DNA

MDPI

Protocol

A Dual-Labeled Multiplex Absolute Telomere Length Method to Measure Average Telomere Length

Sue Rutherford Siegel *, E. Alex Calcagni, Kelsey M. Draughon and Sheree F. Logue

Biomarker Core Laboratory, Department of Biobehavioral Health, The Pennsylvania State University, University Park, PA 16802, USA; calcagni@hawaii.edu (E.A.C.); kmd6785@psu.edu (K.M.D.); sul459@psu.edu (S.F.L.)
* Correspondence: srs40@psu.edu; Tel.: +1-814-865-0563

Abstract: Background/Objectives: Telomeres consist of repetitive nucleotide sequences and associated proteins that safeguard chromosome ends from degradation and fusion with neighboring chromosomes. As cells divide, telomeres shorten due to the end-replication problem and oxidative stress, ultimately contributing to cellular senescence. Telomeres therefore play a role in cellular health and aging. Measuring telomere length has emerged as a significant biomarker in various fields of research, including aging, cancer, and chronic diseases. Accurate measurement of telomere length is critical for interpreting research findings and clinical applications. Variability in measurement techniques can lead to inconsistent results, underscoring the need for standardized protocols. **Methods and Results:** The Telomere Research Network (TRN), an initiative from the National Institute of Aging and National Institute of Environmental Health Sciences, has established recommended guidelines to standardize the measurement of telomere length using qPCR to ensure accuracy and reproducibility in population-based studies. The monochrome multiplex quantitative PCR (MMqPCR) assay has emerged as a robust method endorsed by the TRN for its accuracy and reproducibility in quantifying telomere length in epidemiology ad population based studies. The absolute telomere length (aTL) qPCR assay is currently being evaluated by the TRN for its capability to utilize an oligomer standard, enabling the generation of absolute telomere lengths. The oligomer feature facilitates a more direct comparison of results across experiments and laboratories. **Conclusions**: This paper outlines a novel dual-labeled multiplex aTL method by incorporating dual-labeled multiplex probes to measure average absolute telomere length, providing a clear advantage over the relative telomere length assay, which quantifies the ratio of telomeric repeats to single-copy gene numbers.

Keywords: telomere length; absolute telomere length (aTL); dual-labeled multiplex aTL; quantitative PCR (qPCR); aging; disease

Citation: Rutherford Siegel, S.; Calcagni, E.A.; Draughon, K.M.; Logue, S.F. A Dual-Labeled Multiplex Absolute Telomere Length Method to Measure Average Telomere Length. *DNA* **2024**, *4*, 370–379. https://doi.org/10.3390/dna4040026

Academic Editor: Darren Griffin

Received: 23 September 2024
Revised: 14 October 2024
Accepted: 15 October 2024
Published: 18 October 2024

1. Introduction

Telomeres, located at the ends of eukaryotic chromosomes, are essential for maintaining genomic stability and integrity [1]. In humans, telomeres consist of repetitive nucleotide sequences (TTAGGG) and associated proteins that safeguard chromosome ends from degradation and fusion with neighboring chromosomes and prevent DNA repair mechanisms from mistaking telomeres for damaged DNA [2]. Telomere length (TL), which naturally shortens with each cell division, serves as a molecular clock that reflects cellular aging and replicative history [2]. Furthermore, TL dynamics have been implicated in various age-related diseases, including cancer, cardiovascular diseases, and neurodegenerative disorders [2].

Accurate measurement of TL is crucial for understanding its role in health and disease. Quantitative PCR (qPCR) is a versatile technique that amplifies and quantifies specific DNA sequences based on the principles of PCR. When applied to TL measurement, two qPCR amplifications are performed: one to amplify the telomeric repeat sequences and another to amplify a single-copy gene (e.g., *B2M* or *IFNB1*) as a reference. The ratio of telomeric repeat

copy number to single gene copy number is then calculated to infer relative differences in TL between samples [3]. Among the qPCR methods available to measure TL assessment is the relative telomere, the absolute telomere (aTL), and the monochrome multiplex qPCR (MMqPCR) assays [3–6]. When analyzed by the Telomere Research Network (TRN)—an initiative from the National Institute of Aging and National Institute of Environmental Health Sciences—to establish best practices for the measurement of TL in large-scale epidemiological and population-based studies, it is the MMqPCR assay that provides a robust telomere length measurement even with a variety of sample types [7,8]. This MMqPCR assay has the ability to simultaneously analyze the telomeric repeat and the single-copy gene regions in a single qPCR amplification using monochromatic SYBR Green fluorescence. The multiplex capability of the MMqPCR assay reduces the need for multiple individual assays, minimizing variability and improving the precision and accuracy of the results in different laboratories and conditions, which simplifies the process and reduces potential sources of error.

A previous publication outlined an adaptation to this MMqPCR assay using a dual-labeled multiplex qPCR TL assay [9] based on the same principle as that used in the monochromatic fluorescence assay to quantify telomeric repeat copy number relative to a single-copy gene. The principle behind this dual-labeled multiplex qPCR assay is similar to a Taqman assay that relies on the 5′ nuclease activity of Taq DNA polymerase to detect and quantify specific nucleic acid sequences in a real-time PCR amplification [10,11]. The basis behind a dual-labeled multiplex qPCR TL assay involves the use of two sequence-specific primers that flank the telomeric repeat sequence or the single-copy gene region and a corresponding probe labeled with a fluorescent reporter dye and a quencher molecule. During PCR amplification, the probe hybridizes to the target sequence, and the exonuclease activity by Taq polymerase during the PCR extension phase, cleaves the probe, releasing the fluorescent reporter dye from the quencher molecule, resulting in a measurable increase in fluorescence proportional to the amount of target sequence amplified, allowing for quantitative detection of the target.

The absolute TL (aTL) method differs from the MMqPCR method by introducing two separate qPCR assays using duplex oligomers to construct standard curves to determine either the telomeric repeat sequence or the genome copy number. By introducing serial dilutions of an 84mer duplex oligomer standard of TTAGG repeated 14 times, a telomere standard curve can be constructed where the average number of telomeric repeat sequences can be determined in kilobase pairs (kb). Incorporating another separate qPCR assay using a duplex oligomer standard curve for a single-copy reference gene allows for the measurement of total TL per diploid genome (in kilobases per chromosome). Although the MMqPCR assay does not provide a direct measurement of TL, the aTL qPCR method does. It uses TL standards to mathematically calculate TL to directly measure average TL in the tested sample [5,6]. Even though more time is needed for the aTL qPCR method to be evaluated by the TRN [7,8], an absolute value (in base pairs) is provided for TL, which can be useful for certain applications and comparisons. The aTL qPCR assay can offer detailed quantitative data on TL, which might be beneficial for specific research applications or for understanding subtle variations in TL that might not be easily detected with other methods. Since there is no dual-labeled multiplex aTL assay available, we provide such a method to measure average TL using a modification to an existing monochromatic aTL assay [12].

2. Materials and Methods

The methodology involved in this dual-labeled multiplex aTL assay is an adaptation of a previously published aTL assay using a monochromatic SYBR Green fluorescent dye [5,6,12] and was designed for the ability to multiplex the telomeric repeat and the single-copy gene regions in the one qPCR assay using dual-labeled fluorogenic hybridization probes [10,11]. It relies on using standard curves generated with synthesized oligomers of known lengths and quantities to generate quantitative results for tested samples by incorporating a corresponding dual-labeled fluorogenic probe to track primer amplification

for both the telomeric repeat and the single-copy gene qPCR amplifications in the one assay using the same DNA template.

2.1. Reagents and Supplies Needed

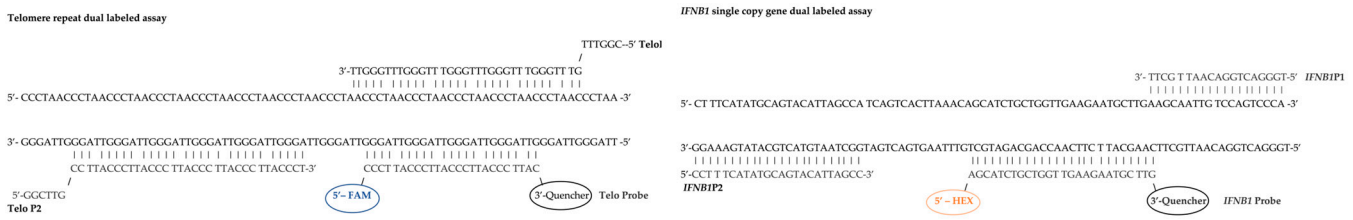
A dual-labeled multiplex aTL assay relies on detecting short amplicon lengths of 50–150 base pairs to ensure efficient doubling at each PCR cycle [13]. The dual-labeled aTL probes and primers were designed to produce an amplicon length of 84mer for the telomeric repeat qPCR assay and an 84mer for the *IFNB1* single-copy gene qPCR assay. Sequences for the telomeric repeat and single-copy gene primers, probes, and duplex oligomer standards are provided in Table 1. Since this assay multiplexes both the telomeric repeat and the single-copy gene in the one qPCR amplification, a double-quenched ZEN PrimeTime qPCR probe using a 5′ FAM fluorescent dye was made for the telomeric repeat assay and a double-quenched ZEN PrimeTime qPCR probe using a 5′ HEX fluorescent dye was made for the *IFNB1* single-copy gene assay to significantly lower background fluorescence [14].

Table 1. List of primers, probes, and standards used in the dual-labeled multiplex aTL assay.

Assay	Oligo ID		Oligo Sequence *
Telomeric Repeat Assay	TeloP1		5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3′
	TeloP2		5′-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3′
	Telo Probe		5′-6′FAM/CCCTTACCCTTACCCTTACCCTTAC/3IABkFQ-3′
Single Copy-Gene Assay	*IFNB1*P1		5′-TGGGACTGGACAATTGCTT-3′
	*IFNB1*P2		5′-CCTTTCATATGCAGTACATTAGCC-3′
	IFNB1 Probe		5′-HEX/AGCATCTGCTGGTTGAAGAATGCTTG/3IABkFQ-3′
Absolute Telomere Length Standards	Telomere Oligomer	Sense	5′-CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA CCC TAA-3′
		Antisense	5′-TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG TTA GGG-3′
	IFNB1 Oligomer	Sense	5′-CCT TTC ATA TGC AGT ACA TTA GCC ATC AGT CAC TTA AAC AGC ATC TGC TGG TTG AAG AAT GCT TGA AGC AAT TGT CCA GTC CCA -3′
		Antisense	5′-TGG GAC TGG ACA ATT GCT TCA AGC ATT CTT CAA CCA GCA GAT GCT GTT TAA GTG ACT GAT GGC TAA TGT ACT GCA TAT GAA AGG-3′

* All primers, probes, and duplex oligomer standards were manufactured using IDT (Integrated DNA Technology). All primers and PrimeTime qPCR probes were HPLC purified while the standards were PAGE purified. 3IABkFQ = 3′ Iowa Black Fluorescent Quencher.

When designing the primers and probes listed in Table 1 and illustrated in Figure 1, we placed the forward and reverse primers (P1 and P2) for both the telomeric repeat and the *IFNB1* single-copy gene as close as possible to each corresponding probe position while avoiding overlap (Figure 1).



(**a**) (**b**)

Figure 1. Schematic representation of the primers and probes annealing to their targeted sequence (**a**) for the telomeric repeat oligomer probe mix and (**b**) for the *IFNB1* single-copy gene oligomer probe mix.

The telomere primers are crafted with overhangs to reduce primer dimer formation while preserving their original length (Figure 1a). Consideration was also provided to the design of the probe too, as it is best to keep the G/C content of each probe at 30–80% [13]. The telomeric repeat probe was designed with 52% G/C content, while the *IFNB1* single-copy gene probe had 46.2% G/C content. Similarly, the current design also ensured that probes should not have a string of identical nucleotides, especially not four or more consecutive G's, and should have more C's than G's [13]. We also considered that probes should not have a G at the 5′ end [13]. For this reason, the telomeric repeat and *IFNB1* single-copy gene probe were designed to bind to the anti-sense strand for each oligomer to limit the number and position of the G's in the probe design especially for the telomeric repeat probe targeting a TTAGGG rich region.

All reagents, supplies, and equipment needed for the dual-labeled multiplex aTL assay are listed in Table 2.

Table 2. List of reagents, supplies, and equipment used to run the dual-labeled multiplex aTL assay.

Material	Vendor	Catalog #
Quant-iT Picogreen dsDNA Assay kit	ThermoFisher, Waltham, MA, USA	P7589
TE Buffer, pH 7.4 (20×)	ThermoFisher, Waltham, MA, USA	J60234.EQE
PrimeTime Gene Expression Master Mix (5 mL)	IDT, Coralville, IA, USA	1055772
pBR322 circular plasmid	ThermoFisher, Waltham, MA, USA	S66763
Jurkat Genomic DNA (human acute T cell leukemia)	ThermoFisher, Waltham, MA, USA	SD1111
1301 Genomic DNA (human T cell leukemia)	Sigma Aldrich, St. Louis, MO, USA	01051619
UltraPure Sterile Water	VWR, Radnor, PA, USA	RLMB-010-0100
Rainin Pipet-Lite LTS Pipettes (0.2–2 µL, 2–20 µL, 20–200 µL, and 100–1000 µL)	Mettler Toledo, Columbus, OH, USA	L-2XLS+; L-20XLS+ L-200XLS+; L-1000XLS+
QIAcuity HEPA/UV pipetting robot	Qiagen, Germantown, MD, USA	9001903
Rotorgene Q 5 plex HRM qPCR machine	Qiagen, Germantown, MD, USA	9001580
Rotor-disc 100 Rotor	Qiagen, Germantown, MD, USA	9081195
Rotor-disc 100 locking ring	Qiagen, Germantown, MD, USA	9018896
100 well rotor disk plate	Qiagen, Germantown, MD, USA	981311
Rotor disk heat sealing film	Qiagen, Germantown, MD, USA	981601
50 µL Conductive Filtered Tips (Qiagility)	Qiagen, Germantown, MD, USA	990512
200 µL Conductive Filtered Tips (Qiagility)	Qiagen, Germantown, MD, USA	990522
200 µL PCR tubes	Qiagen, Germantown, MD, USA	981005
5 mL diluent tube	Qiagen, Germantown, MD, USA	990552

2.2. qPCR Setup

2.2.1. Establishing the Standard Curves

Establishing the dual-labeled multiplex aTL method modified a previous real-time monochromatic aTL qPCR assay [5,6,12]. Standard curves both for the telomeric repeat and

the *IFNB1* single-copy gene are used along with incorporating dual-labeled fluorogenic probes in this multiplex aTL method. The information on generating the standard curves can be found in the Supplementary Material. A telomeric repeat standard curve is established with dilutions of known quantities of a synthesized 84 bp duplex oligonucleotide containing only TTAGGG repeated 14 times with a molecular weight (MW) of 51,779.6 (MW anti-sense = 25,112.4 + MW sense = 26,667.2) for the telomere standard curve. Similarly, the synthesized *IFNB1* duplex oligomer is 84 bp in length with a MW of 51,780 (MW anti-sense = 25,770 + MW sense = 26,010). After combining 80 µL of a 0.15 ng/µL working concentration of the telomeric repeat standard, 80 µL of a 0.00005 ng/µL working concentration of the *IFNB1* single-copy gene standard, and 640 µL of 0.25 × TE buffer, the first A1 multiplex standard curve contains 60 pg of telomeric repeats for the telomere standard and 0.02 pg diploid copy numbers for the *IFNB1* single-copy gene standard when 4 µL of this 800 µL solution is used in qPCR amplification. The calculation to determine telomeric repeat and diploid copy numbers in the first standard curve dilution was described previously [12]. A standard curve is generated by performing ten-fold serial dilutions of this A1 multiplexed telomeric repeat and the *IFNB1* single-copy gene duplex standard solution as listed in Table 3.

Table 3. Standard curve dilutions used in the dual-labeled multiplex aTL assay.

Standard Oligomer ID	Telomere Oligomer [DNA] pg/µL	Total Telomere [DNA] pg	Telomeric Repeat Sequences (kb)	*IFNB1* Oligmer [DNA] pg/µL	Total *IFNB1* [DNA] pg	Diploid Copy Number
STD A1 *	15	60	5.86×10^7	0.005	0.02	1.16×10^5
STD B2	1.5	6	5.86×10^6	0.0005	0.002	1.16×10^4
STD C3	0.15	0.6	5.86×10^5	0.00005	0.0002	1.16×10^3
STD D4	0.015	0.06	5.86×10^4	0.000005	0.00002	1.16×10^2
STD E5	0.0015	0.006	5.86×10^3	0.0000005	0.000002	1.16×10^1

* QIAgility robot pipets 1:10 dilution from STD A1 to generate other duplex standard oligomer dilutions.

2.2.2. DNA Concentration

Each tested sample is analyzed in triplicate. The concentration of each tested DNA sample was determined using the Quant-iT Picogreen dsDNA binding assay following the manufacturer's instructions. All DNA samples were diluted to a uniform concentration of 0.5 ng/µL using 0.25 × TE. A total of 3 ng of DNA (6 µL of 0.5 ng/µL for the tested samples) was analyzed in the dual-labeled multiplex aTL assay. An aliquot of 2 µL of 1.5 ng/µL plasmid DNA (pBR322) concentration was added to each 4 µL standard (A1-E5) to maintain a consistent 3 ng of total DNA per reaction tube.

2.2.3. qPCR Master Mix

A working master mix was constructed, as listed in Table 4, and modified for the number of samples to be analyzed. The QIAgility robotic workstation (Qiagen, Hilden, Germany) pipetted 14 µL of the working master mix (listed in Table 4) with either 6 µL of the tested DNA sample or 4 µL of the standard oligomer with 2 µL of the 1.5 ng/µL pBR322 circular plasmid. All samples were analyzed in triplicate in a 20 µL qPCR volume.

Table 4. Representation of master mix preparation.

Reagent	Volume in Each Sample (µL)	Final Concentration
40× Taqman Telomeric Repeat Assay mix	0.5	1×
40× Taqman Single-Copy Gene Assay (*IFNB1*) mix	0.5	1×
PrimeTime Gene Expression master mix (2×)	10	1×
UltraPure Water	3	

2.2.4. qPCR Amplification Conditions

Real-time qPCR was performed on a Qiagen Rotorgene Q using the cycling conditions listed in Table 5.

Table 5. qPCR cycling temperatures, cycling times, and purposes.

Cycle	Function
Hold	50 °C for 2 min to activate the UDG
Hold	95 °C for 10 min releases and activates the Hot Start AmpliTaq Gold DNA Polymerase
40 cycles	Denature at 92 °C for 15 s Anneal at 60 °C for 30 s Extension at 72 °C for 30 s (FAM and HEX data acquisition at each cycle)
Hold	40 °C for 10 min

Green fluorescence was enabled to capture the telomeric 6-FAM fluorescence while yellow fluorescence was enabled to capture *IFNB1* single-copy gene HEX fluorescence during the extension phase of the three step qPCR cycling conditions.

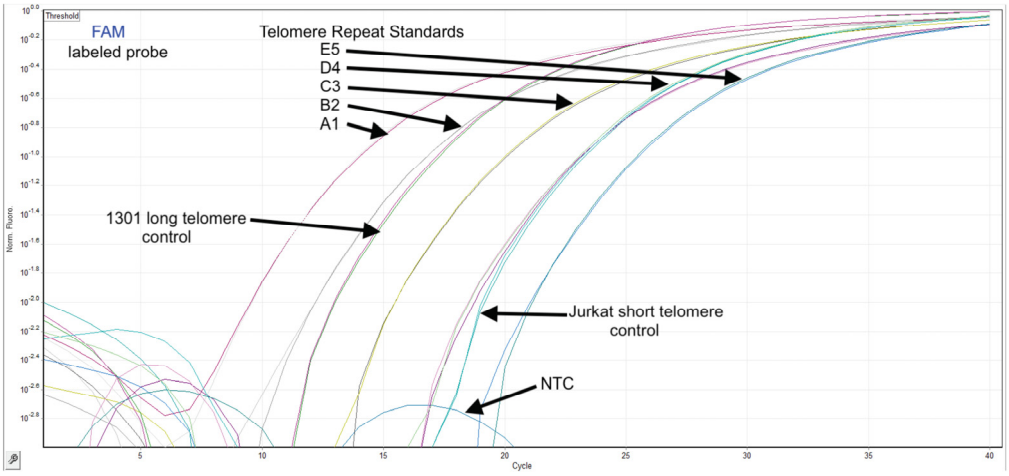
For tested samples, calculation of total TL per diploid genome for each sample was performed by dividing the number of diploid genomes in the sample determined from the *IFNB1* single-copy gene standard curve into the number of telomeric repeats determined from the telomeric repeat standard curve for each sample. Since there are 46 chromosomes with a telomere on the end of each diploid genome, this TL number was then divided by 92 to determine the average length of one telomere in the sample.

3. Results

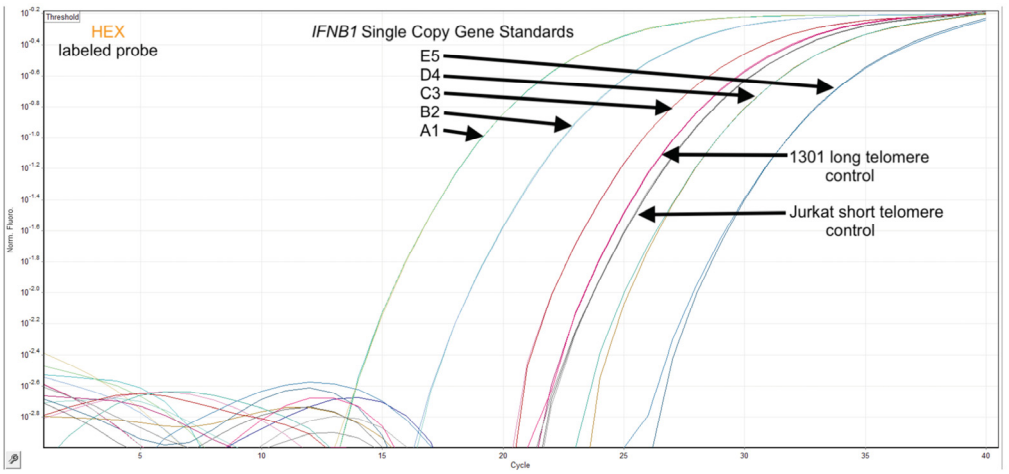
After the amplification steps were completed, the Rotor-Gene Q qPCR machine automatically determined the Ct thresholds for both the telomeric repeat and the *IFNB1* single-copy gene oligomer standard curves. The results obtained following qPCR cycling are shown in Figure 2.

To analyze the telomeric repeat qPCR results, only the 6-FAM fluorescence for the standard curves from A1-E5 were selected, as shown in Figure 2a. One of the triplicate readings was dropped if it deviated 15% or more from the other two. This was repeated with selecting only the HEX fluorescence to generate the *IFNB1* single-copy gene standard curve results, as shown in Figure 2b. Normally, a ten-fold dilution in the standard curve should result in ΔCt values between -3.6 to -3.1 cycles, reflecting a two-fold doubling of the oligomer template in each PCR cycle and a PCR efficiency of between 90 and 110% [15]. Results shown in Figure 1 using this dual-labeled multiplex qPCR aTL assay show a ΔCt of -3.10 with a PCR efficiency of 99.96% for the telomeric repeat assay while a ΔCt of -3.28 and a PCR efficiency of 99.98% is obtained for the *IFNB1* single-copy gene assay.

Figure 2 also shows no amplification crossing the threshold when using a non-template control (NTC). Figure 2a shows no amplification of the NTC crossing the threshold but generates some background fluorescent signal. This is to be expected, since the telomere primers and probes may act on themselves being a hexa-nucleotide DNA repeat region (5′ TTAGGG-3′)n when there is very little template available for the primers and probes to find. Conversely, there is no fluorescent signal obtained for the NTC amplification in the *IFNB1* single-copy gene qPCR assay in Figure 2b.



(**a**)



(**b**)

Figure 2. Dual-labeled fluorescent standard curves (**a**) generated with the telomeric repeat oligomer probe mix and (**b**) generated with the *IFNB1* single-copy gene oligomer probe mix.

We also ran the 1301 and Jurkat DNA samples to show the validity of this assay with tested DNA samples. The 1301 genomic DNA is marketed by Sigma Aldrich (St. Louis, MO, USA) as being derived from a human T cell leukemia cell line with long telomeres and is useful as a control in tests where telomere length is determined. The Jurkat genomic DNA is derived from human T cell leukemia cells and was shown to be capable of maintaining telomere length over time [16]. Table 6 shows the results obtained after analyzing the telomeric repeat and the dual-labeled multiplex aTL assay using these tested samples.

Table 6. Telomere length results for the tested samples using the dual-labeled multiplex aTL assay.

Tested Sample ID	Telomeric Repeat Sequences (kb)	Diploid Copy Number	Average aTL (kb)	Average aTL per Telomere (kb)
1301	2.59×10^6	3.63×10^2	7130	77.5
Jurkat	5.82×10^4	1.39×10^2	418.6	4.55

We obtained a telomere length of 77.5 kb for the 1301 DNA sample and a telomere length of 4.55 kb for the Jurkat DNA sample.

4. Discussion

We show a modification to an existing monochromatic aTL assay [12] to provide a dual-labeled multiplex aTL assay. Accurate measurement of telomere length using qPCR in large-scale epidemiological and population studies is important. The TRN has recommended the use of the MMqPCR telomere length assay, which uses monochromatic fluorescence to multiplex the telomeric and the single-copy gene measurement together in the one assay [4,7]. A previous publication modified this assay to introduce a dual-labeled fluorescence to detect the ratio of the telomeric repeat sequences to a single-copy gene to limit the detection of unspecific products when using the intercalation SYBR Green dye [9]. In a similar method, we modified an existing aTL assay [12] to multiplex both the telomeric sequence and the single-copy gene qPCR in the one assay by incorporating dual-labeled fluorescent probes to minimize time and costs involved when generating aTL results in base pair values. Furthermore, ΔCt values between -3.6 to -3.1 cycles and a PCR efficiency of between 90–110% is obtained with this method, reflecting a 2-fold doubling of the oligomer template in each PCR cycle when a ten-fold dilution of the standard curve for the telomeric repeat and the *IFNB1* single-copy gene qPCR assays are used [15]. Since a multiplex dual-labeled aTL assay was currently unavailable, this method provides a precise way to multiplex the telomeric repeat and the *IFNB1* single-copy gene qPCR assays together in the one PCR amplification, similar to the TRN recommended MMqPCR assay [4,7].

Having a multiplex aTL assay would also provide a cost-effective method when duplicating the TL results to analyze using the TRN recommended intra-class correlation coefficient (ICC) analysis [7,17]. A comparison between the monochromatic SYBR Green aTL assay and the dual-labeled multiplex aTL assay found the dual-labeled multiplex aTL method is half the cost of the monochromatic version. Although there is no difference in time required for analyzing the results, the dual-labeled multiplex aTL assay offers a two-fold time savings in generating the qPCR results as it requires only one qPCR assay. Additionally, this method minimizes the need for double the DNA to analyze by multiplexing two assays into a single qPCR assay. Consequently, ICC calculations can be performed at a comparable cost to the monochrome aTL assay, which necessitates two separate assays.

The primary advantage of a dual-labeled multiplex aTL assay is the significant enhancement in specificity and sensitivity since the reporter dye only emits fluorescence when it is released from suppression by the quencher. The inhibition of fluorescence by the quencher is only removed when the probe is degraded by the 5′ to 3′ exonuclease activity of the Taq DNA polymerase during the extension phase of the PCR cycle. Consequently, the resulting fluorescent signal directly correlates with the amount of target DNA present, leading to more accurate quantification. This can be shown when analyzing the non-template control sample, where no fluorescence was obtained for both the telomeric repeat and *IFNB1* single-copy gene qPCR assay as there is no target to amplify (Figure 2a,b).

The use of dual-labeled fluorescence also helps minimize background noise and reduces false positives, thereby increasing the assay's overall reliability. Dual-labeled fluorescent qPCR offers an extended dynamic range, which is crucial for detecting a wide range of target quantities. The use of a specific probe allows for accurate measurement across low to high copy numbers of the targeted nucleic acid. This is particularly valuable in TL gene expression analysis, where the telomeric DNA is compared to a reference single-copy gene.

The other advantage of using this dual-label multiplex aTL assay is the ability to generate telomere length results in base pair values by using a standard curve of known length. We show how this assay is able to detect both long and short telomere lengths in base pair values. When the 1301 and Jurkat DNA samples were tested with this dual-labeled multiplex aTL assay, a telomere length of 77.5 kb is obtained for the 1301 DNA sample and a telomere length of 4.55 kb for the Jurkat DNA sample. Previous studies also obtained similar telomere length results showing a telomere length of 70–80 kb for 1301 [5,18] and 4.77 kb for Jurkat [19]. The capability to generate absolute telomere length results using a

dual-labeled multiplex qPCR assay should allow for a more direct comparison of result between experiments similar to the MMqPCR method in a cost effective manner to allow for analysis using an ICC method.

Limitation of This Assay

Although this dual-labeled multiplex aTL assay shows a robust qPCR assay by obtaining ΔCt values between -3.6 and -3.1 and PCR efficiencies between 90–110% using a 10-fold dilution of the telomeric repeat and the *IFNB1* single-copy gene standard curves, it should be noted that the telomeric repeat probe will have an affinity and complementarity for binding with the TeloP1 primer, reducing the availability of this primer and probe in the amplification of this hexa-nucleotide DNA repeat telomere region. The complementarity between the telomere probe and TeloP1 primer won't contribute to any expected fluorescence because the telomeric repeat reporter dye isn't cleaved from its 3′ quencher, as there is no amplification occurring with the Taq DNA Polymerase when this primer binds to the probe (see Figure 3).

Figure 3. Primer/probe annealing shows complementarity between the telomere probe and the TeloP1 primer.

5. Conclusions

Using a dual-label multiplex fluorescent absolute telomere length qPCR protocol represents a significant advancement over the current monochrome absolute telomere length assay. The innovative approach outlined in this paper offers several key advantages that enhance both the precision and reliability of telomere length measurements. By utilizing dual-labeled fluorescent probes, the protocol outlined in this paper not only enables the simultaneous quantification of telomeric repeat and *IFNB1* reference sequences in the one reaction but also improves the accuracy of absolute telomere length assessments using qPCR. The multiplex capability reduces the need for multiple individual reactions, thereby conserving valuable samples and minimizing experimental variability. Furthermore, the enhanced sensitivity and specificity of this technique make it an invaluable tool for a cost-effective measurement to analyze telomere length using ICC analyses, providing deeper insights into the role of telomeres in aging, disease, and therapeutic interventions. As the field continues to evolve, the dual-label multiplex fluorescent absolute telomere length qPCR protocol may be presented as a powerful and efficient method that addresses many of the limitations of traditional telomere qPCR techniques, paving the way for more nuanced and impactful scientific discoveries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/dna4040026/s1, Supplementary Methods: Absolute Telomere Length Standard Curve Calculations.

Author Contributions: Conceptualization, S.R.S. and S.F.L.; Data curation, S.R.S., E.A.C., and K.M.D.; Formal analysis, S.R.S.; Methodology, S.R.S.; Supervision, S.R.S. and S.F.L.; Writing—original draft, S.R.S.; Writing—review and editing, S.R.S., E.A.C., K.M.D., and S.F.L. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding. The APC was waived due to this study being an invited manuscript contribution.

Institutional Review Board Statement: This study was conducted following a review from The Pennsylvania State University Institutional Review Board, who determined the samples were not collected for research purposes (protocol code: IBC 48849).

Informed Consent Statement: Not applicable.

Data Availability Statement: Data presented in this article are also freely available by contacting the corresponding author.

Acknowledgments: We would like to acknowledge the Biomarker Core Lab in the College of Health and Human Development at Penn State University for their assistance.

Conflicts of Interest: The authors declare there are no conflicts of interest.

References

1. Pfeiffer, V.; Lingner, J. Replication of telomeres and the regulation of telomerase. *Cold Spring Harb. Perspect. Biol.* **2013**, *5*, a010405. [CrossRef] [PubMed]
2. Vaiserman, A.; Krasnienkov, D. Telomere Length as a Marker of Biological Age: State-of-the-Art, Open Issues, and Future Perspectives. *Front. Genet.* **2021**, *11*, 630186. [CrossRef] [PubMed]
3. Cawthon, R.M. Telomere measurement by quantitative PCR. *Nucleic Acids Res.* **2002**, *30*, e47. [CrossRef] [PubMed]
4. Cawthon, R.M. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res.* **2009**, *37*, e21. [CrossRef] [PubMed]
5. O'Callaghan, N.; Dhillon, V.; Thomas, P.; Fenech, M.A. A quantitative real-time PCR method for absolute telomere length. *Biotechniques* **2008**, *44*, 807–809. [CrossRef] [PubMed]
6. O'Callaghan, N.J.; Fenech, M.A. quantitative PCR method for measuring absolute telomere length. *Biol. Proced. Online* **2011**, *13*, 3. [CrossRef] [PubMed]
7. Lindrose, A.R.; McLester-Davis, L.W.Y.; Tristano, R.I.; Kataria, L.; Gadalla, S.M.; Eisenberg, D.T.A.; Verhulst, S.; Drury, S. Method comparison studies of telomere length measurement using qPCR approaches: A critical appraisal of the literature. *PLoS ONE* **2021**, *16*, e0245582. [CrossRef] [PubMed]
8. Telomere Research Network TRN Recommendations. Available online: https://trn.tulane.edu/resources/study-design-analysis/ (accessed on 21 September 2024).
9. Sethi, I.; Bhat, G.R.; Kumar, R.; Rai, E.; Sharma, S. Dual labeled fluorescence probe based qPCR assay to measure the telomere length. *Gene* **2021**, *767*, 145178. [CrossRef] [PubMed]
10. Arya, M.; Shergill, I.S.; Williamson, M.; Gommersall, L.; Arya, N.; Patel, H.R. Basic principles of real-time quantitative PCR. *Expert Rev. Mol. Diagn.* **2005**, *5*, 209–219. [CrossRef] [PubMed]
11. Heid, C.A.; Stevens, J.; Livak, K.J.; Williams, P.M. Real time quantitative PCR. *Genome Res.* **1996**, *6*, 986–994. [CrossRef] [PubMed]
12. Siegel, S.R.; Ulrich, M.; Logue, S.F. Comparison qPCR study for selecting a valid single copy gene for measuring absolute telomere length. *Gene* **2023**, *860*, 147192. [CrossRef] [PubMed]
13. Premier Biosoft Taqman Probes. Available online: https://www.premierbiosoft.com/tech_notes/TaqMan.html (accessed on 21 September 2024).
14. Integrated DNA Technologies Double Quenched Probes Increase Signal to Noise Ratios by Decreasing Background Fluorescence. Available online: https://www.idtdna.com/pages/education/decoded/article/two-quenchers-are-better-than-one (accessed on 21 September 2024).
15. Biosistemika. Understanding qPCR Efficiency and Why It Can Exceed 100%. Available online: https://biosistemika.com/blog/qpcr-efficiency-over-100/ (accessed on 21 September 2024).
16. Huang, E.E.; Tedone, E.; O'Hara, R.; Cornelius, C.; Lai, T.P.; Ludlow, A.; Wright, W.E.; Shay, J.W. The Maintenance of Telomere Length in CD28+ T Cells During T Lymphocyte Stimulation. *Sci. Rep.* **2017**, *7*, 6785. [CrossRef] [PubMed]
17. Liljequist, D.; Elfving, B.; Skavberg Roaldsen, K. Intraclass correlation—A discussion and demonstration of basic features. *PLoS ONE* **2019**, *14*, e0219854. [CrossRef] [PubMed]
18. Jeyapalan, J.C.; Saretzki, G.; Leake, A.; Tilby, M.J.; von Zglinicki, T. Tumour-cell apoptosis after cisplatin treatment is not telomere dependent. *Int. J. Cancer* **2006**, *118*, 2727–2734. [CrossRef] [PubMed]
19. Zhdanov, D.D.; Pokrovsky, V.S.; Pokrovskaya, M.V.; Alexandrova, S.S.; Eldarov, M.A.; Grishin, D.V.; Basharov, M.M.; Gladilina, Y.A.; Podobed, O.V.; Sokolov, N.N. Inhibition of telomerase activity and induction of apoptosis by Rhodospirillum rubrum L-asparaginase in cancer Jurkat cell line and normal human CD4+ T lymphocytes. *Cancer Med.* **2017**, *6*, 2697–2712. [CrossRef] [PubMed]