Telomere length measurement by a novel monochrome multiplex quantitative PCR method

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ABSTRACT

The current quantitative polymerase chain reaction (QPCR) assay of telomere length measures telomere (T) signals in experimental DNA samples in one set of reaction wells, and single copy gene (S) signals in separate wells, in comparison to a reference DNA, to yield relative T/S ratios that are proportional to average telomere length. Multiplexing this assay is desirable, because variation in the amount of DNA pipetted would no longer contribute to variation in T/S, since T and S would be collected within each reaction, from the same input DNA. Multiplexing also increases throughput and lowers costs, since half as many reactions are needed. Here, we present the first multiplexed QPCR method for telomere length measurement. Remarkably, a single fluorescent DNA-intercalating dye is sufficient in this system, because T signals can be collected in early cycles, before S signals rise above baseline, and S signals can be collected at a temperature that fully melts the telomere product, sending its signal to baseline. The correlation of T/S ratios with Terminal Restriction Fragment (TRF) lengths measured by Southern blot was stronger with this monochrome multiplex QPCR method ($R^2 = 0.844$) than with our original singleplex method ($R^2 = 0.677$). Multiplex T/S results from independent runs on different days were highly reproducible ($R^2 = 0.91$).

INTRODUCTION

Telomere lengths are frequently measured in biomedical research. Recently, we developed a singleplex quantitative polymerase chain reaction (QPCR) assay for relative average telomere lengths, which uses far less DNA and requires much less time to perform than the traditional Southern blot method for measuring Terminal Restriction Fragment (TRF) lengths (1). As the interest in the role of telomere length and telomere length dynamics in disease and health continues to grow, we felt it would be helpful to the research community to further improve the accuracy, raise the throughput and lower the costs of telomere length measurement by QPCR by developing a multiplex version of the assay.

It has been presumed impossible to determine the relative copy numbers of two different DNA sequences in a multiplex QPCR using a single DNA-intercalating dye, because the accumulating fluorescent signal arises from both amplicons. Here we present a simple strategy that, in theory, should allow the signals from any two amplicons to be collected separately in such a system, whenever the two target sequences of interest differ greatly in copy number. The cycle thresholds (C_t) for the first, more abundant target sequence are collected at earlier cycles, when the signal from the second, less abundant target sequence is still at baseline. The values of C_t for the second amplicon are collected at a temperature well above the melting temperature (T_m) of the first amplicon, rendering the first amplicon single-stranded and sending its signal to baseline. Primers are simply designed to make both amplicons small, and to place GC-clamps on both ends of the second amplicon, raising its T_m. Pairs of templates that occur in biological samples as high and low abundance species with no overlap in copy number range within samples are natural targets for this approach. Here we demonstrate success with this monochrome multiplex quantitative PCR (MMQPCR) method when telomere repeats are the high abundance species and a single copy gene (scg) is the low abundance species.

MATERIALS AND METHODS

Research subjects

Genomic DNA was extracted directly from blood samples by standard procedures, and stored long-term in TE^{-4} (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5) at 4°C at a concentration of ∼100 ng/µl. DNA stocks were diluted into pure water just prior to setting up QPCR runs.

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The samples, from 95 Utah individuals (47 females and 48 males, age range 5–94 years), are those analyzed in our previous paper describing telomere length measurement by singleplex QPCR (1).

MMQPCR

PCR reactions were set up by aliquoting 15 μl of master mix into each reaction well of a 96-well plate compatible with the Bio-Rad MyiQ Single Color Real-Time PCR Detection System, followed by 10 μl of each experimental DNA sample, containing approximately 20 ng of DNA diluted in pure water, for a final volume of 25 μl per reaction. Five concentrations of a reference DNA sample (the 'Standard DNA') spanning an 81-fold range of DNA concentration were prepared by serial dilution and analyzed in duplicate in every 96-well plate in this study; these reactions provided the data for the generation of the standard curves used for relative quantitation. All experimental DNA samples were assayed in triplicate.

The final concentrations of reagents in the PCR were $0.75 \times$ SYBR Green I (Invitrogen), 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM $MgCl_2$, 0.2 mM each dNTP, 1 mM DTT and 1 M betaine (U.S. Biochemicals). Each 25 μl reaction received 0.625 U AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.). For multiplex QPCR, the telomere primer pair telg and telc (final concentrations 900 nM each), were combined either with the albumin primer pair albu and albd (final concentrations 900 nM each), or with the beta-globin primer pair hbgu and hbgd, (final concentrations 500 nM each) in the master mix. All primer sequences and the rationale for their design are presented in the Results section.

The thermal cycling profile was Stage 1: 15 min at 95°C; Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C; and Stage 3: 32 cycles of 15 s at 94°C, 10 s at 62°C, 15 s at 74°C with signal acquisition, 10 s at 84°C, 15 s at 88°C with signal acquisition. The 74°C reads provided the C_t values for the amplification of the telomere template (in early cycles when the scg signal is still at baseline); the 88°C reads provided the C_t values for the amplification of the scg template (at this temperature there is no signal from the telomere PCR product, because it is fully melted).

After thermal cycling and raw data collection were complete, the MyiQ software (Bio-Rad iQ5 2.0 Standard Edition Optical System Software) was used to generate two standard curves for each plate, one for the telomere signal and one for the scg signal. The T/S ratio for an experimental DNA sample is T, the number of nanograms of the Standard DNA that matches the experimental sample for copy number of the telomere template, divided by S, the number of nanograms of the standard DNA that matches the experimental sample for copy number of the scg. As each experimental sample was assayed in triplicate, three T/S results were obtained for each sample; the final reported result for a sample in a given run is the average of the three T/S values. Average T/S is expected to be proportional to the average telomere length per cell. Samples with a T/S > 1.0 have an average telomere length greater than that of the standard DNA; samples with a T/S < 1.0 have an average telomere length shorter than that of the standard DNA.

Determination of mean terminal restriction fragment lengths

Mean TRF lengths were determined in duplicate as described previously (1). Briefly, DNA was digested with HaeIII restriction endonuclease, and digested samples were mixed with DNA size standards prior to agarose gel electrophoresis and Southern blotting onto nylon membranes. Following hybridization of the blots with a radioactive telomeric oligonucleotide probe $(TTAGGG)_7$ and capture of the telomere smear images, blots were stripped and hybridized with radioactive probes specific for the DNA size standards. The size standard images and telomere smear images were then superimposed to locate the positions of the size intervals within the telomere smears. Mean TRF length was then calculated as $\Sigma(OD_i)/\Sigma(OD_i/L_i)$, where OD_i is total radioactivity above background in interval i and L_i is the average length of i in basepairs.

RESULTS

Primers that amplify a fixed-length product from telomeric tandem hexamer repeats

Relative average telomere length can be measured by QPCR using primers that hybridize the telomeric hexamer repeats, because the number of binding sites for the primers increases as average telomere length increases. Our original tel1 and tel2 primers for telomere length measurement by singleplex QPCR (1) are both able to prime at multiple locations along the tandem repeats of telomeric DNA. They therefore generate a series of products of various sizes, some of which melt at temperatures high enough to overlap the melting curve of the scg's amplicon. Consequently, 'clean' reads, at a high temperature, of the SYBR Green I fluorescence signal from the scg's double-stranded amplicon, without any interfering signal from double-stranded telomere PCR products, as required for successful MMQPCR, are not possible when tel1 and tel2 are the telomere primers.

To solve this problem, we designed a pair of telomere primers, telg, ACACTAAGGTTTGGGTTTGGGTTTG GGTTTGGGGTTAGTGT and telc, TGTTAGGTATCC CTATCCCTATCCCTATCCCTAACA, that generate a short, fixed-length product (Figure 1). Only telg is able to prime DNA synthesis along native telomeric DNA sequences. The telc primer is blocked from priming native telomeric DNA by a mismatched base at its 3′ terminus. However, telc is able to hybridize along various stretches of the telg primer extension product, and exactly one configuration of those hybridizations allows the priming of DNA synthesis, thereby enabling the generation of a single, fixed-length product. This is achieved by introducing a nucleotide change in telg at the third base from the 3′ end, such that the last three bases of the telg and telc primers overlap with perfect complementarity. This overlap is not sufficient to allow the native telg and telc primers to prime each other efficiently, so primer dimer formation
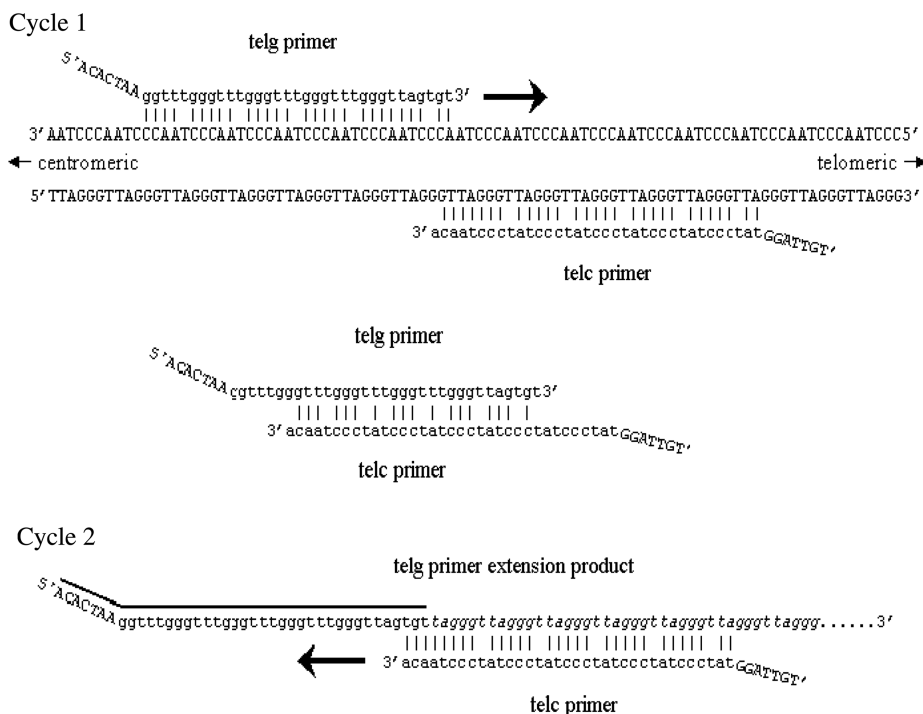
Figure 1. In Cycle 1 the telg primer hybridizes to native telomere sequences and primes DNA synthesis. The telc primer hybridizes native telomere sequences but cannot prime DNA synthesis, due to its 3′ terminal mismatch. When hybridized to each other as shown, and in other configurations not shown, telg and telc have multiple mismatches, including at their 3′ terminal bases, so primer dimer formation is inhibited. The 3′ ends of telg and telc can also align as a perfectly complementary 3 bp overlap, but this is not stable enough to allow efficient primer dimer formation. In Cycle 2, telc can hybridize along telg primer extension products that were synthesized in Cycle 1, but can only prime DNA synthesis when hybridized in the configuration shown, since other configurations produce a mismatch at telc's 3′ terminal base. In the telg extension product, the overbar marks the sequence of the telg primer itself, and the italicized bases mark sequence newly synthesized in Cycle 1 of the PCR. The non-templated capitalized sequences at the 5′ ends of the primers prevent the 3′ ends of the telomere PCR product from priming DNA synthesis in the middle of other copies of the telomere PCR product.

is undetectable over the range of cycles that telomere length quantitation occurs. However, when the telg extension product is hybridized to telc, this three base overlap is the only site where the 3′ end of telc can efficiently prime DNA synthesis. The resulting PCR product is, therefore, of fixed length, and three bases shorter than the sum of the lengths of the two primers used to generate it. The sharp melting curve for this product (green curve in Figure 2) is consistent with specific, fixed-length product formation, and agarose gel electrophoresis in 6% gels revealed only the expected 79 bp product (data not shown). Figure 2 also demonstrates that the melting curve for the telomere PCR product is well separated from the melting curve for the albumin PCR product (blue curve in Figure 2), allowing the SYBR Green I signal from albumin to be read at a temperature that fully melts the telomere PCR product.

Primer design for single copy genes (albumin and beta-globin)

Primers were designed so that the scg amplicon would melt at a much higher temperature than the telomere amplicon. Fluorescent signal from the scg amplicon could then be acquired at a temperature high enough to completely melt the telomere amplicon, eliminating its contribution to the signal, but low enough to keep the scg amplicon double-stranded and therefore able to bind SYBR Green I.
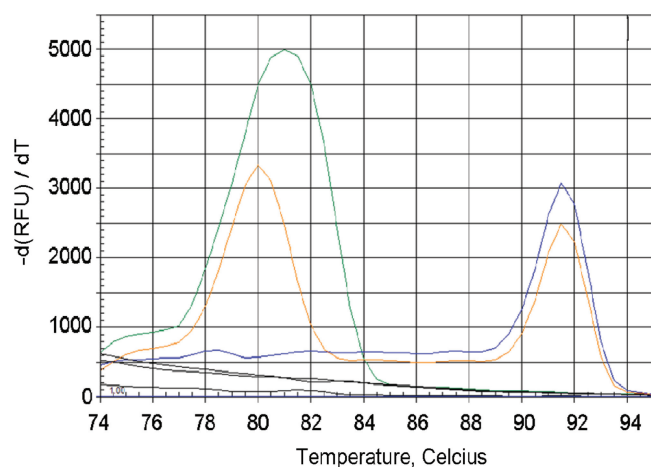

Figure 2. Melting curves following 25 cycles of amplification (thermal profile given in Materials and methods section) of 150 ng of human genomic DNA with telomere primers only (green curve), albumin primers only (blue curve) or both primer sets (orange curve). No template control melting curves are in black. After the final 88°C incubation, reactions were cooled to 72°C, and signal was acquired from 72°C to 95°C, in 0.5°C steps, with a 30 s dwell period per step. There is approximately an 11°C difference in the melting temperatures of the telomere and albumin amplicons. Clearly, 88°C is a good choice for acquiring SYBR Green I signal from the albumin amplicon without interference from the telomere amplicon, which is completely melted at that temperature.

The primers for amplification of the scg albumin are albu: CGGCGGCGGGCGGCGCGGGCTGGGCGGaa atgctgcacagaatccttg and albd: GCCCGGCCCGCCGCG CCCGTCCCGCCGgaaaagcatggtcgcctgtt. The predicted product size is 98 bp. The primers for amplification of the scg beta-globin are hbgu: CGGCGGCGGGCGGCG CGGGCTGGGCGGcttcatccacgttcaccttg and hbgd: GCC CGGCCCGCCGCGCCCGTCCCGCCGgaggagaagtctgc cgtt. The predicted product size is 106 bp. Capitalized bases are non-templated 5′ tag sequences that confer a very high melting temperature on the resulting PCR product. Please note that the 5′ tag sequences for the albumin primers are identical to those used in the beta-globin primers. Note also that the two GC-rich 5′ tagging sequences in each primer set are very different from each other; if they were the same, hairpin formation shutting down amplification would be likely to occur during the PCR.

The addition of a GC-clamp to the 5′ end of a PCR primer to raise the melting temperature of one end of the PCR product is common practice when screening a gene for point mutations by Denaturing Gradient Gel Electrophoresis (2). We reasoned that by attaching 5′ GC-clamps to both of the primers used to amplify the scg, and keeping the targeted genomic sequence short, we would be able to generate a PCR product with a very high melting temperature. Figure 2 shows that the T_m for the doubly GC-clamped albumin PCR product is above 91°C. Agarose gel electrophoresis in 6% gels revealed only the expected size product. Similar results were obtained for the doubly GC-clamped beta-globin PCR product (data not shown).

The 5′ GC-clamps, also ensure that both of the primers used to amplify the scg have T_m values for their amplicon that are higher than the T_m of the telomere PCR product. The benefits of this design are discussed below (see Thermal profile and cycling design section). An analysis using the OligoAnalyzer program (www.idtdna.com) indicated that all four scg primers (albu, albd, hbgu and hbgd) have T_m values greater than 84°C in the buffer composition used in this study.

Thermal profile and cycling design

In Stage 1 of the thermal cycling protocol, the AmpliTaq Gold DNA polymerase is heat-activated, and the genomic DNA sample is denatured. In Stage 2, two cycles of relatively low temperature are needed to effectively anneal and extend the telomere primers, due to the presence in those primers of purposely introduced mutations that prevent formation and amplification of primer dimer PCR products (1).

In Stage 3 the repeating cycle begins with a denaturation, an annealing and an extension step with signal acquisition that are typical of conventional QPCR. These are followed by two unconventional steps: an incubation at 84°C for 10 s and an incubation at 88°C for 15 s with a second signal acquisition. Heating to 84°C melts the early-amplifying telomere product, releasing DNA polymerase (which binds double-stranded, but not single-stranded DNA; 3) for work on the scg PCR product, where DNA synthesis can proceed, due to the high annealing

temperatures (above 84°C) of the scg primers, and the ability of Taq DNA polymerase to maintain robust activity even at 84°C (4).

In conventional multiplex PCR, high concentrations of the earliest amplifying product often inhibit subsequent amplification of less abundant templates, due to the above-mentioned binding up of DNA polymerase by the early product. The usual recommended solution is to limit the primer concentrations for the more abundant target sequence, so that less product is formed, leaving enough DNA polymerase unbound and free to continue copying the less abundant template. But lowering primer concentrations often results in a reduced PCR efficiency, or even a complete failure to amplify the target sequence. Reduced efficiencies also contribute to greater variation in C_t values between replicates. The 84°C incubation step in MMQPCR eliminates the need to limit the primer concentrations for the more abundant template, releasing polymerase from even high concentrations of the corresponding PCR product, so that the second product can be synthesized efficiently.

Heating further to 88°C for the second signal acquisition step ensures that the telomere PCR product is completely melted and unable to interfere with the collection of the rising SYBR Green I fluorescence signal from the accumulating scg amplicon.

Validity of the MMQPCR method over the natural range of telomere lengths

Figure 3 shows amplification curves collected at two different temperatures (74°C and 88°C) for three reference human genomic DNA samples previously shown to have high, middle or low average telomere lengths (approximately a 3-fold range of telomere lengths). Based on the melting profiles presented in Figure 2, the 74°C reads should detect both telomere and albumin PCR products, and the 88°C reads should detect only the albumin product. However, because the albumin template is much lower in copy number than the telomere template in each DNA sample, the 74°C C_t values, all collected when the corresponding albumin signals were still at baseline, are measures of telomere amplification only. (We have confirmed, in reactions without the telomere primers, that the single copy gene signal rises above baseline at essentially the same cycle number whether collected at 74°C or 88°C.) Even the sample with the shortest telomeres (∼1670 bp), and therefore the most right-shifted amplification curve (blue curve), crosses threshold at a cycle number when the albumin gene's amplification signal is still at baseline. In the present study of 95 whole blood DNA samples from subjects aged 5–94 years, each sample's scg amplication signal was at baseline when the C_t for the corresponding telomere signal was collected.

Independent standard curves for telomere and single copy gene

Figure 4 shows two independent standard curves, one for the telomere repeats and another for the scg albumin, determined for the Standard DNA by acquiring the
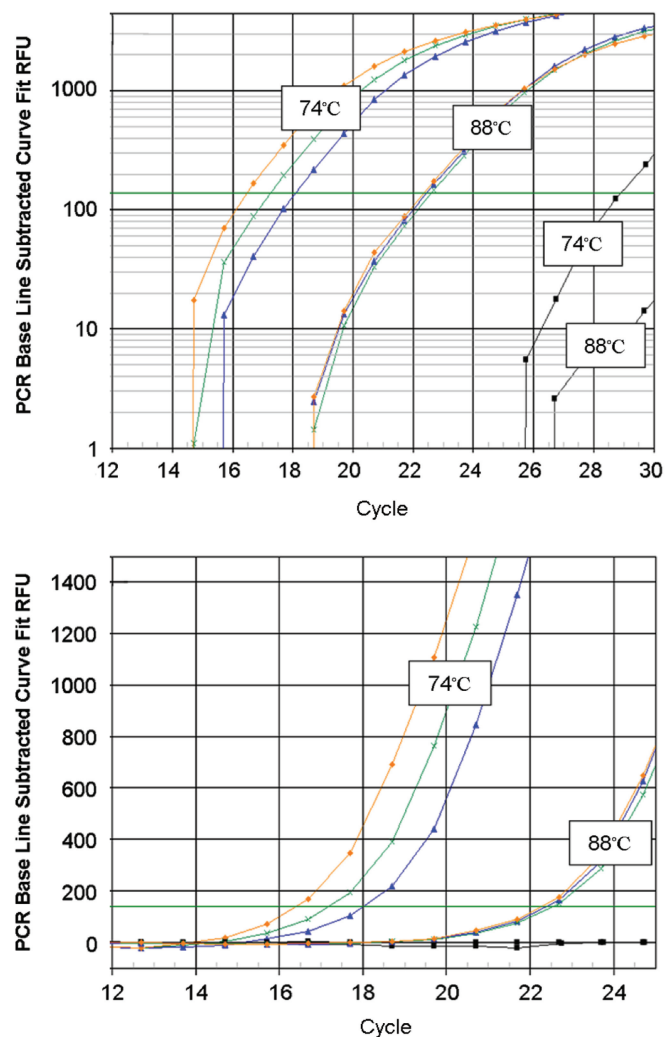
Figure 3. MMQPCR of 20 ng of each of three reference human DNA samples previously shown to have long telomeres (orange curves), middle-length telomeres (green curves) or short telomeres (blue curves). No template control amplification curves are in black. Top panel: semi-log plot and bottom panel: linear plot. Here, special care was taken to input essentially identical amounts of DNA into the reactions (based on OD_{260} UV spectrophotometer readings), so that the differences in C_t observed at 74°C would reflect only differences in telomere length (without influence from variation in the amounts of input DNA). (In normal practice, there is no need to precisely match experimental samples for input DNA, since the procedure of normalizing the T signal to the S signal addresses this issue. A wide range of input DNA amounts are acceptable, as long as both T and S signals fall within the range of the T and S standard curves; see Figure 4.) The nearly perfect overlap of the three amplification curves acquired at 88°C is expected, since only the single copy gene (albumin gene) signal is collected at this temperature. The bottom panel shows that the cycle thresholds for the telomere signals can be collected at 74°C when the albumin signal is still at baseline. (We have confirmed, in reactions without the telomere primers, that the single copy gene signal rises above baseline at essentially the same cycle number whether collected at 74°C or 88°C. Also, we have confirmed, in reactions without the single copy gene primers, that the telomere amplification signal is completely flat and at zero throughout the PCR run when read at 88°C, as would be expected based on the melting profiles shown in Figure 2.) Since the Bio-Rad MyiQ software can display only one temperature's amplification curves at a time, here we have superimposed the displays for the 74°C and 88°C reads.
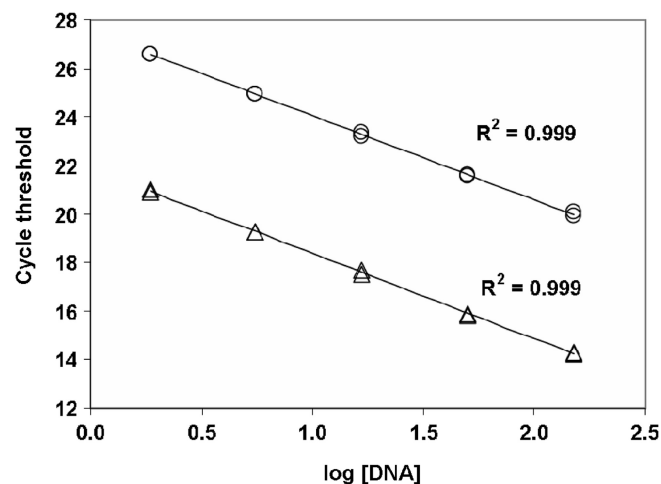

Figure 4. Standard curves used to determine relative T/S ratios. Five concentrations of a standard human genomic DNA sample spanning an 81-fold range were prepared by 3-fold serial dilutions (150 ng, 50 ng, 16.7 ng, 5.55 ng and 1.85 ng per well), and aliquoted in duplicate to a 96-well PCR plate. Both target and reference fluorescent signals were collected from each reaction well. Circles, data for the single copy gene albumin, acquired at 88°C and triangles, data for telomere repeats, acquired at 74°C. The same standard DNA was used to set up standard curve reactions in every plate in the study.

SYBR Green I fluorescence signal at two different temperatures (74°C for the telomere signal and 88°C for the albumin signal) in each cycle of Stage 3 of the cycling protocol. This same DNA sample was used to generate two standard curves for each separate PCR reaction plate in this study. In this semi-log plot of DNA concentration versus cycle threshold, both curves are linear over the 81-fold DNA concentration range. The PCR efficiencies for both telomere and albumin amplifications were greater than 90% and approximately equal. For this particular Standard DNA sample, at each DNA concentration the C_t for albumin occurred approximately six cycles later in cycling than the C_t for the telomere repeats.

Correlation between mean TRF lengths and relative T/S ratios

To test the validity of the MMQPCR approach to telomere length measurement, we compared the relative telomere lengths (average T/S ratios) in whole blood DNA samples from 95 individuals, aged 5–94 years, measured in triplicate by MMQPCR, to the mean TRF lengths of these same DNA samples as measured by the traditional Southern blot approach (1). Figure 5 shows the strong correlation in relative telomere lengths measured by these very different techniques ($R^2 = 0.844$). This correlation is higher than the correlation we reported previously (1) for T/S ratios measured in these same samples by singleplex QPCR versus their mean TRF lengths ($R^2 = 0.677$).

Reproducibility of T/S ratio measurements

To examine the intra-assay reproducibility of T/S measurements by MMQPCR, we determined the coefficient
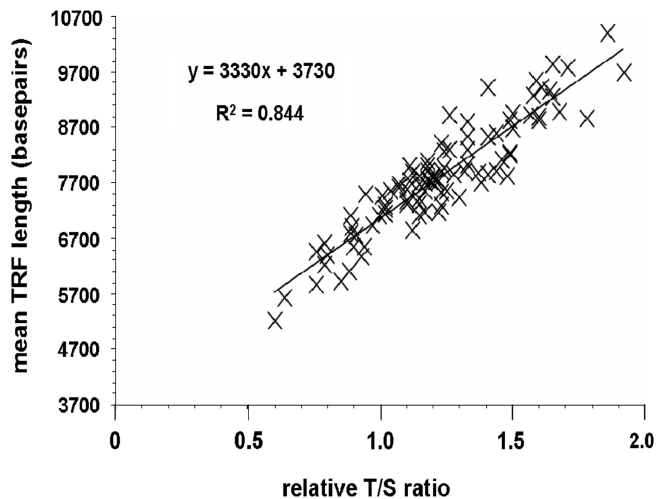
Figure 5. Correlation of relative T/S ratios measured by MMQPCR with albumin as the single copy gene, and mean TRF lengths determined by Southern blot analysis, in whole blood DNA samples from 95 individuals. Each T/S value is the average of triplicate measurements; each mean TRF length is the average of duplicate measurements. The linear regression equation and correlation coefficient were determined using Microsoft Excel.
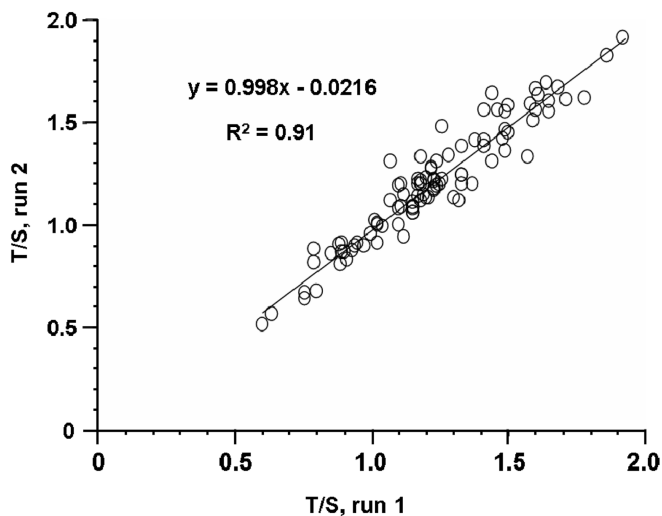

Figure 6. Reproducibility of relative T/S ratios in independent runs of the MMQPCR assay. The same 95 DNA samples assayed in Figure 5 were assayed again the next day, taking care that the specific MyiQ PCR machine and reaction well positions occupied by each DNA sample were different from the previous day. The linear regression equation and correlation coefficient were determined using Microsoft Excel.

of variation (standard deviation divided by the mean) for T/S for each of the 95 DNA samples assayed in triplicate in a single run of the MMQPCR assay, using albumin as the scg. The intra-assay geometric mean of the coefficient of variation was 5.22%. To examine inter-assay reproducibility, we repeated the measurements of T/S in the same 95 DNA samples, again in triplicate, on another day, taking care that the specific MyiQ PCR machine and reaction well positions occupied by each DNA sample were different in these two independent runs of the assay. Figure 6 shows the strong correlation between the average
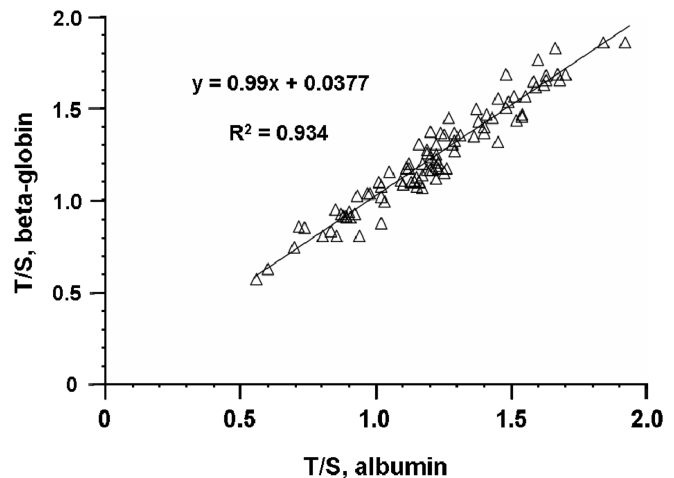

Figure 7. Correlation between T/S ratios obtained with albumin as the single copy gene versus beta-globin as the single copy gene. Relative T/S ratios were measured in the same 95 DNA samples, in triplicate, in two separate runs, substituting the beta-globin primers for the albumin primers. For each sample, the average T/S from the two separate runs with albumin as the single copy gene (x-axis) is plotted against the average T/S from the two runs with beta-globin as the single copy gene (y-axis). The linear regression equation and correlation coefficient were determined using Microsoft Excel.

T/S ratios determined by the first and second runs ($R^2 = 0.91$). The slope of the linear regression line through the data was near unity, and the y-intercept near zero, as expected. The coefficient of variation for each of the 95 pairs of average T/S values from the two independent runs was determined. The inter-assay geometric mean of the coefficient of variation was 3.13%.

T/S ratios are independent of the single copy gene used

To test whether using beta-globin, instead of albumin, as the scg might alter apparent relative telomere lengths, we repeated the measurements of T/S in the same 95 DNA samples, in triplicate, in two separate runs, substituting the beta-globin primers for the albumin primers. Figure 7 plots the average T/S values from the two runs with albumin as the scg (x-axis) versus the average T/S values from the two runs with beta-globin as the scg (y-axis). The T/S values obtained with albumin correlated highly with those obtained using beta-globin ($R^2 = 0.934$).

DISCUSSION

Relative telomere lengths (T/S ratios) measured in 95 DNA samples by the novel MMQPCR method described here were very highly correlated with relative TRF lengths measured by Southern blot. The T/S ratios measured in these same samples by our original singleplex QPCR assay were not as highly correlated with the TRF lengths. These results suggest that telomere length measurement by MMQPCR is more accurate than telomere length measurement by singleplex QPCR. Furthermore, T/S results obtained by MMQPCR were highly reproducible in

independent runs of the assay. Multiplexing the telomere QPCR assay will allow increased throughput and lower costs for epidemiologic studies of telomere length. Furthermore, the usual additional cost associated with converting to a multiplex assay, of having to synthesize or purchase expensive custom-made multi-color fluorescent probes is also avoided by adopting this method.

We expect that MMQPCR can be easily adapted for the study of many pairs of DNA templates that naturally occur at very different copy numbers, e.g. mtDNA copies versus single copy genes, rDNA copies versus single copy genes, and Alu DNA copies versus single copy genes. Similarly, pairs of RNA species with very different copy numbers may be quantifiable by this method, following reverse transcription into cDNA. For most pairs of targets, standard principles of primer design can be followed, with the only additional guidelines being that the primers for the more abundant template should generate a relatively short product (40–80 bp) so that its T_m will be appropriately low ($<83°C$), and the primers for the less abundant template should contain the 5′ GC-clamps presented here (or similar ones) and generate a short product so that its T_m will be sufficiently high ($>90°C$). Furthermore, the nuisance and attendant difficulties, in conventional multiplex QPCR, of having to limit the primer concentrations used to amplify the more abundant template, is eliminated in MMQPCR. (The additional arcane and very unusual design features of the telg and telc telomere primers in the current study were only needed due to the special problem of amplifying short tandem repeats with primers that hybridize to those repeats.) In addition to measuring telomere lengths by MMQPCR, we have already measured mtDNA to nDNA ratios by this approach, and it worked well on the first attempt (manuscript in preparation). Even pairs of templates with similar copy numbers may be studied by this approach by applying primer and thermal profile designs that delay the amplification of one amplicon (manuscript in preparation). We expect that researchers will find MMQPCR to be an attractive, generally useful alternative to conventional multicolor multiplex QPCR.

REFERENCES

1. Cawthon,R.M. (2002) Telomere measurement by quantitative PCR. *Nucleic Acids Res.*, **30**, e47.
2. Sheffield,V.C., Cox,D.R., Lerman,L.S. and Myers,R.M. (1989) Attachment of a 40-base-pair G + C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc. Natl Acad. Sci. USA*, **86**, 232–236.
3. Kainz,P. (2000) The PCR plateau phase—towards an understanding of its limitations. *Biochim. Biophys. Acta.*, **1494**, 23–27.
4. Lawyer,F.C., Stoffel,S., Saiki,R.K., Chang,S.Y., Landre,P.A., Abramson,R.D. and Gelfand,D.H. (1993) High-level expression, purification, and enzymatic characterization of full-length Thermus aquaticus DNA polymerase and a truncated form deficient in 5′ to 3′ exonuclease activity. *PCR Methods Appl.*, **2**, 275–287.