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Relative Telomere Length Measurement by Quantitative PCR

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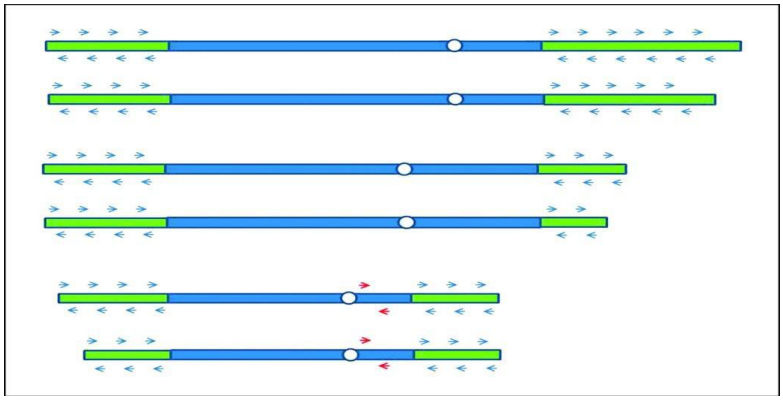
Abstract

*Strategy for determining relative telomere length by quantitative PCR was to measure for each DNA sample the factor by which sample differed from a reference DNA sample (36B4 gene) in its ratio pf telomere repeat copy number (telomere C_t) to single copy gene (36B4 gene Telomere IC) .**This ratio should proportional to the Average telomere length (Ref. Nucleic Acid Research 2002 May 15; 30(10) e47 Richard M. Cawthon[a]***

Principle:

The strategy was adopted from Cawthon et.al.[66] The principle realize on the Direct comparison of the average telomere length (T) of the sample, in Comparison to the Single Copy reference(s) gene (SCG) which serves as the reference for data normalization. Hence the increase in the control value of telomere or increase in the ratio of T/S indicates decreased telomere repeats. [**Nucleic Acids Research Richard M. Cawthon 2002 May 15;30(10): e47].**

Figure 1



Telomere length measurement by qPCR Three pairs of chromosomes are shown (A, B, C) where the circles represent the centromere, the blue region the genomic DNA and the green the telomeric (TTAGGG)n repeats. The telomeric repeat length as shown differs between the different chromosomes as well as the same arms of individual chromosomes. The blue arrows represent the telomere primer pairs, where there are multiple binding sites along the telomeric repeat sequence and the number able to bind is proportional to the length of the telomere, thus is a measure of length. The red primer pairs (C) are the single copy gene primers, which only bind at one position in the genome, allowing average telomere length to be determined using a ratio of the telomere product to the single copy gene (T/S) ***(Adapted from Ref- Thesis Establishment and validation of a telomere length assay A thesis submitted for the degree of Bachelor of Biomedical Science with Honors at the University of Otago, Christchurch, New Zealand. Sarah Jodczyk November 2011).***

ISSN : 2321- 4708
Oct. 2019, Year - 6 (78)
Paper ID : RRJ673031

Instruments, Reagents & consumables

Following reagents, chemicals, used were of analytical grade and sourced from well-known companies. High quality Nuclease free consumables &calibrated instruments from various companies are also listed in table below

Reagent/Chemicals name	**Brand/ Model Name**	**Source**	**Storage**
Blood Genomic DNA Extraction kit	NA	IBI Scientific	Ambient Room Temperature
100% Absolute Ethanol	NA	Merck Inc.	Ambient Room Temperature
Real time PCR Reagents kit	2X Syber fast Q-PCR reagent	Kappa Biosciences	-20 deep freezer
Oligonucleotide primers	NA	Sigma	-20 deep freezer
Instruments			
Real Time Q-PCR Machine	Quant Studio-5 systems Sr No. 272520630	Applied Biosystems	NA
Nanodrop	Nano Vue	GE Healthcare	
-20 Deep Freezer	Celfrost	Samsung	
Micropipettes	NA	Eppendorf	
Consumables			
0.2 ml DNAse, RNAse free PCR tubes	8 well strips with lids	Genexy	
1.5 ml graduated DNAse, RNAse free microfuge tubes	1.5 ml MCT	Genexy	
St. DNAse, RNAse free microtips (1000ul, 200ul & 10ul)		Prefect	
Autoclave			
1% Sodium Hypochlorite		Local	
Reagent/Chemicals name	**Brand/ Model Name**	**Source**	**Storage**
Nuclease free water		AMBION	
Agarose powder	LE Agarose	Sisco Research Laboratories	
Ethidium Bromide Solution		Merck	
Horizontal agarose		Technosource	

ISSN : 2321- 4708
Oct. 2019, Year - 6 (78)
Paper ID : RRJ673031

Gel electrophoresis system			
UV Transilluminator		Techno-source	
Dry Heat Block		Neolab	
High speed centrifuge with 1.5 ml tube rotor		Thermo Scientific	

Control & Study subjects sample source:

Total 300 subjects were studied (controlled diabetes mellitus 150 & Uncontrolled Diabetes Mellitus 150) age group of participant is 30 to 60 years.

DNA Extraction & Quantification: Method Verification

The Genomic DNA samples from the control and study subjects used for the project had been extracted using IBI scientific Blood genomic DNA extraction kit and stored at -20^0 C prior to the commencement of the Q-PCR assay. The extracted DNA was quantified on Nanodrop spectrophotometer (NanoVue, GE Healthcare). The ratio of 260/280 was determined for quality and quantity of DNA extracted using IBI kit. The integrity of DNA was also checked on 0.8% agarose gel using ethidium bromide as DNA visualizing dye under UV Transilluminator. All the DNA samples were quantified as µg/ml on Nanovue using elution buffer from DNA extraction kit as a blank. The record of which was required in further Q-PCR assay.

Genomic DNA Extraction and quantification:IBI Scientific Blood DNA extraction protocol

Step 1: RBCLysis
Step 2: Cell Lysis
Step 3:DNA Binding
Step 4: Washing

Oligonucleotide Reconstitution:

Oligonucleotide primers for single copy internal control gene 36B4 and Telomere region were ordered from Sigma. All the 4 lyophilised primers procured from Sigma were dissolved in DNAse,RNAse free water (Ambion Inc.) to a final stock concentration of 100µM and aliquots of 10µlit are stored at -20^0 C. A working stock of 10pmole/µL (10uM) is prepared by 10-fold dilution of each 100µM. The working stock concentration is stored in a separate box at -20^0 C.A working stock of 10pmole/µL (10uM) is prepared by 10-fold dilution of each 100µM. The working stock concentration is stored in a separate box at -20^0 C.

- **Table 2: Primers used in the study**

Primer name	Primer Sequence	Amplicon size
Tel1	5'GGT TTT TGA GGG TGA GGG TGA GGG TGA GGG TGA GGG T 3'	76 bp
Tel 2	5' TCC CGA CTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA 3'	

ISSN : 2321- 4708
Oct. 2019, Year - 6 (78)
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36B4 (U)	5'**CAG CAA GTG GGA AGG TGT AAT CC 3'**	75 bp
36B4 (D)	5' CCC ATT CTA TCA TCA ACG GGT ACA A 3'	

Real Time Polymerase Chain Reaction (Q-PCR) set up:

Real Time PCR is one of the most sensitive and reliable quantitative methods for gene expression, targeted mutation analysis (Genotyping) and copy number analysis. Every PCR has three phases: Exponential phase, linear phase and plateau phase (Fig 4).

- **Exponential phase** is the earliest segment in the PCR in which product increases exponentially since reagents are not limited.
- **Linear phase** is characterised by a linear increase in product as PCR reagents become limited.
- **Plateau Phase**: PCR will ultimately reach the plateau phase during later cycles because amount of reagent is depleted and the amount of product will not change.

The graph of florescence intensity Vs cycle no is plotted in Log_2 scale to yield a linear range at which the log of florescence correlates with original template amount. A baseline and a threshold can then be set for each experiment which takes care of the noise and the background florescence generated. The cycle number at which the amplification curve crosses the set threshold is called as cycle Threshold (Ct).

The real Time data can be quantified in 2 ways:

1. Absolute Quantification requires internal or external calibrator with known concentration values from which the target template copy number or concentration can be derived.
2. Relative quantification relies on the comparison between expressions of target gene Vs a reference gene & the expression of target in study population Vs reference population. Relative quantification is sufficient for most physiological & pathological studies. Several data analysis procedures have been developed for relative quantification. ΔΔ Ct method will be used in the present study for relative telomere length measurement.

Table 3: PCR reaction set up for Telomere gene amplification;

TEL PCR			**PCR Profile**
PCR Reagent	**Volume/ reaction**	**Final Conc. /reaction**	**95^{0}C 10 min**
2x SYBR green universal Q-PCR MM with low ROX	**12.5**µL		**95 ^{0}C 15 sec** **60 ^{0}C 1 min data collection** (40 cycles)
Tel1 (Forward primer)	**0.027**µL	270 nM	**Melt curve**

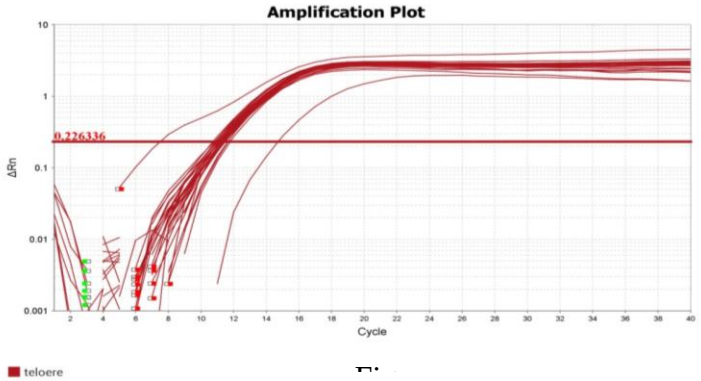
ISSN : 2321- 4708
Oct. 2019, Year - 6 (78)
Paper ID : RRJ673031

Tel2 (Rev primer)	**0.04**µL	900 nM	**Cooling**
Genomic DNA	**Variable**	20 ng	
Nuclease free water	**Variable**		
Total Volume	**25** µL		

Table 4: Real time PCR set up for reference / Single copy gene/ Internal control gene amplification

36 B4 PCR			**PCR Profile**
PCR Reagent	**Volume/ reaction**	**Final Conc. / reaction**	**95^{0}C 10 min**
2x SYBR green universal Q-PCR MM with low ROX	**12.5** µL		**95 ^{0}C 15 sec** **60 ^{0}C 2 min data collection** (40 cycles)
36B4 U (Forward primer)	**1.4** µL	300nM	**Melt curve**
36 B4 D(Rev primer)	**1.4** µL	500 nM	**Cooling**
Genomic DNA	**Variable**	20 ng	
Nuclease free water	**Variable**		
Total Volume	**25** µL		

Total 300 Type 2 Diabetes Mellitus patients [150 controlled T2DM and 150 Uncontrolled T2DM] (Ct , Ic, and ratio detailed value) are seen in Master Chart



ISSN : 2321- 4708
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