Relative Human Telomere Length Quantification by Real-Time PCR: Methods and Protocols

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Chapter 5

Relative Human Telomere Length Quantification by Real-Time PCR

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Abstract

Telomere measurement by quantitative PCR amplification is a well-known simple method to detect telomere length that involves large numbers of samples. The method has been developed by Cawthon in 2002 (Cawthon, Nucleic Acids Res 30:47e–47, 2002) and remains the most frequently used technique either in original or modified version. Telomere length is estimated by comparing the amount of telomere repeat amplification product (T) to a single copy gene (S) product. The T/S ratio correlates with the average telomere length. Cawthon suggested and recommended the use of 36B4 (*RPLP0*) as a single copy gene. However, Cawthon's suggestion was no longer considered a single copy gene and the gene was not suitable and appropriate for normalization.

We thereby introduced a simple method for relative measurement of average human telomere length using quantitative real-time PCR. Our protocol was based on Cawthon's initial technique (Cawthon, Nucleic Acids Res 30:47e–47, 2002), modified by single-copy gene (SCG) primers and optimized.

This technique is rapid, low cost, not demanding on DNA amount (or live cells), and can be used for a high-throughput screening and time monitoring.

Key words Telomere length, Quantitative PCR, Real-time PCR, Telomere, Telomere measurement, IFNB1, 36B4, Primers, Single-copy gene

1 Introduction

Since Alexei Olovnikov formulated an elegant telomere theory in 1971 [1, 2] a new era has been opened and it became necessary to accurately measure the telomere length. In 2002, R. Cawthon [3] developed the simple protocol of telomere length measurement using real-time PCR technology. To amplify telomere repeats, Cawthon further proposed a special design of primers. This was due to the impossibility to use fully complementary telomeric primers. The complete complementarity leads to the formation of primer dimers and their effective amplification to the detriment of the telomere amplification. To reduce the probability of primer dimer amplification, Cawthon proposed using primers that bind to the G/C-rich segments of telomeric repeats with one mismatch

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at the other bases with telomeres and two mismatches between themselves. The essence of the method is that the relative average telomere length is estimated by determining the ratio of a copy number of telomere repeat sequences to a copy number of the reference gene with a known fixed number of copies per genome. As a reference gene, Cawthon suggested using the *RPLP0* (coding ribosomal protein lateral stalk subunit P0), which was mentioned as 36B4 in his protocol.

At the time of protocol development in 2002, 36B4 was considered a single-copy gene. However, it has been recently demonstrated that *RPLP0* (36B4) is a typical ribosomal gene with multiple processed pseudogenes in the human genome [provided by RefSeq, Jul 2008]. Primers to 36B4 initially described in Cawthon paper are completely complementary to genomic sequences on chromosomes 12, 2, and 5, and also highly homologous to chromosome 1, 14, and 18. On the melting curve the PCR product gives multiple peaks. Our method relies on using another gene as an SCG reference, *IFNB1*. *IFNB1* (gene coding interferon beta 1) belongs to interferon genes' cluster on chromosome 9 and is represented by single copy in the haploid genome. We provide here a detailed protocol on how to measure telomere length by using *IFNB1*-primers, which are strictly specific to SCG and do not form dimers.

2 Materials

All the procedures need to be prepared very carefully in separate work areas (hoods) for DNA extraction, PCR reaction setup, adding the template, and handling the amplification product with regular decontamination by UV irradiation and 10% bleach. All solutions need to be prepared using ultrapure water and aliquoted (*see* **Note 1**).

2.1 DNA Isolation

1. DNA extraction kit. Any extraction method/kit that provides a high level of DNA purity and integrity can be used.
2. UV–visible spectrophotometer.
3. Sterile, DNase-free tubes and filter tips.

2.2 qPCR

1. HPLC-purified PCR primers. The sequences of the primers used in this protocol:

   TEL-F 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′.
   TEL-R 5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′.
   IFNB1-F 5′-GGTTACCTCCGAAACTGAAGA-3′.
   IFNB1-R 5′-CCTTTCATATGCAGTACATTAGCC-3′.

Primers for telomere amplification were designed by R. Cawthon [3, 4]. Primers for *IFNB1* amplification were designed using Primer-Blast and Oligoanalyzer 3.1 software.

2. Sterile DNase-free PCR tubes, strips or 96-well plates with optically clear caps or seals.
3. Sterile DNase-free filter tips.
4. Maxima SYBR Green qPCR Master Mix (2×) 5 μL (Thermo Scientific) (*see* **Note 2**).
5. Nuclease-free water.
6. Real-time PCR machine and software (e.g., 7500 Real-Time PCR System and SDS v1.2 software, Applied Biosystems).
7. gDNA (0.5–10 ng per reaction).

   Reaction Mix:

   Maxima SYBR Green qPCR Master Mix (2×) 5 μL.
   Forward primer (1 μM TEL/2 μM IFNB1) 1 μL.
   Reverse primer (1 μM TEL/2 μM IFNB1) 1 μL.
   gDNA 2 μL (0.5–10 ng per reaction) (*see* **Note 3**).
   Nuclease-free water to 10 μL.

3 Methods

3.1 Sample Collection

1. Collect samples of any human biological material from which DNA can be isolated.

3.2 DNA Isolation

1. Extract total DNA from your sample.
2. Determine DNA quantity and purity by spectrophotometry. A260/A280 and A260/A230 values should be greater than 1.8.
3. Store at +4 °C until use.

3.3 qPCR Reaction

1. Set up a reaction mix.

   Perform each reaction in triplicates per DNA sample.
   Include a non-template control (NTC) for each primer pair (*see* **Note 4**).
   Include reference DNA in each run (for evaluation of reproducibility between runs).
   Prepare a separate master mix for each primer pair. Be sure to make some extra mix to allow for pipetting error.
   Pipet 8 μL of master mix into wells.
   Pipet 2 μL of DNA into each well.

2. Use the following cycling conditions: 10 min at 95 °C, 35 cycles of 95 °C for 15 s, 60 °C for 1 min (data collection) followed by a dissociation stage.

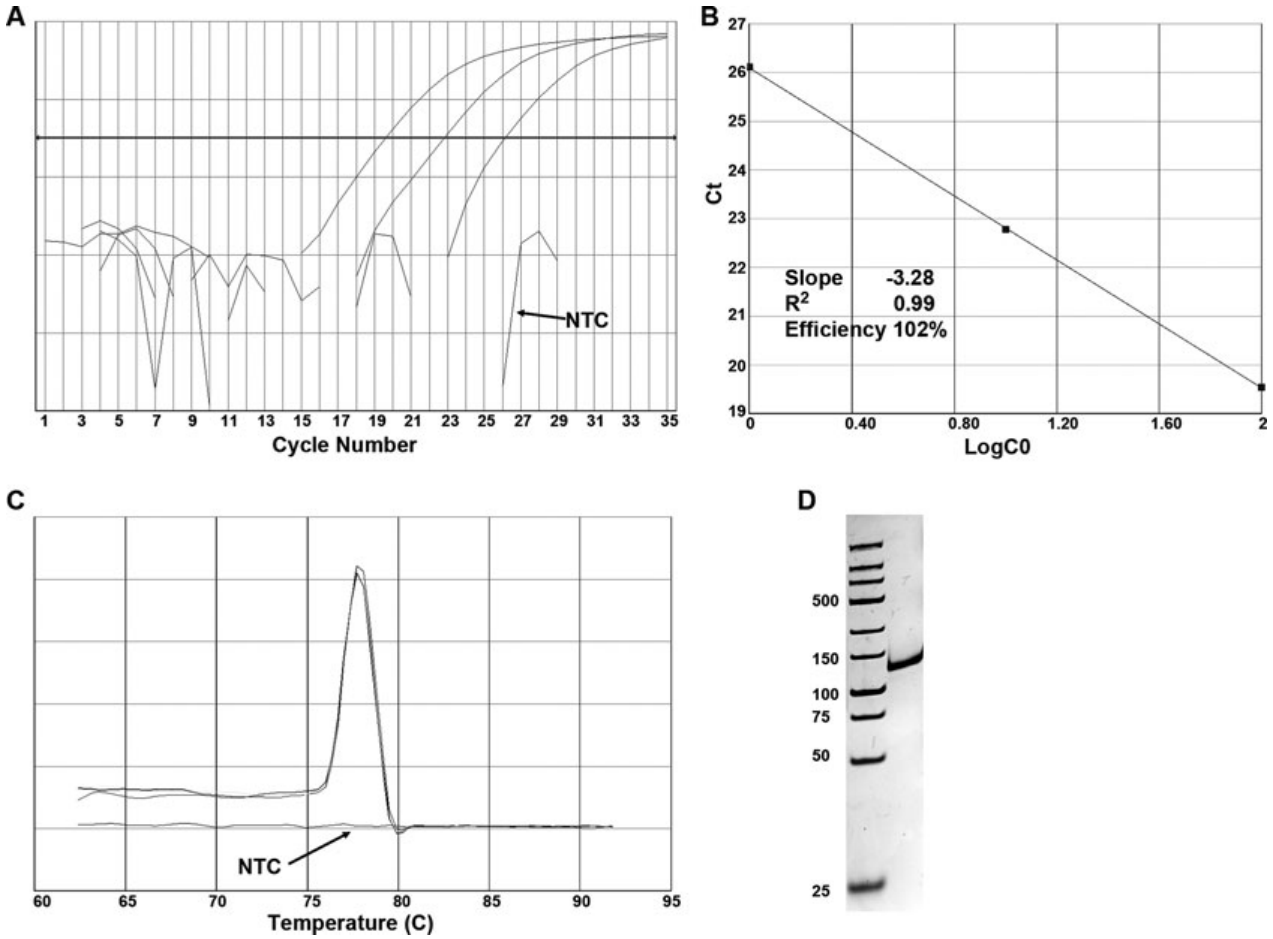

Fig. 1 qPCR results using SCG (IFNB1) primers. (**a**) Cord blood gDNA 25 ng, tenfold serial dilutions. NTC (no template control)—no amplification. (**b**) Standard curve, PCR efficiency. (**c**) Melting curve. NTC (no template control)—no peak. (**d**) PAGE. The specific PCR-product 123 bp

3.4 Determination of PCR Reaction Efficiencies (Optional) (See Note 5)

1. Set up serial dilutions of DNA in the concentration range of 50–0.01 ng/μL (3–5 dilutions) (Figs. 1a, and 2a).
2. Use the absolute quantification program on Real-time PCR machine.
3. Plot the mean of the Ct values for each dilution against the quantity of template to create a standard curve.
4. Calculate PCR efficiencies using the linear regression slope of the standard curve using the following formula: efficiency $= -1 + 10^{(-1/\text{slope})}$.

   Efficiency should be 100 ± 5% (Figs. 1b, and 2b).

3.5 Evaluation of Relative Telomere Length

1. Calculate mean Ct values for TEL and for SCG for each sample.
2. Calculate $\Delta Ct = Ct_{TEL} - Ct_{SCG}$ for each sample.
3. Calculate T/S ratio $(=2^{-\Delta Ct})$ for each sample.

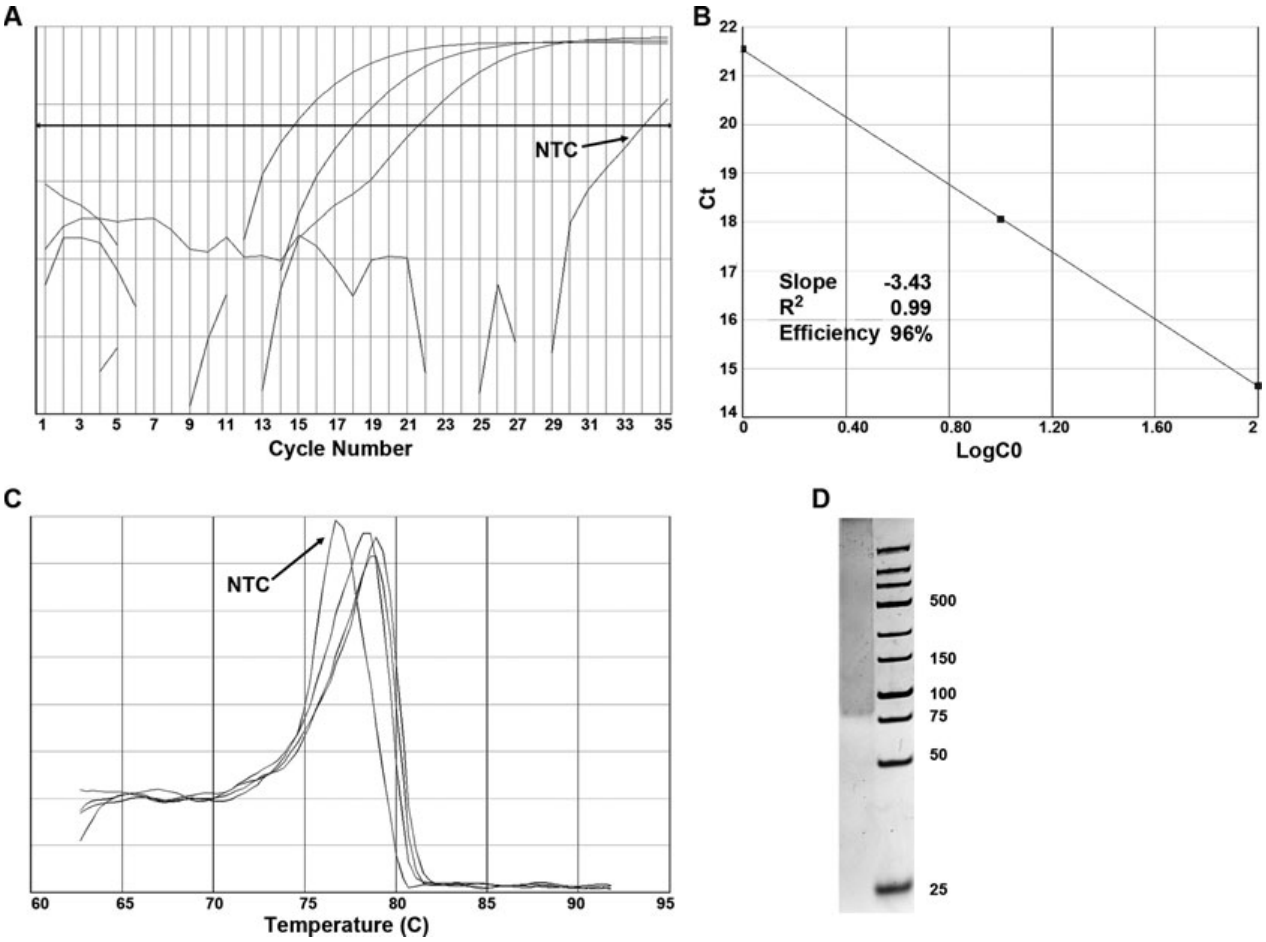

Fig. 2 qPCR results using telomere primers. (**a**) Cord blood gDNA 25 ng, tenfold serial dilutions. (**b**) Standard curve, PCR efficiency. (**c**) Melting curve. (**d**) PAGE. There is a smear starting from 78 bp, which corresponds to minimal telomere amplification product can be obtained using described telomere primers

4. Use the reference DNA sample for normalization:

   $\Delta\Delta Ct = \Delta Ct_{sample}{}^{-}\Delta Ct_{reference}$.

   Fold Change $= 2^{-\Delta\Delta Ct}$ (Fig. 3).

4 Notes

1. This method is very sensitive to contamination.
2. Protocol is optimized using Maxima SYBR Green qPCR Master Mix (Thermo Scientific). Use of other qPCR master mixes with SYBR Green and hot-start polymerase is possible but may require optimization.
3. Large amount of DNA may inhibit PCR and change efficiency.
4. NTC for *IFNB1* primers should give no amplification. Amplification of NTC for telomere primers may occur beyond 33–34 cycles that does not influence results of telomere length

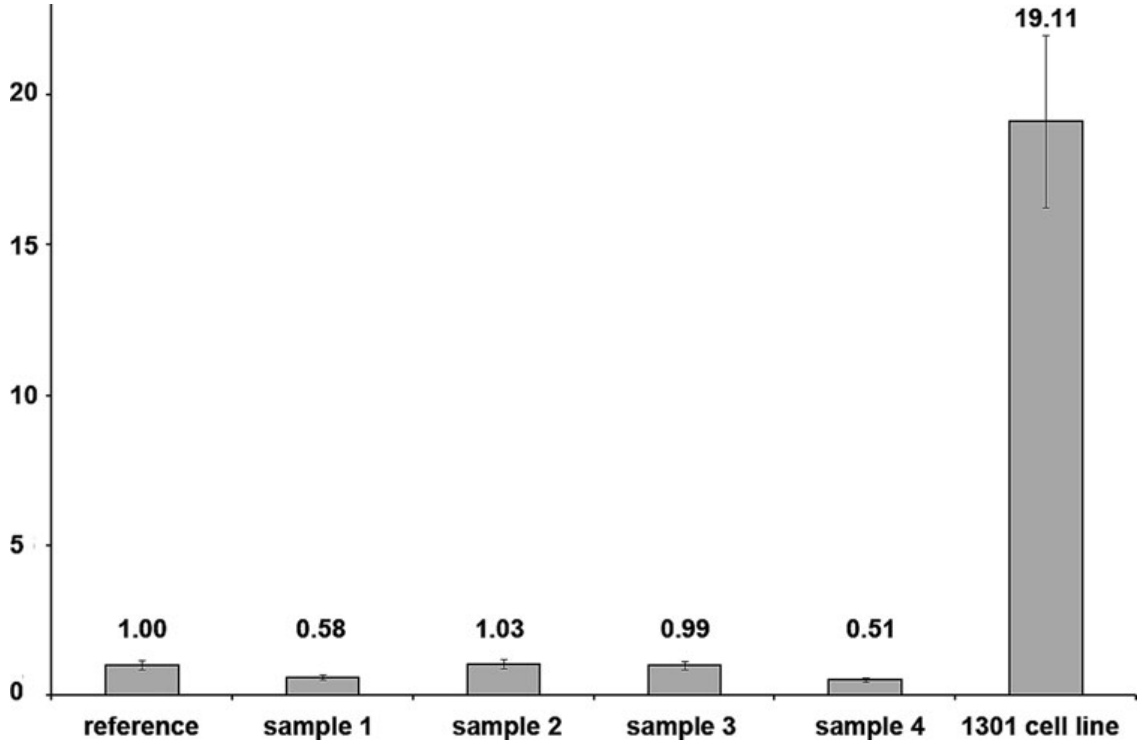

Fig. 3 Relative telomere measurement results: reference sample—gDNA from blood of 40-year-old male, sample 1–4—gDNA from four human blood samples, gDNA from the 1301 lymphoblastic cell line which was used as a long telomere control (telomere length of 70–90 kb) [5]

evaluation (Fig. 2a). It could be due to primer dimer amplification which can appear as a peak shifted to low temperature on a melt curve (Fig. 2c).

5. This step is optional and needed to verify master mix and primer efficiency, to optimize the protocol, to refine the optimal range of DNA concentrations.

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