetic oligonucleotides or cell line DNA with known TL can enable absolute quantification of TL using this technology.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.G. Kibriya
Development of methodology: M.G. Kibriya, S. Roy
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): F. Jasmine, H. Ahsan, B. Pierce
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.G. Kibriya, F. Jasmine, H. Ahsan
Writing, review, and/or revision of the manuscript: M.G. Kibriya, F. Jasmine, H. Ahsan, B. Pierce
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Roy
Study supervision: M.G. Kibriya, H. Ahsan

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