DISSERTATION

ON

TELOMERE LENGTH A BIOMARKER FOR AGEING

Submitted by:

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In partial fulfilment for the award of the degree of

MASTER OF SCIENCE IN BIOTECHNOLOGY

Department of Biotechnology

THAPAR UNIVERSITY, PATIALA

JULY, 2015

ABBREVIATIONS

TL	-	Telomere Length
DNA	-	Deoxyribo Nucleic Acid
RNA	-	Ribo Nucleic Acid
PCR	-	Polymerase Chain Reaction
RT-PCR	-	Real Time Polymerase Chain Reaction
PCNA	-	Proliferation Cell Nuclear Antigen
DDR	-	DNA damage response
NHEJ	-	Non-Homologous End Joining
ROS	-	Reactive Oxygen Species
ALT		Alternative Lengthening of Telomeres
TRF	-	Telomere Restriction Fragment
STELA	-	Single Telomere Length Analysis
NAC	-	N-acetyl-l-cysteine
POT	-	Protection of Telomeres
TRF	-	Telomerase repeat binding factor
µl	-	Micro litre
NaCl	-	Sodium Chloride
TE	-	Tris EDTA
EDTA	-	Ethylene Diamine Tetra Acetic acid
mM	-	mili Molar
w/v	-	Weight / volume
rpm	-	Rotation per minute
EtBr	-	Ethidium Bromide

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Date- 10th July, 2015

CERTIFICATE

This is to certify that **Ms. Taruna Gupta**, student of Thapar university, Patiala, Punjab, has successfully completed her project in **"Telomere length a Biomarker for Ageing"** with us at *Allele Life Sciences Pvt. Ltd. Noida*, (U.P.) from 5th January, 2015 to 10th July, 2015. She has been very hard working and sincere in her entire period.

We wish her all the best for future.

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This is to certify that the dissertation entitled "Telomere length a biomarker for ageing" submitted by Taruna Gupta in partial fulfilment of requirement for the ward of degree of Master of Science in Biotechnology to Thapar University, Patiala, is a record of students own work carried out by her under my supervision and guidance. The report has not been submitted for the ward of any other degree or certificate in this or any other university.

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I hereby declare that work present in dissertation entitled **"Telomere length a biomarker for ageing"** in partial fulfilment of the requirement for the award of degree of Master of Science in Biotechnology is an authentic record.

Taruna Gupta

Place: PATIALA

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Date : July, 15

301301018

ACKNOWLEDGEMENT

I acknowledge with gratitude, the invaluable opportunity, the care and constant encouragement which Mr. Mritunjay Kr Singh has provided me. I am deeply indebted for his constant support, time and guidance. Without his guidance I could not complete my thesis.

I certainly want to thank Mrs. Vasundhara Reddy who always encouraged me to do my work.

I deeply like to thank Mr. KB Singh who has always been a mentor for me. He showed different ways to approach a research problem and helped me to be persistent to accomplish any goal.

I also want to acknowledge the continued help given by Miss Roma Lal and Miss Rashmi Negi for their direct supervision when handling the laboratory experiments. A special thanks to my fellow mates for their constant support.

Last but not the least, I thanks my family for their unconditional love and support and immense faith in me at times when I doubted myself, without them completion of this thesis would not be possible.

ABSTRACT

Telomeres are nucleoprotein structure located at the end of chromosomes that plays a major role in protecting it. Telomeres shorten with each round of cell division and this mechanism limits proliferation of human cells to a finite number of cell divisions by inducing replicative senescence, differentiation, or apoptosis. Moreover, shortening of telomere during ageing and smoking is associated with increasing cardiovascular disease risk as we know smoking increases the thickening of blood. In this review we summarize our current knowledge on the role of telomeres shortening in humans by comparative analysis of TL by using blood samples of different person with different age groups, smokers and non-smokers & sports and non-sports of same ages with the help of different techniques like PCR, RT-PCR and Southern Blotting.

Keyword: Telomere, ageing, senescence, apoptosis, TL (Telomere Length), PCR (Polymerase Chain Reaction), RT-PCR (Real Time- Polymerase Chain Reaction), Southern blotting.

LIST OF TABLES AND FIGURES

1. INTRODUCTION

1.1 General Introduction

Ageing is the process of becoming older. In broader sense, ageing can refer to single cell within an organism. In human, ageing represents the accumulation of changes in a human being over time, encompassing physical, psychological, and social change. Ageing is among the largest known risk factors for most human diseases. The rate of ageing varies substantially across different species, and this, to a large extent, is genetically based. Every nucleus of a eukaryotic cell is packed with chromosomes, the long, thread-like DNA molecules that carry the genes of living organisms. In the end of the chromosomes there is a region of repetitive nucleoprotein structure called telomeres, which prevent chromosomal ends from end to end fusion and degradation.

1.2 Telomere Structure

Telomeres consist of stretches of repetitive DNA with high guanine and cytosine content. In humans, the telomere terminus consists of 4-15 kbp (kbp = kilobase pairs) of the hexanucleotides 5'-TTAGGG-3'. The G-rich DNA strand runs 5' to 3' toward the terminus and protrudes 100-150 nucleotides beyond the complementary C-rich strand.

Telomere repeat sequences in different organisms:-

- Humans - TTAGGG
- Plants - TTTAGGG
- *S.cerevsiae* - TGGTGTGTGTG

1.2.1 Shelterin

Telomeres are stabilized by a protein complex called shelterin. It helps in formation of t-loops and regulation of telomere length. It is composed of six protein. Figure 1.1 – telomeric repeat binding factor 1and 2 (TRF1andTRF2), protection of telomeres 1 (POT1), repressor/activator protein 1 (Rap1), POT1-TIN2 organizing protein (TPP1, also referred to as TINT1, PIP1, and PTOP), and TRF1-interacting protein 2 (TIN2). Three of these proteins

(TRF1, TRF2, and POT1) directly bind to telomeric repeats. TRF1 and TRF2 binds to double-stranded repeats via their SANT/Myb domains with high specificity and affinity and each have a flexible hinge that enables the formation of the t-loop. POT1 binds directly to the single-stranded 3'overhang via its OB folds, which is important in the formation of the d-loop. Unlike human cells that have only one POT1 gene, mice have two variants that are highly homologous (POT1a and POT1b) but have distinct roles at telomeres. The other three proteins (Rap1, TPP1, and TIN2) are recruited by TRF1 and TRF2 to assist in stabilizing telomeres. TIN2 acts as the bridge between different shelterin components by binding TRF1 and TRF2 in separate domains and recruiting the TPP1-POT1 complex Furthermore, a novel isoform of TIN2 (TIN2L) tethers telomeres to the nuclear, which may play a role in chromosome stability.

TPP1 binds TIN2 and POT1 in separate domains and is thought to be central in recruiting POT1 to telomeres. Lastly, Rap1 is recruited by TRF2 and forms a tightly associated complex that is essential for Rap1's binding to telomeres. Overall the shelterin complex is involved in telomere stability and telomere length regulation.

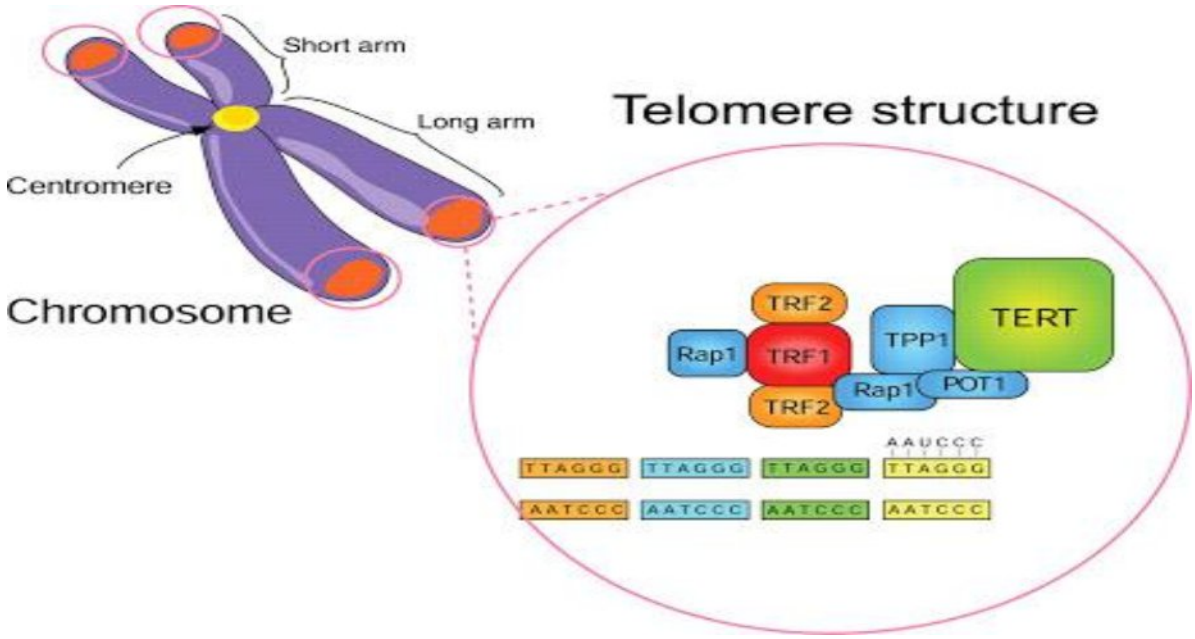


Figure 1 shows structure of Telomere

1.3 Telomere Function

Telomeres are considered as a telomeric cap because of the important functions it serves for linear DNA. Over the years scientists have uncovered that telomeres play a fundamental role in the stability and mobility of the genome and prevent erosion of coding DNA.

1.3.1 Genomic Stability

The chromosome ends were first proposed to be important for chromosome stability by the combined work of Barbara MClintock and Hermann Muller on their work with maize and fruit flies, respectively (McClintock, 1939; Muller, 1938). Muller coined the term telomere from the Greek words telos (end) and meros (part). Both scientists observed that without telomeres, chromosomes would fuse together and break off during mitosis, which was catastrophic to cellular physiology.

Telomere dysfunction either by critically shortened telomeres or missing Shelterin components can activate the DNA damage response (DDR) or signal up-regulation of cell cycle checkpoints such as p53. In some cells, cell cycle checkpoints can be by passed, propagating breakage fusion bridge cycles and genomic rearrangements with risk of neoplastic transformation. Thus cells require telomeres as a way to protect natural ends from being recognized as double strand breaks, in order to suppress the DNA repair machinery and maintain genomic stability.

Furthermore, during meiosis telomeres form bouquet structures that are integral to the coordination of the spindle apparatus to ensure proper alignment and segregation of genetic material

1.3.2 End-replication problem

One of the most important functions of telomeres was discovered by Olovnikov and Watson who noticed that linear chromosomes cannot replicate their very ends, termed the “end-replication problem”. The end-replication problem leads to inevitable loss of telomeres at every cell division. DNA polymerase can only replicate in the 5’ to 3’ direction and requires a short RNA primer for initiation. Because DNA is double stranded, one strand will be synthesized in short “okazaki” fragments. However, at the 5’ end of the lagging strand, there will be a gap due to removal of the last RNA primer. Currently, it is not known whether or not the last RNA primer is placed at the very end of telomeres or is variable. Telomere ends undergo further processing following DNA replication to ensure there is a 3’ overhang with a

precise end sequence. Interestingly, the length of this overhang is highly variable between different cell types and is closely associated with telomere length.

Initially, cells were thought to lose telomeres at a constant rate, keeping track of the number of cell divisions like a "mitotic clock". However, there is considerable heterogeneity in the rate of telomere shortening and in the number of divisions a cell can undergo. Notably, the end-replication problem directly limits the number of possible cell divisions, later known as replicative senescence.

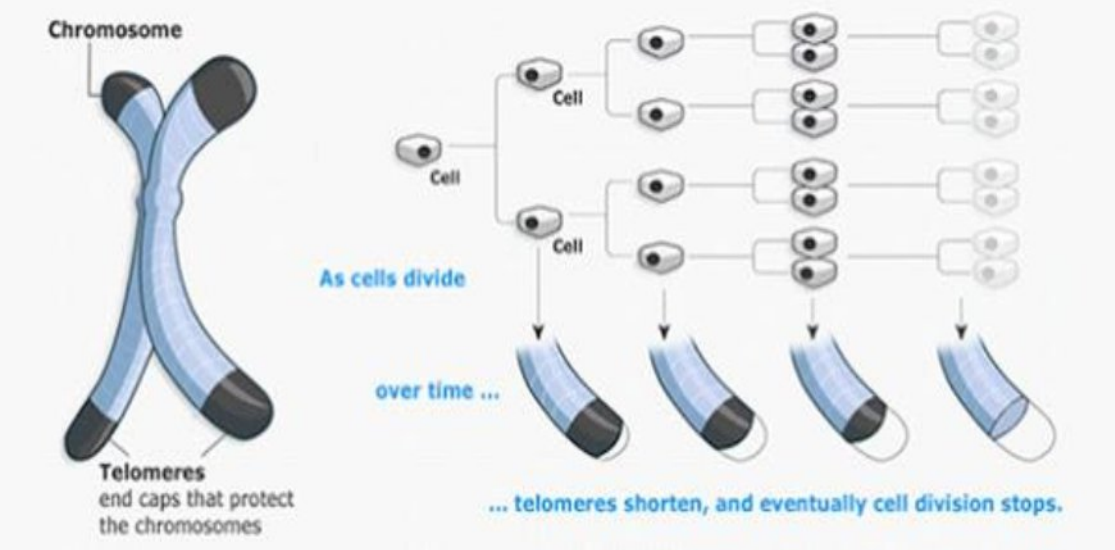


Figure 2 shows what we lose with Age

1.4 Telomeres and cell senescence

There are several ways in which cells senesce but one of the main mechanisms is telomere shortening and dysfunction, specifically referred to as replicative senescence. During each round of cell division, telomeres shorten by ~50-200 base pairs due to oxidative stress and the end replication problem – where DNA polymerase cannot replicate the very ends of the lagging strand. In cells, this shortening eventually causes telomeres to reach a critical length ("Hayflick limit") that signals a DNA-damage response (DDR), which involves protein kinases (ATM and CHK2), adaptor proteins (53BP1 and MDC1) and chromatin modifiers (γ-H2AX). Telomere dysfunction induced foci (TIF), defined as cells with more than 50% of 53BP1 foci colocalised with telomere repeats, and are often used to identify telomere-dependent senescent cells. Telomere-dependent activation of DDR further leads to the activation of p53 via its phosphorylation and upregulation of its downstream target p21.

Expression of p21 maintains pRB in a hypo phosphorylated active state, which inactivates the transcriptional factor E2F. E2F is responsible for inducing the expression of genes that encode proteins responsible for cellcycle progression (i.e. replication-dependent histones, c-FOS, cyclin A and B, and proliferation cell nuclear antigen (PCNA). Therefore, repressing E2F activity causes cells to enter a state of permanent cell cycle arrest (senescence). If any of these cell cycle checkpoints are by-passed (most commonly p53), perhaps due to mutations, the cell will continue to divide and telomeres will continue to shorten until a crisis phase (M2 phase) is reached. In M2 phase, telomeres are virtually non-existent and chromosome fusion events occur due to activation of the non-homologous end joining (NHEJ) pathway.

One of the key factors in triggering senescence or apoptosis in aging cells is shortened telomeres that can no longer form a closed t-loop. In fact, inhibition of TRF2 from shelterin in vitro causes the activation of DDR and senescence. In a similar way, human cells transfected with short telomere oligonucleotides that mimic the effect of an open t-loop also trigger a p53 dependent cell cycle arrest. It is noteworthy that cellular senescence can also be induced by telomere-independent mechanisms such as damage induced by reactive oxygen species (ROS), which follows an alternate senescence pathway through the upregulation of p16 and pRB. Murine fibroblasts in culture will senesce after 15-20 population doublings despite having exceptionally long telomeres (>20 kb) when exposed to atmospheric oxygen concentrations of 20%, which is above biological levels of 3%. In contrast, human fibroblasts with shorter telomeres (10-15 kb) senesce between 50-60 population doublings, suggesting better protective mechanisms against ROS compared to mouse cells. To some degree this argues against the relationship between telomere shortening and aging, particularly in mice, however, in vitro studies do not fully translate in vivo. Furthermore, the shortest mice telomeres are just as short to those in human cells and a single short telomere is sufficient to set off the cascade of events leading to senescence. Finally, it is not known whether the threshold telomere length that induces senescence in humans and in mice is different. Perhaps in mice that have an average T-loop size 6-7 times greater than in humans, require longer telomeres to maintain a closed T-loop structure.

1.5 Telomere length (TL) regulation

TL equilibrium is established by the net result of telomere shortening and lengthening events. The frequency of each of these events varies depending on the cell type and organism involved.

1.6 Factors involved in Telomere length maintenance

The progressive attrition of telomeres caused by the end-replication problem and oxidative damage posed a problem for certain cell types, especially in the germ line. If telomeres continue to shorten across generations, species with linear chromosomes (eukaryotes) would soon become extinct. Cells had to develop ways to overcome this problem by mechanisms of telomere lengthening. The most common method, discovered by Nobel Prize laureates Dr. Elizabeth Blackburn and Dr. Carole Greider, is by the activity of a reverse transcriptase called telomerase. A second, less common method, alternative lengthening of telomeres (ALT), was discovered in telomerase negative cancer cells.

1.6.1Telomerase

Telomerase exists to circumvent the end-replication problem by catalyzing the addition of telomeric repeats de novo to the ends of DNA. Telomerase is a ribonucleoprotein composed of a core reverse transcriptase (TERT) and an RNA component (TERC), which acts as a template for addition of telomeric repeats to the 3' end of telomeres.

Expression and activity of telomerase are highly regulated at various levels, which include transcription, splicing, post-translational modifications, assembly, and subcellular localization. Many proteins function in concert to orchestrate recruitment of telomerase, telomere length itself being a signal. In fact, telomerase preferentially elongates the shortest telomeres, which seems to be a function of shelterin proteins. Longer telomeres contain more shelterin proteins, which represses access of telomerase to telomere ends. However, the rate of telomere elongation by telomerase can be increased by inhibition of various shelterin components, such as POT1 or TRF1.

Telomerase expression in somatic cells also varies between species. Mice continue to express telomerase to some degree in somatic tissues, whereas humans do not. Furthermore, recent evidence suggests that decreased somatic telomerase expression correlates with increased body size of a species, proposing an evolutionary mechanism to offset the increased risk of cancer due to increased body mass.

Ultimately, telomerase expression is highly associated with risk of cancer as expressed in over 85% of human cancers. Therefore, there is a trade-off between replicative capacity and risk of cancer in the regulation of telomerase activity, which varies between species and cell types.

1.7 Telomere length measurement

Several methods of measuring telomere lengths have been developed over the years. The main two groups of measurements are those that measure average telomere length (TRF and qPCR) and those that measure telomere lengths of individual chromosomes (Q-FISH, Flow FISH, and STELA). All methods use binding of nucleic acid probes or primers specific to telomeric repeats. Discrepancies between telomere lengths measurements of the various methods arise from variability in the amount of extra-telomeric region included in some techniques and less in others. The two most popular methods in the literature are telomere restriction fragment (TRF) analysis and quantitative polymerase chain reaction (qPCR).

1.7.1 TRF analysis

TRF analysis by southern blotting was the first method to be described by Harley et al, in 1990. This technique uses a pair of frequent cutting restriction enzymes (i.e. Hinf1 and Rsa1) to cut DNA into small fragments, specifically excluding telomeric repeats as substrates. Frequent cutting restriction enzymes are used to minimize the size and variation of extra-telomeric region. The fragmented DNA is then separated by agarose gel electrophoresis, transferred to a nitrocellulose or nylon gel and hybridized to telomere sequences. Average telomere length of the resulting smear is estimated based on a DNA ladder and normalization to a reference sample to correct for inter-experimental gel effect. The length and intensity of the smear needs to be accounted for in the calculation of average telomere length. Although this is a well-established technique, variations between studies have procured over time because the technique was not standardized in terms of restriction enzymes used, DNA quantity and quality, and blot analysis. There are several main pitfalls to this method. First, a relatively large amount of pure and unfragmented DNA (0.5-10 μg) is required and small differences in telomere lengths are difficult to detect, which limits its use to broad research questions.

1.7.2 qFISH

Telomere length can also be measured by quantitative fluorescence in situ hybridization (qFISH), which combines use of image cytometry and metaphase spreads through use of peptide nucleic acid (PNA) probes. These probes have a greater affinity than DNA oligonucleotides and bind specifically to denatured telomere repeats. The fluorescent signal is acquired using specific qFISH analysis software and telomere lengths are measured relative to standards of known length. Karyotyping of metaphase spreads allows telomere lengths to

be matched with its corresponding chromosome arm. It's the method of choice to determine telomere length of specific chromosome ends. The analysis requires 15-20 metaphases per sample to obtain reliable results due to the high variation in the technique. Some advantages are that qFISH can detect chromosome fusion events and ends with virtually no repeats (<0.5 kb). This technique is a good choice to study telomere biology in rare cell types and can be used to ask more specific scientific questions because it requires so few cells. However, some disadvantages are that qFISH cannot be used to measure telomeres in cells that cannot divide such as senescent cells and highly aberrant cells. Furthermore, the technique is labour intensive and takes a long time, and requires specialized equipment.

1.7.3 Flow-FISH

Similar to qFISH, flow-FISH also uses PNA probes, however hybridization is done with cells in suspension and median telomere length of individual cells is measured using flow cytometry. This technique can be used to measure telomere lengths of specific cell populations (i.e. granulocytes and lymphocytes) in a single sample by cell sorting prior to flow-fish or staining for antibodies(limited to a few cell surface markers that are retained after hybridization). A semi-automated adaptation of this technique uses a 96-well plate and robotic micro dispenser to reduce tedious work and increase reproducibility. It is currently the method of choice for measuring telomere length in specific subsets of blood cells. Some drawbacks of this technique are that it requires a suspension of living cells, which is very fragile. This technique is generally limited to use of fresh blood. Like qFISH this method requires specialized equipment, is technically challenging and costly.

1.7.4 STELA

One of the newest techniques available is Single Telomere Length Analysis (STELA), which uses PCR to amplify a specific chromosome arm of telomere. STELA takes advantage of the fact that all chromosome arms end in a 3' overhang, which is targeted as a template to anneal an oligonucleotide linker at the 5' end of the telomere. A linker-specific primer and subtelomere specific primer is then used to amplify a precise length of a single telomere tract. PCR amplicons are then separated by gel electrophoresis, southern blotting and probed with specific subtelomere sequences. Resulting banding patterns are intricate sets of discrete bands of individual telomeres, which can be measured individually or pooled based on size according to a DNA ladder of known lengths. One drawback of STELA is its limitation to well characterized chromosome arms (XpYp, 2p, 11q, 12q and 17p) because not all

chromosomes have adequate sequences for the design of unique chromosome primers. Therefore, caution must be taken when analysing results because a subset of telomere measurements may not be reflective of the overall telomere status of cells. Although no specialized equipment is required, STELA is technically challenging and requires intensive initial preparation and optimization. Furthermore, STELA has an upper detection limit of 20kb, which limits its use mostly to human samples, and it cannot be used on model organisms with long telomeres such as *Mus musculus* strains of mice. Benefits of STELA are in its precision of telomere length measurement, its ability to detect subtle changes in telomere length, and minimal starting material required (as few as 50 cells) to produce reliable results. Like qFISH, STELA is well fit to study telomere lengths in rare cell populations, and it can also detect short telomere outliers, those responsible for chromosome fusion and senescence.

1.7.5 QPCR

QPCR based telomere length measurement has become widely used in large clinical and epidemiological studies because of its rapid design, ease of use, and low cost. This technique relies on specific amplification of telomeric repeats (T) normalized to a single-copy gene (S) to produce an average telomere length (T/S ratio). A sample calculation can be viewed in appendix B. A special set of telomere specific primers were designed that contain mismatches every six base pairs, different in both the forward and reverse primer. These mismatches are important to minimize primer dimer formation and maximize primer-template hybridization. Telomere-specific primers bind along telomeric repeats at each chromosome arm and amplify in fragments of at least 76 bp and up to ~500 bp long. SYBR green, a dye that fluoresces upon binding to double stranded DNA, is used to monitor the amplification of DNA using Real-Time PCR. The longer the average telomeres are in a given sample, the greater the fluorescent signal.

A setback of qPCR is in the variability between experiments. Proper controls are required to minimize this variability. In every experiment, a set of inter-plate control samples and a standard curve must be produced to offset the variability. An important quality control of this technique is in the preparation of DNA samples. It is critical that all DNA samples are of equivalent quality so that there are no variations in amplification efficiencies. Another drawback of this technique is that it is not standardized across different laboratories, and therefore results between laboratories cannot be compared. A multiplex version of this

technique has recently been developed that further reduces variation, which amplifies telomere repeats and single-copy-gene in the same PCR reaction. Although qPCR technique is prone to variation, with proper controls it can produce quick and reliable results at a low cost, which is beneficial for most large-scale experimental designs.

2. REVIEW LITERATURE

2.1 Factors that cause telomere shortening

Telomere shortening rates can be modified by several lifestyle factors that include mental stress levels, cigarette smoking and dietary intake. Baseline telomere shortening due to the end-replication problem will always exist, but efforts to live a healthy and balanced lifestyle can reduce any additional loss of telomeres. Even poor sleep quality has been associated with shorter telomere lengths in leukocytes among midlife women, independent of perceived psychological stress.

There are several factors that causes telomere shortening in a cell as listed in Figure below. The two most common causes are due to the end-replication problem, and oxidative damage to DNA.

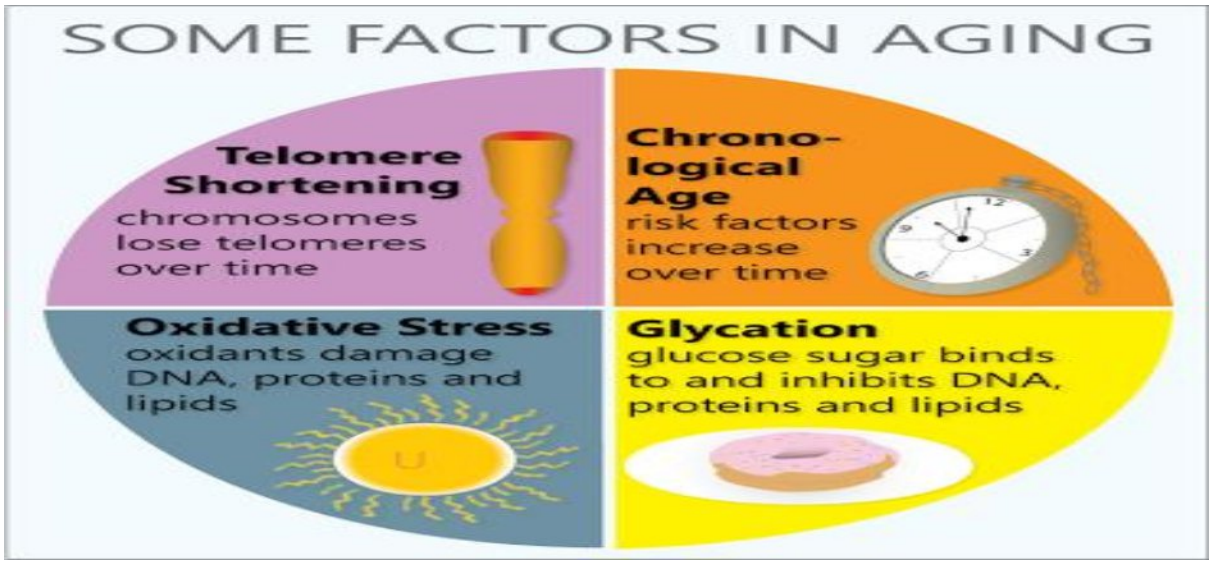


Figure 3: Shows factors affecting ageing

During DNA replication telomeres are in an open structure to allow DNA polymerase to extend the entire length of the chromosome. However, various factors contribute to telomere shortening that are propagated during replication such as the end-replication problem, oxidative DNA damage, replication errors, and telomere trimming. As cells divide and telomeres reach a critical length, they can no longer form a closed t-loop structure which signals cell cycle checkpoints such as ATM, ATR and p53, causing cells to enter a state of

senescence or apoptosis. Telomere lengthening mechanisms, such as telomerase and alternative lengthening of telomeres can extend the replicative capacity of cells by lengthening their telomeres.

2.1.1 Oxidative DNA damage

While the end-replication problem sets the baseline of telomere shortening, oxidative stress is the best recognized modifier. The minimal rate of telomere shortening observed in culture is 10-20 bp per cell division in fibroblasts with high antioxidant capacity or those treated with an agent that reduces oxidative stress in cells. This lower rate of attrition is likely set by the end-replication problem, which differs significantly from the average rate of telomere attrition in human cells (50-200 bp). This difference is primarily due to oxidative stress.

The major source of reactive oxygen species (ROS) in cells comes from mitochondria, as a metabolic by-product. Burden of ROS in a cell induces various types of DNA damage (oxidized bases, single and double strand breaks), which accumulates over time. Telomere shortening is largely accelerated by accumulation of oxidative DNA damage; in particular, by single strand breaks. Those damages that are not repaired or replaced before the next round of DNA replication will be lost due to stalling of the replication fork. Therefore, telomere loss due to oxidative damage is dependent on DNA.

Cells engage antioxidant defenses to protect against the harmful effects of oxidative stress by neutralizing free radicals. The strength of these defenses varies between cell types and species. Some strains of human fibroblasts have higher antioxidant levels than others, reflective in lower telomere attrition rate. The degree of damage at a given level of oxidative stress is highly variable simply due to chance, which may explain differential rates of telomere shortening observed between populations of cell clones in vitro and between individuals in vivo.

2.1.2 Other factors that contribute to Telomere shortening

Telomere shortening can be caused by DNA replication errors caused by mutations or malfunction of various proteins such as RecQ protein-like helicases (WRN and BML) and RTEL. These helicases function in the resolution of higher order telomeric structures to allow for efficient replication of telomeres and prevent stalling of the replication fork.

A recently discovered and less understood mechanism of telomere shortening is called telomere trimming, which involves resolution of t-loops from telomeres by recombination.

This mechanism has been observed in human and mouse cells and is believed to set an upper limit on telomere length and contribute to a length equilibrium set-point.

2.1.3 Mental stress

The amount of stress experienced is a key factor in variations in telomere shortening among the adult population. Reproductive stress displayed shorter telomeres compared to controls. In humans, psychological stress has been linked to short telomeres. Leukocyte telomere lengths were analysed in mother's caring for their chronically sick child and revealed that duration of care was associated with higher levels of oxidative stress, lower levels of telomerase and shorter telomeres. In fact, the level of stress perceived by the caregiver was proportional to their telomere lengths. Telomere shortening due to stress does not occur only in adults but also in children.

2.1.4 Smoking, Obesity and Dietary Intake

There are several studies that link cigarette smoking to short leukocyte telomeres. Studies have also shown a dose-effect relationship between tobacco exposure and telomere length. Accelerated telomere shortening in smokers is linked to increased oxidative stress and DNA damage. In fact, an in vitro model of early mice embryos revealed that exposure to cigarette smoke condensate or cadmium caused increased oxidative stress and telomere shortening and associated with chromosomal instability and apoptosis. However, treatment with the antioxidant N-acetyl-l-cysteine (NAC) was able to greatly reduce oxidative stress and toxic effects of cigarette smoke. Similar to smoking, obesity also causes an increase in oxidative stress and inflammation. Short leukocyte telomere length in type-2 diabetic men was shown to correlate with triglyceride and total cholesterol levels, suggesting a link between BMI and telomeres in aging. A diabetes intervention study on obese individuals with glucose intolerance revealed that by promoting a healthy lifestyle leukocyte telomere length can improve overtime. Even a moderate amount of physical activity is associated with longer leukocyte telomere lengths. Proper dietary intake can also improve telomere length. For instance, vitamin D intake was linked to increased telomerase activity in leukocytes of overweight individuals and improved telomere length in haemodialysis patients treated with vitamin D compared to those not treated. Since oxidative stress accelerates telomere shortening, intake of antioxidants, such as berries, could reduce rates of telomere attrition. As with mental stress, exercise can also modify telomere length attrition by improving overall health and by reducing BMI and oxidative stress levels.

OBJECTIVE

The objectives of this thesis are

- To measure the length of the telomere with the help of conventional Polymerase chain reaction and Southern blotting in people with different age groups.
- To find the impact of smoking and sports on the length of the telomere in blood cells of people having a record of smoking, sports and to that of normal individuals of different age groups Real Time Polymerase Reaction.

MATERIAL AND METHODS

3.1 Sample Collection, Storage

Source: Nutech Mediworld Hospital, Delhi

Age Group**:** 13 to 55 years

3.1.1 Type of Samples**:** Smoker & Non-Smoker Person; Sports & Non-sports person, Normal person with different age groups.

The first step to study the telomere length is collection and storage of samples for subsequent DNA extraction. While TL measurement techniques vary in the amount of DNA they require, the need for high-quality DNA is consistent across all techniques. Likewise, there are many available methods for extracting DNA; it is important to select one that will maximize DNA quality and yield and to store the extractions in such a way that they will not degrade. It is also important to be consistent in the method used for DNA extraction and to ensure that it is appropriate for the telomere measurement method being used.

3.1.2 Tissue Type

The tissue that can be sampled for telomere measurement will depend on many things, not least the size and life stage of the organism, the study environment, and the degree to which invasive or destructive sampling is ethically justifiable and feasible. Telomere length and dynamics vary and the degree of telomere restoration, most commonly through the action of the enzyme telomerase. For instance, tissues with low replication rates and minimal telomerase expression (e.g. central nervous system, muscle) may show little telomere change during adult life. Most in vivo studies of TL in vertebrates uses blood, for which small samples can be taken routinely and repeatedly from the same individuals, usually with little adverse effect. Importantly, blood cells generally have a high turnover rate leading to the expectation and widespread observation of TL loss in blood cells with age and over time. However, blood cells comprise a rich composite of cell types with very different functions, proliferation patterns and levels of telomerase expression.

3.2 GENOMIC DNA ISOLATION FROM BLOOD SAMPLES

3.2.1 Preparation of Chemicals**:**

1. SATURATED PHENOL

Materials Required:

- Redistilled Phenol, molecular biology grade. (Stored in aliquots at -20℃).
- 8-hydroxyquinoline
- 0.5 M Tris.Cl buffer (pH 8.0)
- 0.1 M Tris.Cl buffer (pH 8.0)

Note: *Phenol is volatile and caustic.* .

Protocol for preparation of Saturated Phenol:

- Heat a water bath to 65°C in the fume hood. Place the bottle of phenol in the fume hood to warm to room temperature.
- Place the bottle of phenol in the 65°C water bath to melt the crystals.
- Add 8-hydroxyquinoline to a final concentration of 0.1 % w/v to the phenol. Mix to dissolve the 8-hydroxyquinoline.
- Add an equal volume of 0.5 M Tris.Cl (pH 8.0) to the phenol. Mix for 15 minutes. Return the bottle to the 65°C water bath. Allow the phases to separate. Siphon off the top layer and discard. Repeat the procedure as in Step 4 twice
- Add an equal volume of 0.1 M Tris.Cl (pH 8.0) to the phenol. Repeat the procedure as in Step 4.
- Repeat the extractions with 0.1 M Tris.Cl (pH 8.0) until the aqueous phase is ~pH 7.8 (measure with pH paper). Repeat the procedure as in Step 4. Leave approx. ~1 cm layer of 0.1 M Tris.Cl (pH 8.0) over the phenol. Add 2-mercaptoethanol to a final concentration of 0.2% w/v to the 0.1 M Tris.Cl (pH 8.0).
- The buffer saturated phenol may be stored at 4°C for 1 month for DNA extraction. Test the pH periodically and do not use if the pH is <7.5.

2. LYSIS BUFFER
    - 50M Tris.cl
    - 20 mM EDTA

- 1% SDS
- 10 µL β-merceptoethanol

3. TE BUFFER
    - 10mM Tris
    - 1 mM EDTA

3.2 Protocol for Genomic DNA Isolation:

- Approximately 250µl of previously separated blood cells were taken in a 700µl of lysis buffer. The mixture was incubated at 55°C overnight for digestion. Incubated for 37°C for 6 hrs with occasion are mixing by gentle swirling in waterbath.
- Dissolve solution was centrifuge to seperate cell debris at 10000 rpm for 10 minutes. Aqueous phase was transfer to another tube.
- Equal volume of s-phenol was added to precipitate the protein and centrifuge at 10,000 rpm for 10 minutes. Aqueous phase was taken and transfer to another tube, without disturbing protein complex precipitated as an interphase.
- Equal volume of s-phenol:Chloroform (1:1) was added and centrifuged at 10,000 rpm for 10 minutes to ensure emulsification of the phases. Aqueous phase was transfer to another tube.
- Equal volume of chilled chloroform were added and centrifuged at 10,000 rpm for 10 minutes. Aqueous phase was taken and transfer to another tube.
- 150-170µl of 5M NaCl was added to the mixture and double the volume of chilled absolute isopropanol and is allowed to stand for incubation at -20°C for 45 minutes.
- DNA pellete was collected by centrifugation at 10,000 rpm for 10 minutes.
- The tube were inverted and drained on a paper towel. The pellete was washed with 500 µl of 70% ethyl alcohol and centrifuged at 10,000rpm for 10 minutes. Discard the supernatant and air dried the pellete.
- The pellete was resolved in 50µl of TE buffer.

After isolation of DNA, quantification and analysis of quality are necessary to ascertain the approximate quantity of DNA obtained and the suitability of DNA sample for further analysis. This is important for many applications including digestion of DNA by restriction enzymes or PCR amplification of target DNA. The most commonly used methodologies for quantifying the amount of nucleic acid in a preparation are as follow:

(3.2.1) Electrophoretic run along with standard DNA

(3.2.2) Spectrophotometric estimation

3.2.1 Agarose Gel Electrophoresis for DNA Quantification and Quality Analysis

This method of quantification is based on the ethidium bromide fluorescent staining of DNA. Ethidium bromide is a fluorescent dye, which intercalates between the stacked bases. The fluorescent yield of the dye: DNA complex is much greater than the unbound dye. UV irradiation at 254nm is absorbed by the DNA and transmitted to the dye and the bound dye itself absorbs radiation at 302nm and 366nm. This energy is retransmitted at 590nm, the reddish-orange region of the visible spectrum. In case of blood DNA, the nucleic acids are electrophoretically separated on a 0.7-0.8% agarose gel containing ethidium bromide at a final concentration of 0.5 ?g/ml. The quantity of DNA can be estimated by comparing the fluorescent yield of the samples with a series of standards, for instance, lambda (λ) DNA at varying known concentrations. This provides a very rapid and sensitive means of estimating the nucleic acid concentration. A large number of samples with as little as 5ng of DNA can be quantified. Besides quantification, it also allows provides the advantage of analyzing the quality of the DNA preparation. Native DNA, which migrates as a tight band of high molecular weight (40 kb), presence of RNA, and degraded/sheared DNA, if any, can be visually identified on the gel.

Instruments used:

- Gel casting tray
- Electrophoresis chamber
- Voltage
- UV trans illuminator

Chemicals used:

- Agarose gel

- EtBr
- TAE Buffer
- 6X DNA loading dye

Preparation of chemicals**:**

1. Agrose gel
    - 0.8gm to 2gm Agrose
    - TAE Buffer

2. 50X TAE Buffer (100 ml)
    - Tris - 24.2gm
    - Acetic Acid - 5.71 gm
    - .5 M EDTA -10 ml

3. 6X DNA loading dye
    - 10mM Tris Cl (pH – 7.6)
    - 0.03% Bromophenol blue
    - Xylene cynol
    - 60mM EDTA

Procedure

1. Prepare a 0.8% agarose gel.
2. Add 3 µl of 6X gel loading dye to 7 µl of each DNA sample before loading the wells of the gel. Addition of dye allows us to note the extent to which the samples might have migrated during electrophoresis, so that it can be halted at an appropriate stage.
3. Load at least 1 or 2 wells with ladder DNA or any previously quantified DNA samples (50ng and 100ng) as molecular weight standards.
4. Run the submarine electrophoretic gel at 70V till the dye has migrated one-third of the distance in the gel.
5. DNA can be visualized using a UV transilluminator and quantified in comparison with the fluorescent yield of the standards.

3.2.2 DNA quantification by UV spectroscopy Analysis of UV absorption by the nucleotides:

This method provides a simple and accurate estimation of the concentration of nucleic acids in a sample. Purines and pyrimidines in nucleic acid show absorption maxima around 260nm (eg., dATP: 259nm; dCTP: 272nm; dTTP: 247nm) if the DNA sample is pure without significant contamination from proteins or organic solvents. The ratio of OD260/OD280 should be determined to assess the purity of the sample. This method is however limited by the quantity of DNA and the purity of the preparation. Accurate analysis of the DNA preparation may be impeded by the presence of impurities in the sample or if the amount of DNA is too little. In the estimation of total genomic DNA, for example, the presence of RNA, sheared DNA etc. could interfere with the accurate estimation of total high molecular weight genomic DNA.

Procedure

1. Take 1 ml TE buffer in a cuvette and calibrate the spectrophotometer at 260nm as well as 280nm.
2. Add 10 µl of each DNA sample to 900µl TE (Tris-EDTA buffer) and mix well.
3. Use TE buffer as a blank in the other cuvette of the spectrophotometer.
4. Note the OD260 and OD280 values on spectrophotometer.
5. Calculate the OD260/OD280 ratio.
6. The amount of DNA can be quantified using the formula:

$$\text{DNA concentration } (\mu g/ml) = \frac{\text{OD 260 x 100(dilution factor) x 50}\mu g/ml}{1000}$$

Inferences:

- A ratio between1.8-2.0 denotes that the absorption in the UV range is due to nucleic acids.
- A ratio lower than 1.8 indicates the presence of proteins and/or other UV absorbers.
- A ratio higher than 2.0 indicates that the samples may be contaminated with chloroform or phenol. In either case (<1.8 or >2.0) it is advisable to re-precipitate the DNA.

Spectrophotomteric Conversions for Nucleic Acids:

1 A 260 of ds DNA = 50 □g/ml

1 A 260 of ss oligonucleotides = 33 □g/ml

1 A 260 of ss RNA = 40 □g/ml

3.3 PCR Amplification

3.3.1 PCR Cocktail

Components	Volume
ddH_2O	14.5µl
10X Buffer	5 µl
dNTP	1 µl
$MgCl_2$	3 µl
DNA	3 µl
Taq DNA Polymerase	0.5 µl
Primer- Reverse	1 µl
Primer – Forward	1 µl

Primers used for conventional PCR:

Forward Primer	CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
Reverse Primer	GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT

3.3.2 PCR Programme:

95°C	1 minute
Loop [40 cycles	
98°C	1 minute
60°C	1 minute
72°C	2 minute
Loop]	
72°C	5 minutes
4°C	1 hour

3.4 Southern Blotting

3.4.1 Materials Required:-

DNA samples to be analysed

0.25 M HCl

Denaturation solution: 1.5 M Nacl/0.5 M Tris.Cl

Neutralization solution: 1.5 M NaCl/0.5M Tris.Cl, pH 7.0

20x and 2x SSC Buffer

Oblong sponge slightly larger than the gel being blotted,

Whatman 3MM filter paper sheets

Nylon membrane

UV – Transparent plastic wrap

UV transilluminator

Additional reagents and equipment for restriction endonuclease digestion and agarose gel electrophoresis

Preparation of 20x SSC Buffer (For One Litre)

NaCl – 175.3gm

Trisodium Citrate – 88gm

Store at room temperature

SSC Buffer is used as transfer buffer. It provides high ionic strength to promote binding of the DNA to the membrane.

Protocol for Southern Blotting

3.4.2 Preparation for the gel:

- Digest the DNA samples with appropriate restriction enzymes, run in an agrose gel with appropriate DNA size markers, and stain with ethidium bromide.
- Rinse the gel in distilled water and place in a clean glass dish containing ~10 gel volumes of 0.25M HCl. Shake slowly on a platform shaker for 30 minutes at roomtemperature.
- Pour off the HCl and rinse the gel with distilled water. Add ~10 volume denaturation solution and shake as before for 20 minutes. Replace with fresh denaturation solution and shake for further 20 minutes.
- Pour off the denaturation solution and rinse the gel with distilled water. Add ~10 volume neutralization solution, shake as before for 20 minutes, then replace with fresh neutralization solution and shake for 20 minutes.

3.4.3 Set up the transfer:

- Place an oblong sponge, slightly larger than the gel, in a glass dish. Fill the dish with enough 20X SSC buffer to leave the soaked sponge about half – submerged in buffer.
- Cut three pieces of Whatman 3MM paper to the same size as the sponge. Place these on the sponge and wet them with 20X SSC.
- Place the gel on the filter paper and squeeze out air bubbles by rolling a glass pipet over the surface.
- Cut a piece of nylon membrane just large enough to cover the exposed surface of the gel. Pour distilled water ~ 0.5 cm deep in the glass dish and wet the membrane by placing it on the surface of the water. Allow the membrane to submerge, then leave for 5 minutes.

- Place the wetted membrane on the surface of the gel. Try to avoid getting air bubbles under the membrane; remove any that appear by carefully rolling a glass piped over the surface.
- Flood the surface of the membrane with 20X SSC. Cut five sheets of Whatman 3MM paper to the same size as the membrane and place these on the membrane.
- Cut paper towels to the same size as the membrane and stack these on the top of the Whatman 3MM paper to a height of ~4 cm.
- Lay a glass plate on top of the structure and place a weight on top to hold everything in place. Leave over night.

3.4.4 Disassemble the transfer pyramid:

- Remove the paper towels and filter papers and recover the membrane. Mark in pencil the position of the wells on the membrane and ensure that the up-down and back-front orientation are recognizable.
- Rinse the membrane in 2XSSC buffer.
- Then place it on a sheet of Whatman 3MM paper and allow to dry.

3.4.5 Immoblize the DNA:

- Wrap the membrane in UV transparent plastic wrap, place DNA down sided on a UV transilluminator (254nm wave length) and irradiate for 2 hours at 80°C.
- Visualize the bands.

3.5 RNA ISOLATION FROM BLOOD

3.5.1 Preparation of Chemicals:

1. TRIZOL REAGENT

- S-Phenol - 3.8ml.

- 0.8M Guanidium thio cyanate.
  Guanidium thiiocyanate

= 0.8×118.16×10\1000 = 0.9gm

- 0.4M Ammonium thio cyanate pure

  Ammonium thio cyanate

  = 0.4×76.12×10\1000 = 0.3gm

- 0.4M Sodium acetate

  Sodium acetate [3M]

  C1V1 = C2V2

  3×V1 = 0.1×10

  V = 0.1×10\3 = 1\3

  = 0.33ml×1000

  = 330µl

- Glycerol - 500µl.
- Distilled Water - 10ml

Protocol for RNA ISOLATION :

1. Take 500µl blood sample and 1000µl trizol reagent.
2. Keep it on ice for 20 minutes.
3. Centrifuge at 13000 rpm for 10 minutes at 4°C.
4. Collect the supernatant in fresh tube.
5. Add 700µl chloroform chilled at -20°C and 100µl isoamyl alcohol.
6. Invert and mix thoroughly for 10 seconds.
7. Keep it on ice for 15 minutes.
8. Centrifuge at 13,000 rpm for 15minutes at 4°C.
9. Collect the upper aqueous phase in a new tube add equal volume of ice chilled Isopropanol to the supernatant.Mix thoroughly by mixing it 6 – 8times.
10. Incubate it at -20°C for 30-40minutes.
11. Centrifuge at 13,000 rpm for 10minute at 4°C discard the supernatant.
12. Add 50µl of TE buffer

3.5.2 SPECTROPHOTOMETRIC ANALYSIS:

1. Dilute 20 µl of RNA with 980 µl of DEPC-treated water in cuvette, take OD at 260 nm and 280 nm to determine sample concentration and purity. The A260/A280 ratio should be above 1.7. Apply the convention that 1 OD at 260 equals 40 µg /ml RNA.

$$\text{Total RNA Concentration} = \frac{\text{O.D.at 260 X 40 X Dilution factor}}{1000}$$

3.6 cDNA Synthesis

3.6.1 First strand synthesis

Reverse Transcription is a process in which single stranded RNA is reverse transcribed into complementary DNA(cDNA) by using total cellular RNA, a reverse transcriptase enzyme, a primer, dNTPs, and an RNase inhibitor. The resulting cDNA can be used in RT-PCR reaction.

Steps for synthesis of first strand

S. No.	Components	Quantity (micro-litre)
1.	Double distilled water	12.0
2.	Random Primer	3.0
3.	RNA	8.0

Incubate at 65°C for 5 minutes.

Incubate on ice for 5 minutes.

Vortex it and add further components listed below.

S. No.	Components	Quantity (micro-litre)
1.	10X Buffer	4.0
2.	DTT	2.0
3.	dNTPs	2.0
4.	Reverse Transcriptase	1.0

Incubate at 42°C for 1 hour.

Incubate at 70°C for 5 minutes.

3.6.2 Second Strand Synthesis**:**

S. No.	**Components**	**Quantity (micro-litre)**
1.	Double distilled water	7.675
2.	10 X Buffer	2.5
3.	Primer (Beta-actin) forward	1.25
4.	Primer (Beta-actin) reverse	1.25
5.	Taq Polymerase	0.2
6.	DNTPs	0.125
7.	RT mix DNA/First strand of cDNA	2
	Total	**15**

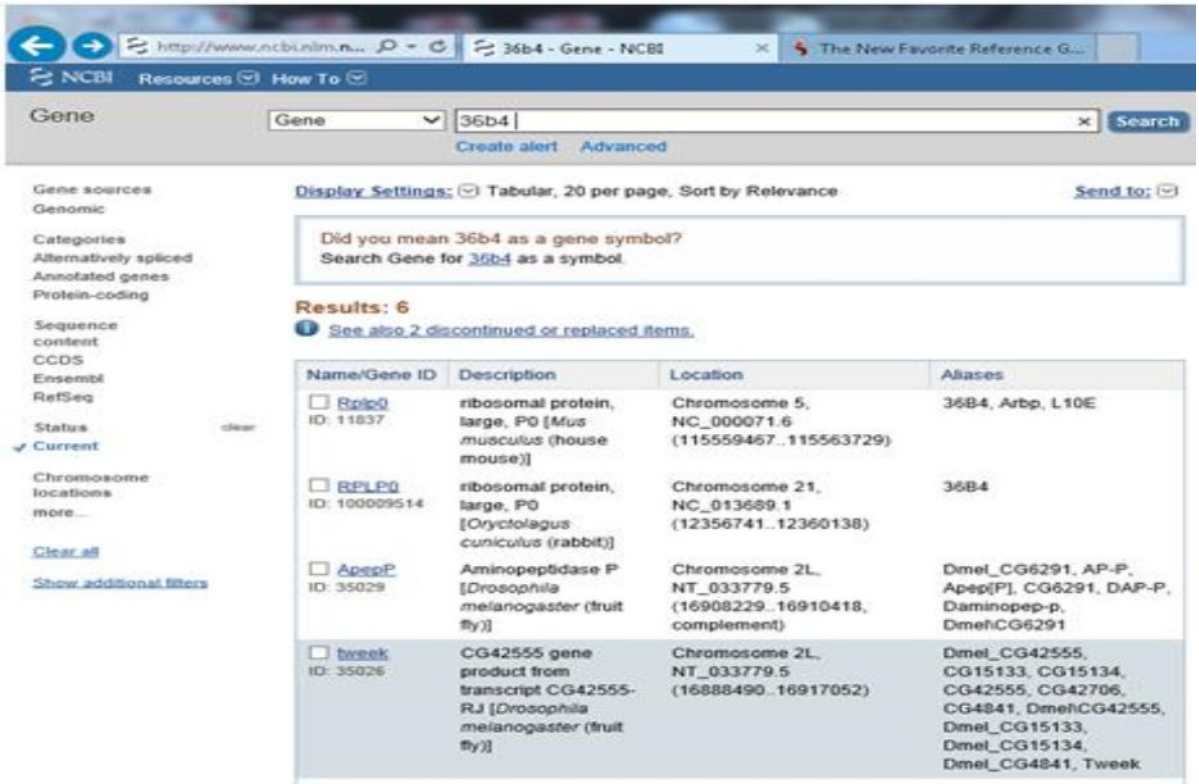
3.63 PCR Program for Second Strand Synthesis:

Step No. 1	Temperature	95 ^{O}C
	Time	7 minutes
Step No. 2	Temperature	94 ^{O}C
	Time	1 minutes
Step No. 3	Temperature	42 ^{O}C
	Time	1 minutes
Step No. 4	Temperature	72 ^{O}C
	Time	2 minutes
Step No. 5	Temperature	72 ^{O}C
	Time	5 minutes
Step No. 6	Temperature	4 ^{O}C
	Time	For 1 hour
(Step 2 to step 5 has 40 cycles)		

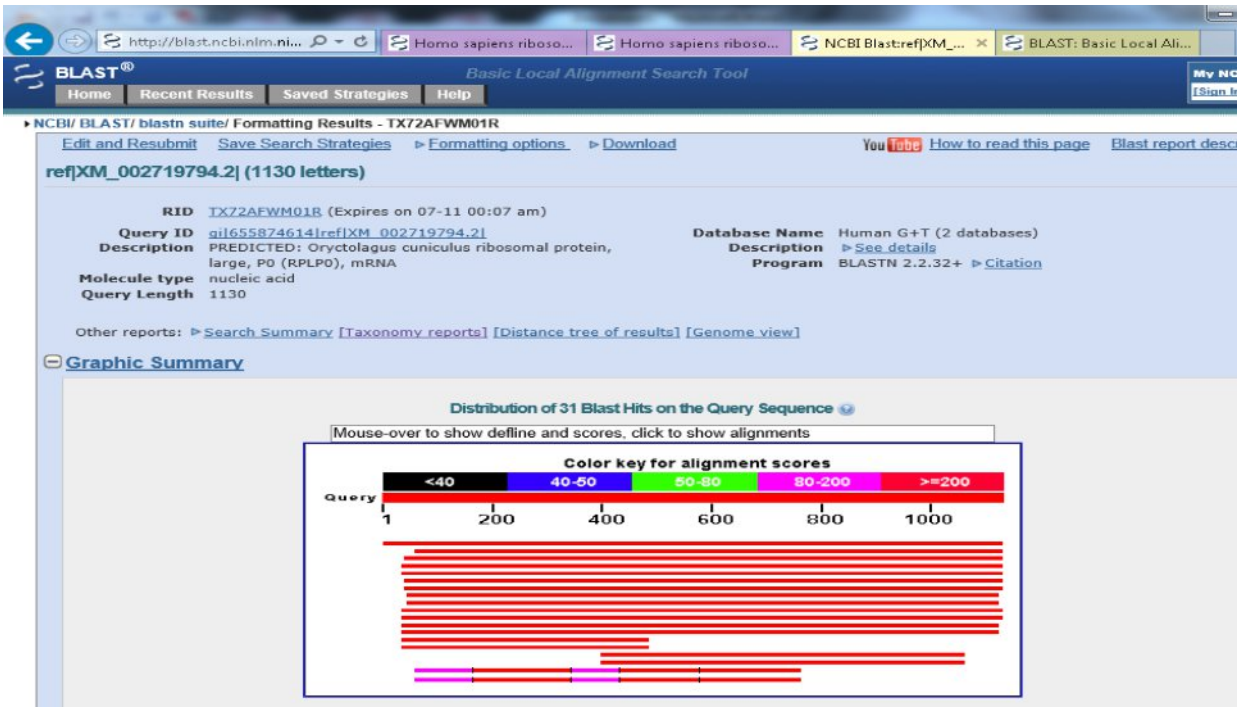
3.7 **PRIMER DESIGNING**

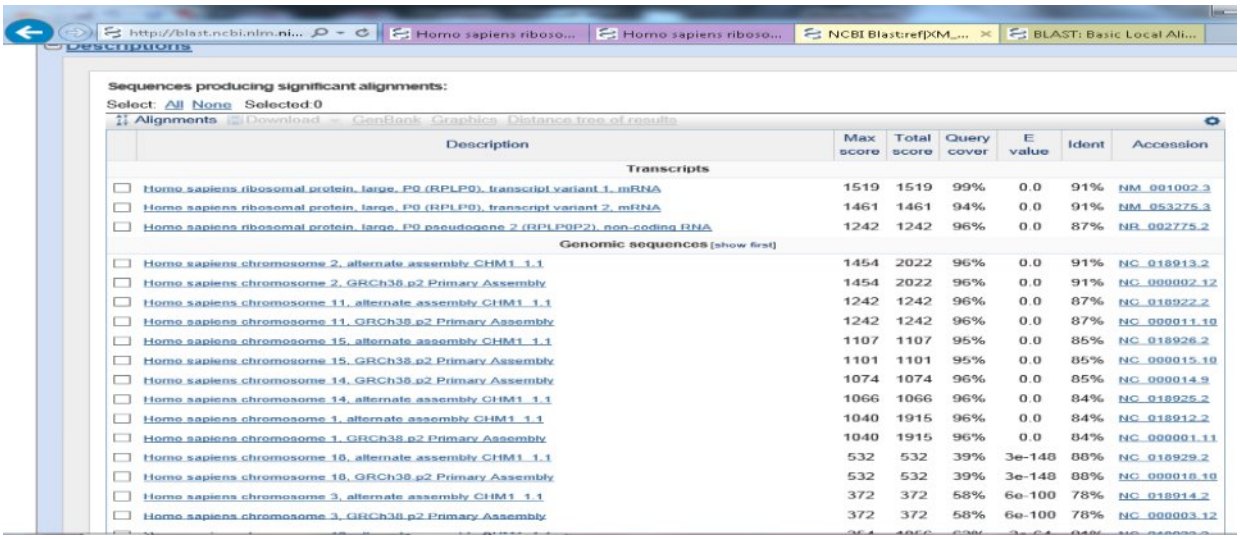
The 36B4 gene encodes an acidic ribosomal phosphoprotein P0 (RPLP0), which forms tight associations with the smaller 40S protein (L12). 36B4 protein is part of a pentameric complex that forms a stem-like structure, protruding into the cytoplasm, off of the 60S subunit. This protrusion functions to support the GTPase steps in the translocation of protein synthesis.

The 36B4 cDNA nucleotide sequence has highly conserved regions in the 5-prime end of its open reading frame that cross tissue and species boundaries. When compared to the transcript levels of other common reference genes, such as beta-actin and cyclophilin, 36B4 proved to be a very reliable and consistent standard for use in gene expression analysis among multiple tissues including: brain, heart, liver, kidney, muscle and lung. Furthermore, the nucleotide sequence homology between species is remarkably high. For canis familiaris (dog) the homology to the human sequence is 94%; for pan troglodytes (chimpanzee), 99%; and for bos taurus (bovine), 94%. In basic science the most commonly used species are mus musculus (mouse) and rattus norvegicus (rat) whose 36B4 maintains a homology with the human gene of 88% and 89% respectively. As with other highly expressed genes, 36B4 has 8 related pseudo-genes in the human genome, found on chromosomes 1-3, 11, 14, 15 and 18.



36b4 gene mRNA sequence of Oryctolagus cuniculus (Rabbit) accession No: XM_002719794.2 in fasta format was retrieved from NCBI site. Homologues Sequence was then searched in Human Genomic + Transcript (Human G+T) using BLAST tool.





Result shows 99% similarities to Homo sapiens ribosomal protein, large, P0 (RPLP0), transcript variant 1, mRNA (Accession No.: NM_001002.3), 94 % sequence similarity to Homo sapiens ribosomal protein, large, P0 (RPLP0), transcript variant 2, mRNA (Accession No.: NM_053275.3) and 96% similarity to Homo sapiens ribosomal protein, large, P0 pseudogene 2 (RPLP0P2), non-coding RNA (Accession No.: NR_002775.2)

mRNA sequence of NM_001002.3, NM_053275.3 and NR_002775.2 were retrieved from NCBI site and were aligned to search a conserved region using CLUSTALW Multiple Alignment Tool.

Conserved region:
>gi|49087144|ref|NM_001002.3|/1-1229
TGACATCGTCTTTAAACCCTGCGTGGCAATCCCTGACGCACCGCCGTGATGCCCA
GGGAAGACAGGGCGACC
TGGAAGTCCAACTACTTCCTTAAGATCATCCAACTATTGGATGATTATCCGAAAT
GTTTCATTGTGGGAGCA
GACAATGTGGGCTCCAAGCAGATGCAGCAGATCCGCATGTCCCTTCGCGGGAAG
GCTGTGGTGCTGATGGGC
AAGAACACCATGATGCGCAAGGCCATCCGAGGGCACCTGGAAAACAACCCAGCT
CTGGAGAAACTGCTGCCT
CATATCCGGGGGAATGTGGGCTTTGTGTTCACCAAGGAGGACCTCACTGAGATC
AGGGACATGTTGCTGGCC
AATAAGGTGCCAGCTGCTGCCCGTGCTGGTGCCATTGCCCCATGTGAAGTCACTG
TGCCAGCCCAGAACACT
GGTCTCGGGCCCGAGAAGACCTCCTTTTTCCAGGCTTTAGGTATCACCACTAAAA
TCTCCAGGGGCACCATT
GAAATCCTGAGTGATGTGCAGCTGATCAAGACTGGAGACAAAGTGGGAGCCAGC
GAAGCCACGCTGCTGAAC
ATGCTCAACATCTCCCCCTTCTCCTTTGGGCTGGTCATCCAGCAGGTGTTCGACA
ATGGCAGCATCTACAAC
CCTGAAGTGCTTGATATCACAGAGGAAACTCTGCATTCTCGCTTCCTGGAGGGTG
TCCGCAATGTTGCCAGT
GTCTGTCTGCAGATTGGCTACCCAACTGTTGCATCAGTACCCCATTCTATCATCA
ACGGGTACAAACGAGTC
CTGGCCTTGTCTGTGGAGACGGATTACACCTTCCCACTTGCTGAAAAGGTCAAGG
CCTTCTTGGCTGATCCA
TCTGCCTTTGTGGCTGCTGCCCCTGTGGCTGCTGCCACCACAGCTGCTCCTGCTGC
TGCTGCAGCCCCAGCT
AAGGTTGAAGCCAAGGAAGAGTCGGAGGAGTCGGACGAGGATATGGGATTTGGT
CTCTTTGACTAATCACCA
AAAAGCAACCAACTTAGCCAGTTTTATTTGCAAAACAAGGAAATAAAGGCTTAC
TTCTTTAAAAAGTAAAAA
AAAAAAAAAAAAAAAAAAAA

Real Time Primer designing using Primer Express 3.0

Primer sequence		Length	%GC	Tm	Amplicon
CCACGCTGCTGAACATGCT TCGAACACCTGCTGGATGAC	Forward Reverse	19 20	58 55	60 58	67

- NOTE: Primer Specificity was checked by using online line tool MFE primer 3.0.

3.8 RT-PCR

Summary of the general key steps in the TaqMan®assay to help the understanding of following discussions.

1. RNA preparation: total or specific type of RNA are extracted from cell lines, tissues, biopsies, etc.
2. RNA is Reversed Transcribed into DNA, which is also known as the RT-reaction.
3. qPCR probes (sometimes also known as 'primers') are added to the transcribed cDNA sample and the polymerase chain reaction takes place. This probe is an oligonucleotide with a reporter dye attached to the 5' end and a quencher dye attached to the 3' end. Till the time the probe is not hydrolized, the quencher and the fluorophore remain in proximity to each other, which does not completely quench the fluorescence of the reporter dye and therefore only background fluorescence is observed.
4. During PCR, the probe anneals specifically between the forward and reverse primer to an internal region of the PCR product. The polymerase then carries out the extension of the primer and replicates the template to which the TaqMan®is bound. The 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter molecule away from the close vicinity of the quencher. The fluorescence intensity of the reporter dye, as a result increases. This process repeats in every cycle.
5. As the cycle number increases, the detected fluorescence also increases. And when the fluorescence crosses an arbitrary line, the device recodes the cycle number until then, which is known as the CT value.

In principle one could also report the CT values of the housekeeping gene and the sample gene(s) in the form of barplots to show their relative relation.

3.8.1 The ddCt Algorithm

These methods make so many assumptions.

Deviation

The exponential amplification of the polymerase chain reaction (PCR) can be describedby the equation 1.

$$\mathbf{X_n = X_0 x (1 + E_X)^n} \quad (1)$$

Where, X_n is the number of target molecules at cycle n of the reaction,

X_0 is the numberof target molecules initially.

Ex is the amplication efficiency of target amplication,

n is the number of cycles.

The threshold value (CT) records the fractional cycle numberat which the fluorescence reaches a fixed threshold. Therefore

$$\mathbf{X_T = X_0 \ x \ (1 + E_X)^{CT;X} = K_X} \quad (2)$$

Where, X_T is the threshold number of target molecules,

C_T.X is the readout CT value,

K_X is a constant.

Similarly we can express the equation 2 for the endogenous referencegene (house-keeping genes) as

$$\mathbf{R_T = T_0 \ x \ (1 + E_R)^{CT.R} = K_R} \quad (3)$$

Where, R_T is the threshold number of the reference molecules,

R_0 is the initial number of reference molecules,

E_R is the efficiency of reference amplification,

CT.R is the CT readoutfor the reference,

K_R is a constant.

On dividing equation (2) by (3), we get

$$X_T/R_T = \mathbf{(X_0 / R_0) \ x \ (1+E)^{CT.X-CT.R}} = K_X/K_R = \mathbf{K}$$

For qRT-PCR using TaqMan®probes, the exact values of X_T and R_T depend on several factors including the chemistry of reporter dye, the sequence context effects on the fluorescence properties of the probe, the efficiency of probe cleavage, purity of the probe, and the setting of the fluorescence threshold. Therefore, the constant K does not have to be equal to one.

Assuming efficiencies of the target and the reference are the same,

$$\mathbf{E_X = E_R = E}$$

So, $$\mathbf{(X_0 / T_0) \ x \ (1+E)^{CT;X-CT;R} = K} \quad (4)$$

Or

$$\mathbf{X_N \ x \ (1 + E)^{dCT} = K} \quad (5)$$

Where X_N is equal to the normalized amount of target (X_0/R_0) the dCT is equal to the difference in the CT for target and reference (CT;X - CT;R).

Equation (5) can be rearranged as

$$\mathbf{X_N = K \ x \ (1 + E)^{-dCT}} \quad (6)$$

The final step is to divide the X_N in the equation (7) for any sample q by the referencesample (also known as the calibrator, cb):

$$\mathbf{X_{N;q} = K \ x \ (1 + E)^{-dCT.\,q}} \quad (7)$$

$$X_{N;cb} = K \times (1 + E)^{-dCT.\,cb} \quad (8)$$

On dividing eqn (7) by (8), we obtained,

$$(X_{N;q} / X_{N;cb}) = (1+E)^{-ddCT}$$

For amplicons designed to be less than 150 basepairs and for which the primer andMg2+ concentration have been optimized, the efficiency E is close to one. Therefore,the amount of target, normalized to the endogenous reference and relative to a referencesample, is given by

$$\text{Amount of target} = 2^{-ddCT} \quad (9)$$

Attention: Note that for the ddCT calculation to be valid, the amplification efficienciesof the target and reference must be approximately equal.

File I/O setup

We have attached two SDS output_les, 'Experiment1.txt' and 'Experiment2.txt', in the package directory. The sample annotation information is also provided as the tab-delimited text_le 'sampleData.txt'. Any warning information (for example Undetermined in reference sample) is saved as a text _le speci_ed by the parameter 'warningFile'.

```
> library(ddCt)
> datadir <- function(x) system.file("extdata", x, package="ddCt")
> savedir <- function(x) file.path(tempdir(), x)
> file.names <- c(datadir("Experiment1.txt"),datadir("Experiment2.txt"))
> info <- datadir("sampleData.txt")
> warningFile <- savedir("warnings.txt")
```

Reference sample and housekeeping gene

For the sake of simplicity, we choose Sample1 and Sample2 as reference samples (calibrators), and Gene2 as the reference gene (housekeeping gene) respectively. This could happen, for example, if the Sample1 and Sample2 are untreated samples while the Sampple3 has been treated with certain drugs. And Gene2 is a housekeeping gene which weassume is expressed constantly in all the samples.

```
> name.reference.sample <- c("Sample1", "Sample2")
> name.reference.gene <- c("Gene2")
```

Note that more than one reference sample or reference gene can be specified.

Read in data

SDMFrame function is called to read in experiment data.Optionally one could also read inthe sample annotation, which is the sampleInformation object in the example.

```
> library(Biobase)
> CtData <- SDMFrame(file.names)
```

>sampleInformation

<- read.AnnotatedDataFrame(info,header=TRUE, row.names=NULL)

Note that SDMFrame is able to accept one or more as input.

Apply the ddCt method

Next step we call ddCtExpression to perform ddCt method on the data.

> result <- ddCtExpression(CtData,

+ calibrationSample=name.reference.sample,

+ housekeepingGene=name.reference.gene,

+ sampleInformation=sampleInformation,

+ warningStream=warningFile)

Two main drawbacks of RT-PCR are as follows:

- This is only applicable in cases where more than one genes are compared in the same sample. In case of mutilple samples one has to calculate the relative expression to a specified reference sample.
- CT value is exponential. In case of a ideal ampli_cation e_ciency of 1, increase ofthe CT value by 1 indicates a two{fold expression. Therefore, it maybe misleading to illustrate the expression with the raw CT value.

Real Time PCR protocol

1st step:

- RNA 5 ul
- ddH2O15 ul
- Incubate at $45^{0,}$ C for 60^{0} C
- Incubate at 95^{0} C for 5 minutes.
- Dilute it with 30ml Nuclease free water.

2nd Step:

RT PCR ABI7000

95^{0} C for 5minutes	Initial Denaturation
35 cycles	
95^{0} C for 30 seconds	Denaturing temperature
55^{0} C for 1 minute	Annealing temperature
72^{0} C for 30 second	Extension temperature

Primers

Beta Actin (Control)	
Forward	5'-CCC ATT GAA CAC GGC AGG G-3'
Reverse	5'-GTA CGA CCA GAG GCA TAC A-3'

QPCR SET UP ABI7000

ABI7000 Absolute Quantification (standard curves)

a. Turn on computer

i. Check Work Station Only box

ii. Username: Administrator (no password)

iii. Create a monthly personal folder

b. Turn on ABI7000

c. **Launch ABI7000 software**.

d. **File → New** choose as follows:

i. Assay: Absolute Quantification

ii. Container: 96 well clear plate

iii. Template: Blank Document

iv. Click **Next**

e. Create detectors

i. Click **new detector**.

ii. Name and highlight your detector as required, assign a dye and color code and either **create another** or **OK**.

iii. Create as many detectors as you have genes for that run.

iv. Highlight your detectors and click **Add → Finish**.

f. Wait until you hear the ABI 7000 instrument initialize by hearing a 'clunk'. If you don't hear this then turn off the instrument, close the software, turn the instrument back on and re-open the software.

g. A 96 well template will open.

h. Go to **View → Well Inspector**.

i. The Well Inspector window will open showing your chosen detectors.

j. Highlight the wells on the grid and assign a detector to them by ticking the Use box.

k. Sample locations:

i. Click on **Task** and define your samples as No Template Controls (**NTC**), Standards (**S**) or Unknowns (**U**) which are your samples.

ii. To create a standard curve **quantify the Standards** by clicking on **Quantity** and enter the values.

iii. To **name the wells**, highlight them accordingly and type into **Sample Name**.

l. Any wells without a sample of any kind leave blank.Click **Close** on the **Well Inspector** window

m. Go to the **Instrument Tab**.

n. Change your **Sample Volume** if necessary - usually to 25 µl.

o. If using SYBR green click Add Dissociation Stage.

p. Save the file as a template (**.sdt**) as this will allow you to come back and use this setup in any subsequent runs.

q. Save the .sdt file onto a disk and re-open on the ABI7000 and check all input is correct.

r. **Re-save** the .sdt file as a **.sds** file into your personal folder on C:/07 (*month)* RESULTS. (**The ABI7000 will not run an .sdt file**)

s. Go to the **Instrument Tab** and click **Start**. Wait for the instrument to 'clunk' and wait for the run time to appear.

t. At the end of the run shut down the software, save your data onto disk, or login to Novell to transfer it, remove your plate from the ABI7000 and dispose of it in the Yellow Waste bin and turn off the instrument.

Real-time polymerase chain reaction of the 36b4 gene was performed, using the following primers:

TCGAACACCTGCTGGATGAC **(forward primer) and**
CCACGCTGCTGAACATGCT **(reverse primer).**

Cocktail

Components	Volume (ul)
SYBR Green	12.5
Primer (Forwrad)	1.0
Primer (Reverse)	1.0
Template cDNA sample	1.0
ddH_2O	9.5

Real time thermal cycler program

Temp	95	10 min
40 cycles of next two steps		
Temp	95	15 sec
Temp	60	60 sec

RESULT AND DISCUSSION

Isolation of DNA- DNA isolated from the blood from different age group using DNA isolation protocol and were analysed in 0.8% Agarose gel electrophoresis.

Quantification for DNA isolated from blood samples:

Samples	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
1	0.396	0.246	1.60	1.98
2	0.325	0.192	1.69	1.62
3	0.252	0.148	1.70	1.26
4	0.286	0.166	1.72	1.43
5	0.185	0.111	1.66	0.92

Table 1: Quantification of Genomic DNA isolated from blood of different age groups.

Samples	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
1	0.245	0.147	1.66	1.22
2	0.421	0.245	1.71	2.10
3	0.325	0.198	1.64	1.62
4	0.215	0.121	1.77	1.07
5	0.315	0.196	1.60	1.57
6	0.184	0.105	1.80	0.92

Table 2: Quantification of Genomic DNA isolated from smokers and non-smokers blood

Samples	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
1	0.121	0.078	1.55	1.72
2	0.194	0.121	1.60	1.76
3	0.235	0.131	1.79	1.26
4	0.298	0.185	1.61	1.62
5	0.321	0.179	1.79	1.44
6	0.285	0.158	1.80	1.44

Table 3: Quantification of Genomic DNA isolated from sports and non-sports blood

Agarose Gel Electrophoresis Image: DNA

L1 L2 L3 L4 L5

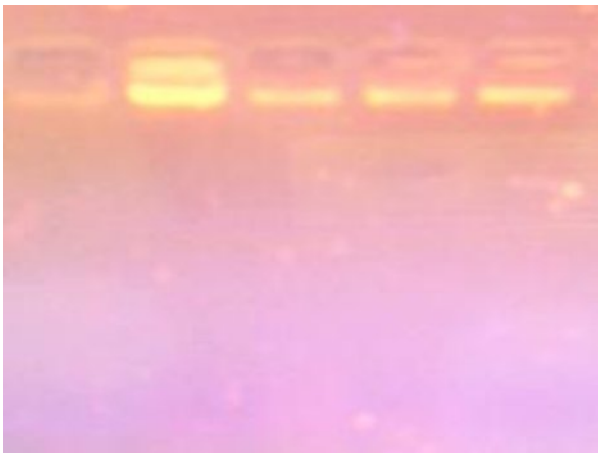


Figure 4: Agarose Gel Electrophoresis image for different age groups.

Descriptions of the Lane is as follows:

Lane No.	Sample
L1	Blood sample 1 (19 years)
L2	Blood sample 2 (23 years)
L3	Blood sample 3 (33 years)
L4	Blood sample 4 (46 years)
L5	Blood sample 5 (56 years)

L1 L2 L3 L4 L5 L6 L7 L8

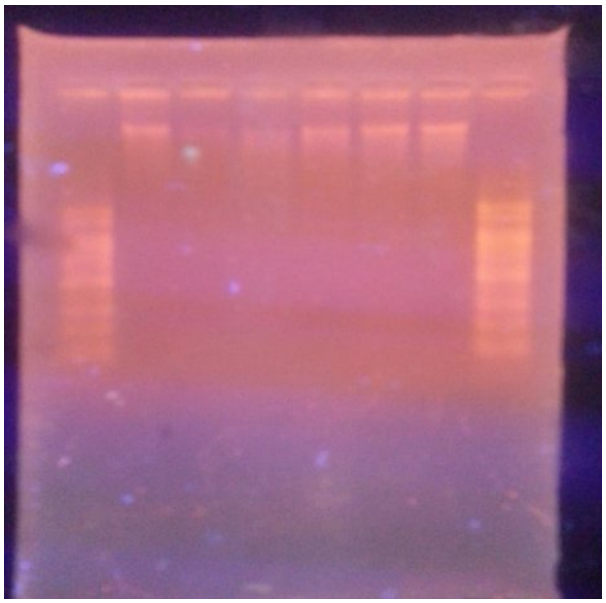


Figure 5: Agarose Gel Electrophoresis image for Sports and Non-sport's

Descriptions of the Lane is as follows:

Lane No.	Sample
L1	DNA Ladder (100 to 1000 base pairs)
L2	Sports person blood sample (25 years)
L3	Non-Sports person blood sample (25 years)
L4	Sports person blood sample (35 years)
L5	Non-Sports person blood sample (35 years)
L6	Sports person blood sample (45 years)
L7	Non-Sports person blood sample (45 years)
L8	DNA Ladder

L1 L2 L3 L4 L5 L6

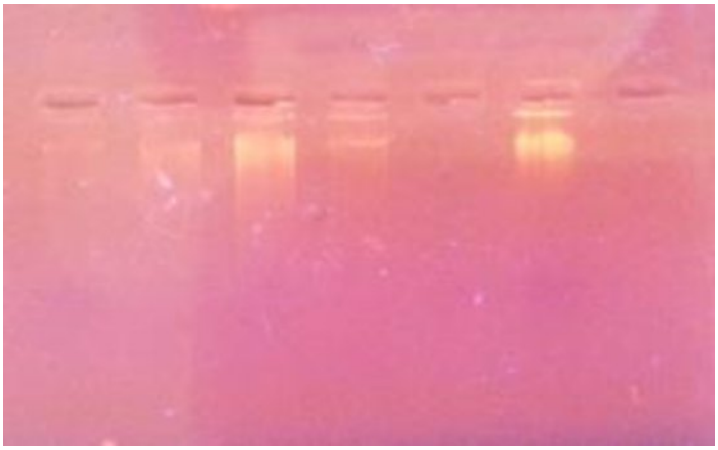


Figure 6: Agarose Gel Electrophoresis image for Smokers and Non-smoker's

Descriptions of the Lane is as follows:

Lane No.	**Sample**
L1	Smoker blood sample (27 years)
L2	Non-smoker person blood sample (27 years)
L3	Smoker person blood sample (38 years)
L4	Non-smoker person blood sample (38 years)
L5	Smoker person blood sample (47 years)
L6	Non-smoker person blood sample (47 years)

Agarose Gel Electrophoresis for Conventional PCR product of different age groups.

L1 L2 L3 L4 L5 L6

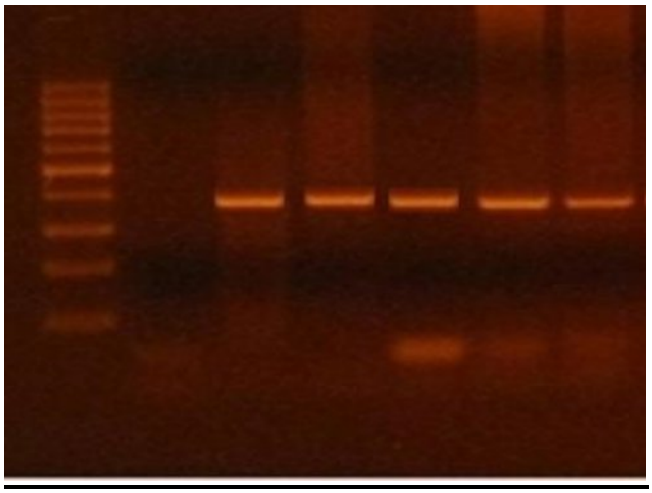


Figure 7: Agarose Gel Electrophoresis image for Conventional PCR product of different age groups.

Descriptions of the Lane is as follows:

Lane No.	Sample
L1	DNA Ladder
L2	Blood sample 1 (19 years)
L3	Blood sample 2 (23 years)
L4	Blood sample 3 (33 years)
L5	Blood sample 4 (46 years)
L6	Blood sample 5 (56 years)

The PCR result shows that, the length of conserved region in telomere which has been identified by different bands does not change within the same age group, and remains constant in normal individuals.

L1 L2 L3 L4 L5

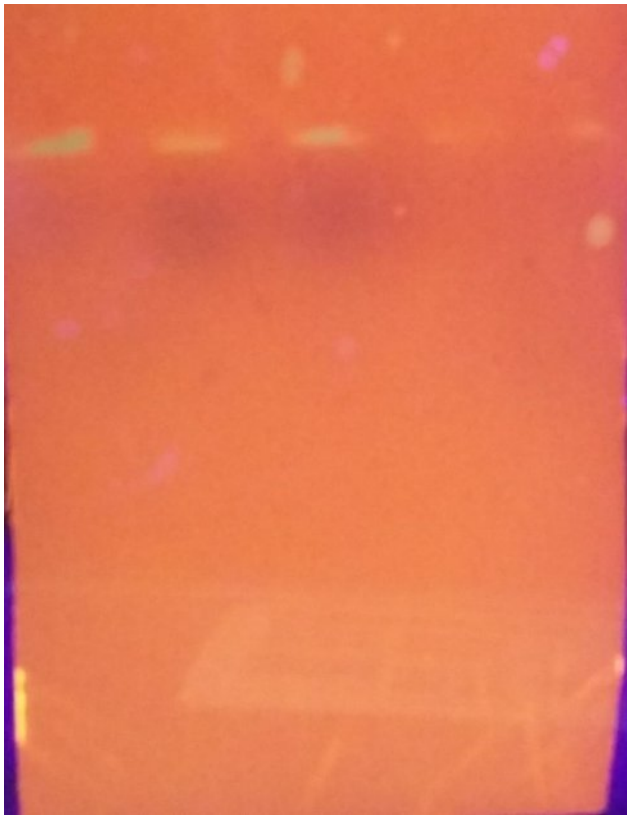


Figure 8: Southern Blotting result for different age groups

Descriptions of the Lane is as follows:

Lane No.	**Sample**
L1	Blood sample 1 (19 years)
L2	Blood sample 2 (23 years)
L3	Blood sample 3 (33 years)
L4	Blood sample 4 (46 years)
L5	Blood sample 5 (56 years)

The Southern blotting result shows that, the length of conserved region in telomere which has been identified by different bands does not change within the same age group, and remains constant in normal individuals.

Quantification of RNA isolated from blood samples:

Samples (total RNA)	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
S1	0.060	0.042	1.420	0.264
S2	0.229	0.141	1.624	1.007
S3	0.086	0.051	1.686	0.378
S4	0.097	0.049	1.979	0.426
S5	0.071	0.038	1.868	0.312

Table 4: Quantification of RNA isolated from blood of different age groups.

Samples (total RNA)	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
S1	0.083	0.044	1.880	0.365
S2	0.074	0.041	1.800	0.325
S3	0.114	0.065	1.753	0.501
S4	0.124	0.065	1.904	0.545
S5	0.212	0.115	1.842	0.932
S6	0.312	0.150	2.021	1.372

Table 5: Quantification of RNA isolated from smokers and non-smokers blood

Samples (total RNA)	O.D. AT 260nm	O.D. AT 280nm	Purity 260/280	Conc.(µg/µl)
S1	0.181	0.099	1.828	0.796
S2	0.243	0.135	1.800	1.069
S3	0.143	0.089	1.606	0.692
S4	0.093	0.042	2.214	0.409
S5	0.176	0.098	1.790	0.774
S6	0.301	0.174	1.729	1.324

Table 6: Quantification of RNA isolated from sports and non-sports blood

Agarose Gel Electrophoresis: RNA from different age groups:

L1 L2 L3 L4 L5

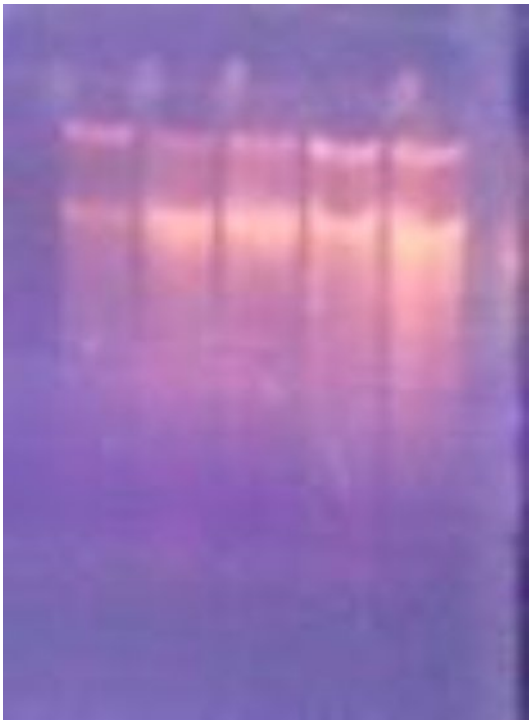


Figure 9: Agarose Gel Electrophoresis RNA image for different age groups.

Descriptions of the Lane is as follows:

Lane No.	Sample
L1	Blood sample 1 (19 years)
L2	Blood sample 2 (23 years)
L3	Blood sample 3 (33 years)
L4	Blood sample 4 (46 years)
L5	Blood sample 5 (56 years)

L1 L2 L3 L4 L5 L6 L7

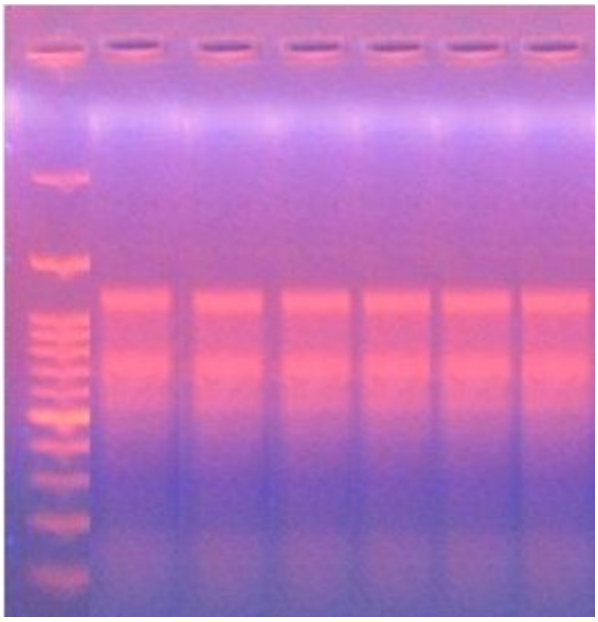


Figure 10: Agarose Gel Electrophoresis RNA image for Sports and Non-sport's

Descriptions of the Lane is as follows:

Lane No.	**Sample**
L1	DNA Ladder
L2	Sports person blood sample (25 years)
L3	Non-Sports person blood sample (25 years)
L4	Sports person blood sample (35 years)
L5	Non-Sports person blood sample (35 years)
L6	Sports person blood sample (45 years)
L7	Non-Sports person blood sample (45 years)

L1 L2 L3 L4 L5 L6

Figure 11: Agarose Gel Electrophoresis RNA image for Smokers and Non-smoker's

Descriptions of the Lane is as follows:

Lane No.	**Sample**
L1	Smoker blood sample (27 years)
L2	Non-smoker person blood sample (27 years)
L3	Smoker person blood sample (38 years)
L4	Non-smoker person blood sample (38 years)
L5	Smoker person blood sample (47 years)
L6	Non-smoker person blood sample (47 years)

Real Time PCR RESULT:

Smokers and Non-smokers: Real time pcr result

18S **Real Time PCR: Standard curve and PCR Efficiency**

Dilution	cDNA input cDNA (ng or µl)	lncDNA	cycle # crossing threshold Ct (or CP)
1x	1	0	10.33
3x	0.45	-0.7985077	13.55
9x	0.3	-1.2039728	15.22
27x	0.2	-1.60943791	17.25
81x	0.1	-2.30258509	19.87
	linearregr.:	K	-4.189868
		D	10.287803
		y = kx + d	
	slope (calc. for 10x dil.)		-9.6475
	PCR efficiency		**1.2696**
			logarithmic fit:

PCR-efficiency = $10^{(-1/slope)}$

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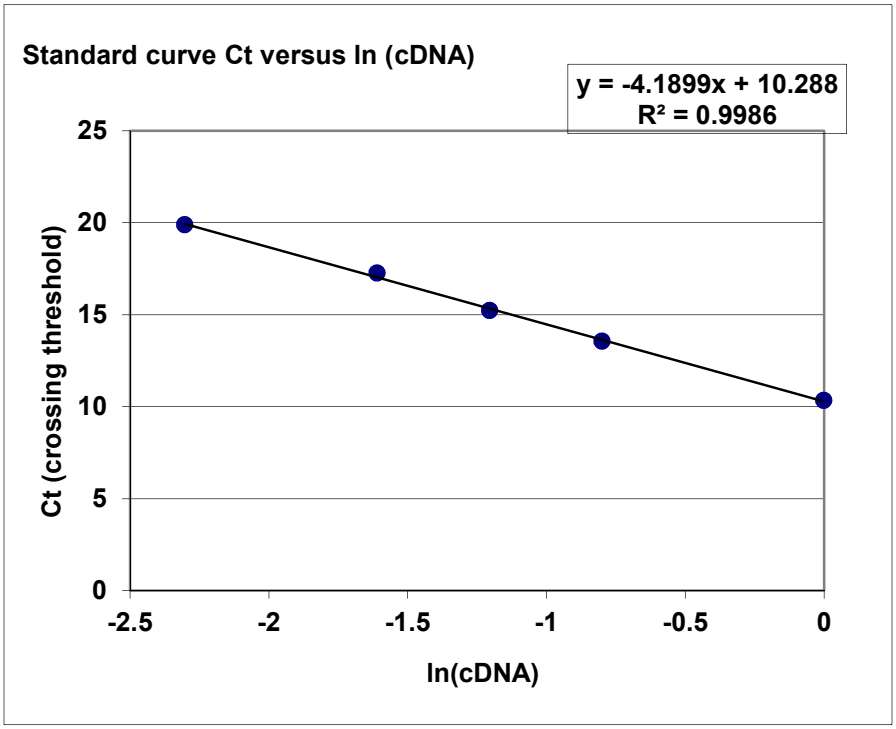
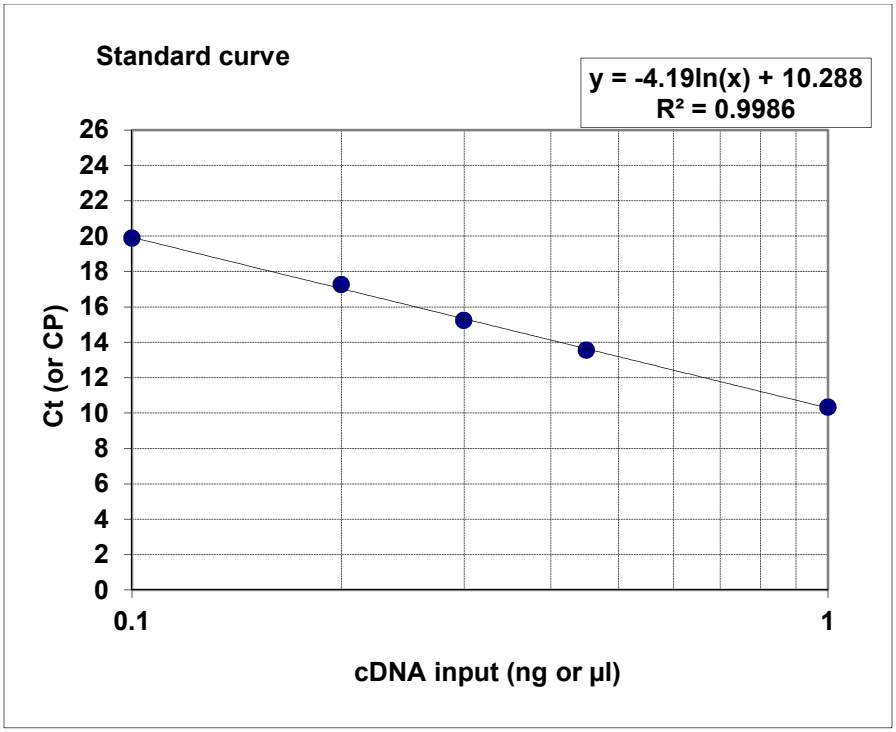


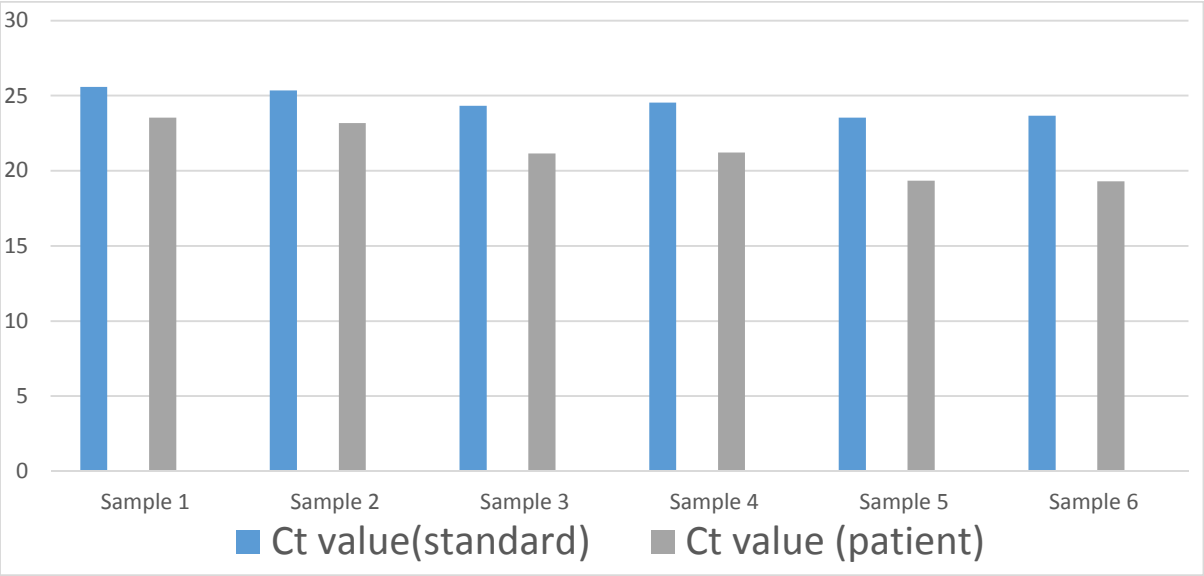


Sample	Ct- value	Mean	Patient	Mean
S-1	26.69 25.75 24.13	25.59	24.26 23.25 22.21	23.54
S-2	26.65 25.35 24.06	25.35	24.15 23.14 22.23	23.17
S-3	24.36 25.33 23.33	25.33	21.22 22.02 22.21	21.15
S-4	25.53 24.52 23.51	24.54	20.10 22.23 21.20	21.21
S-5	23.61 22.51 24.50	23.54	19.01 20.02 19.02	19.35

S-6	22.72 23.31 24.43	23.66	19.42 20.83 20.62	19.92

Real time quantitative result

FIGURE 12: **Comparative study of Ct value of standard and Ct value of telomere length in smokers and non- smoker's samples**



Different Age Groups: Real time pcr result

18S **Real Time PCR: Standard curve and PCR Efficiency**

Insert your data in yellow cells only, the rest is calculated

dilution	cDNA input cDNA (ng or µl)	lncDNA	cycle # crossing threshold Ct (or CP)
1x	2	0.69314718	9.25
3x	0.666666667	-0.40546511	11.63
9x	0.222222222	-1.5040774	13.25
27x	0.074074074	-2.60268969	14.66
81x	0.024691358	-3.70130197	15.98
	linearregr.:	k	-1.500984
		d	10.696403
		y = kx + d	
	slope (calc. for 10x dil.)		-3.4561
	PCR efficiency		**1.9469**
			logarithmic fit:

PCR-efficiency = $10^{(-1/slope)}$

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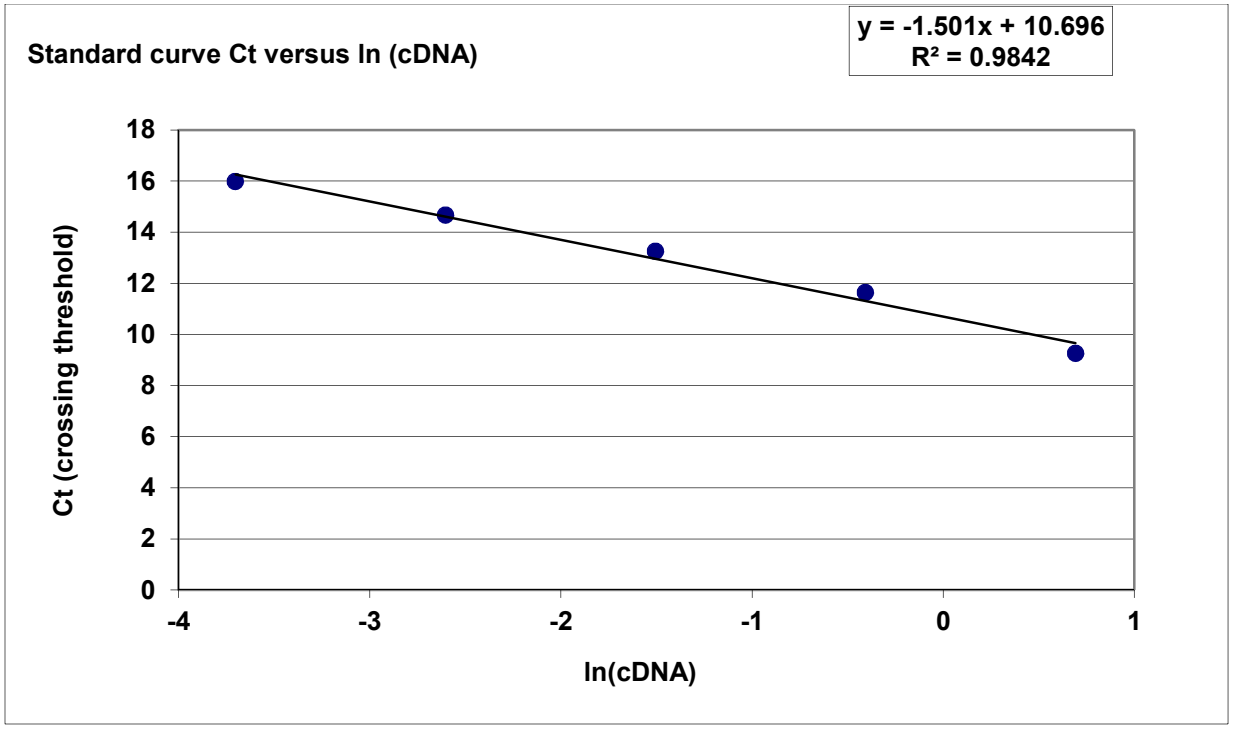
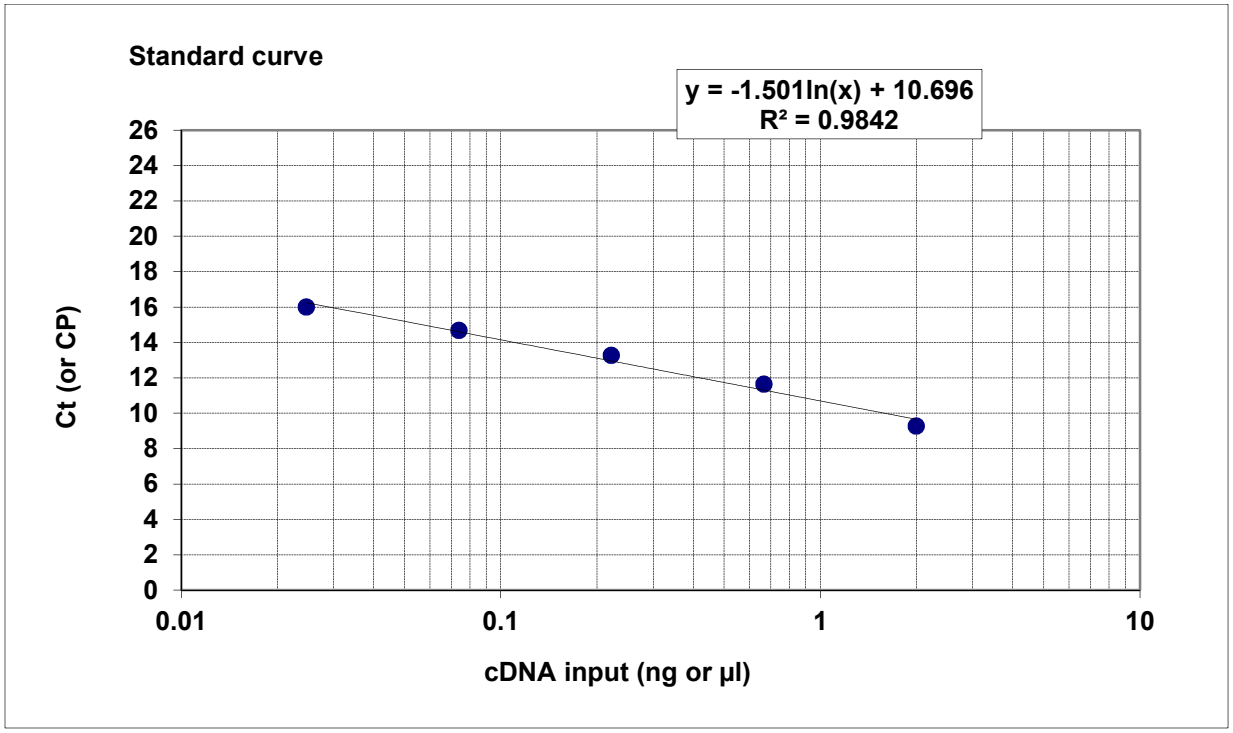
The PCR efficiency calculated for the reference gene standard curve in this sheet is directly used in sheets 3 and 4: fold induction and fold induction (tripl.) to calculate up- or downregulation of a certain gene (normalized to a reference gene) for a given treatment as compared to a control.

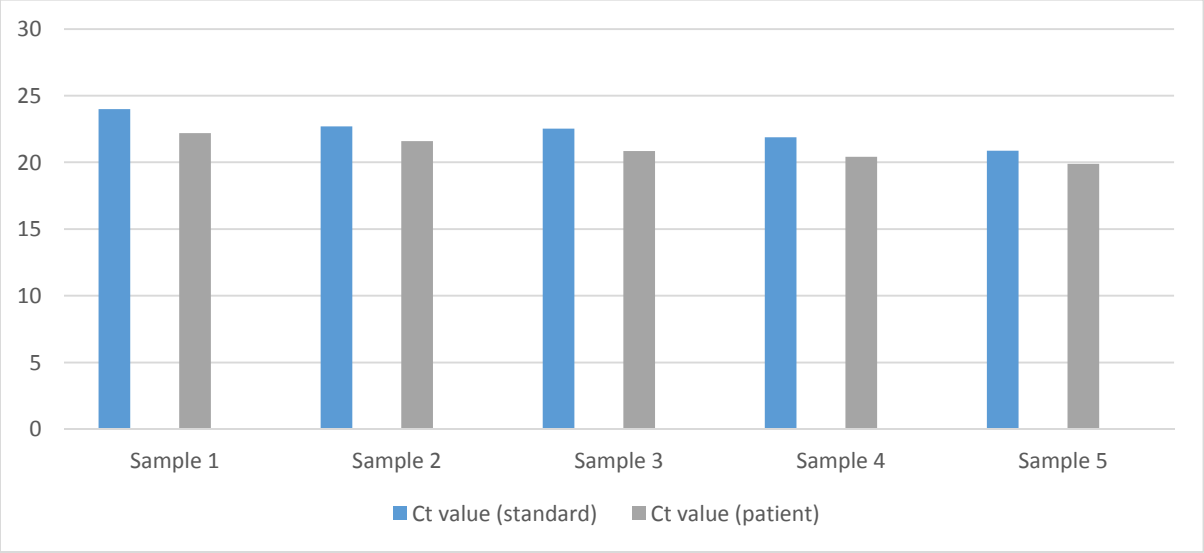

Standard curve Ct versus ln (cDNA)
y = -1.501x + 10.696
R² = 0.9842
Ct (crossing threshold)
18
16
14
12
10
8
6
4
2
0
-4
-3
-2
-1
0
1
ln(cDNA)


Standard curve
y = -1.501ln(x) + 10.696
R² = 0.9842
Ct (or CP)
26
24
22
20
18
16
14
12
10
8
6
4
2
0
0.01
0.1
1
10
cDNA input (ng or µl)

Real time quantitative result

Sample	Ct- value (standard)	Mean	Ct- value (Patient)	Mean
S-1	25.82 23.53 22.62	23.99	23.16 22.23 21.12	22.20
S-2	22.12 24.39 21.58	22.69	21.45 23.14 20.22	21.60
S-3	22.86 23.38 21.35	22.53	21.12 21.12 20.23	20.82
S-4	24.36 21.14 20.11	21.87	22.25 20.25 20.02	20.84
S-5	21.22 20.25 21.15	20.87	20.22 19.22 20.22	19.88

Comparative study of Ct.value of standard and Ct value of telomere length in different age group's samples:



Sports and Non-sports: Real time pcr result

18S **Real Time PCR: Standard curve and PCR Efficiency**

Insert your data in yellow cells only, the rest is calculated

dilution	cDNA input cDNA (ng or µl)	lncDNA	cycle # crossing threshold Ct (or CP)
1x	1.3221	0.27922138	12.213111
3x	0.3111	-1.16764088	15.213221
9x	0.2222	-1.5041774	17.021364
27x	0.1235	-2.09151412	18.627031
81x	0.0963	-2.34028696	19.891602
	linearregr.:	k	-2.883488
		d	12.657652
		y = kx + d	
	slope (calc. for 10x dil.)		-6.6395
	PCR efficiency		**1.4145**
			logarithmic fit:

PCR-efficiency = $10^{(-1/slope)}$

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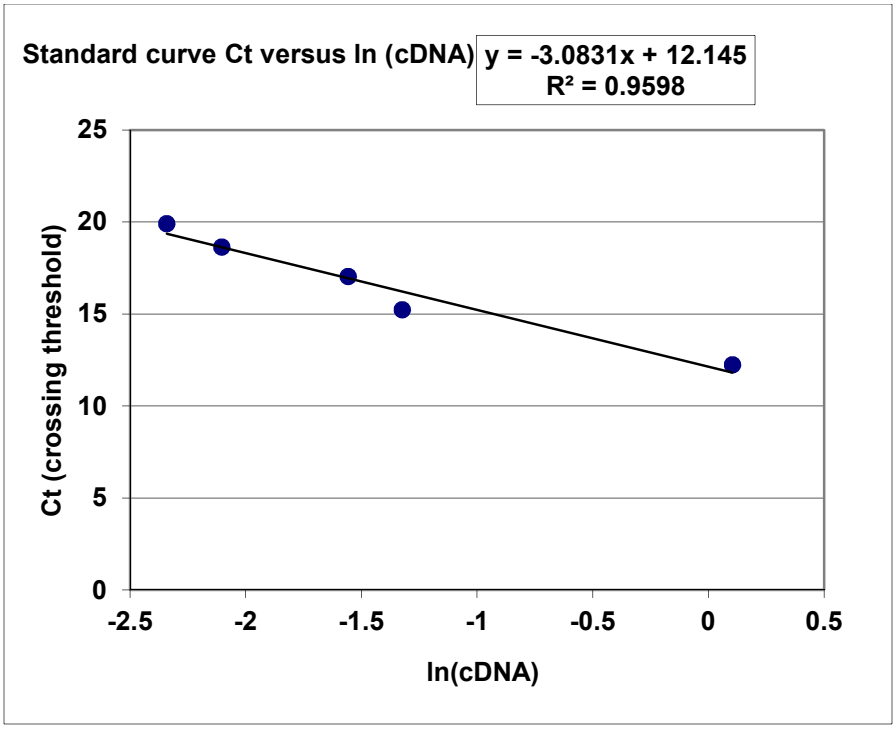
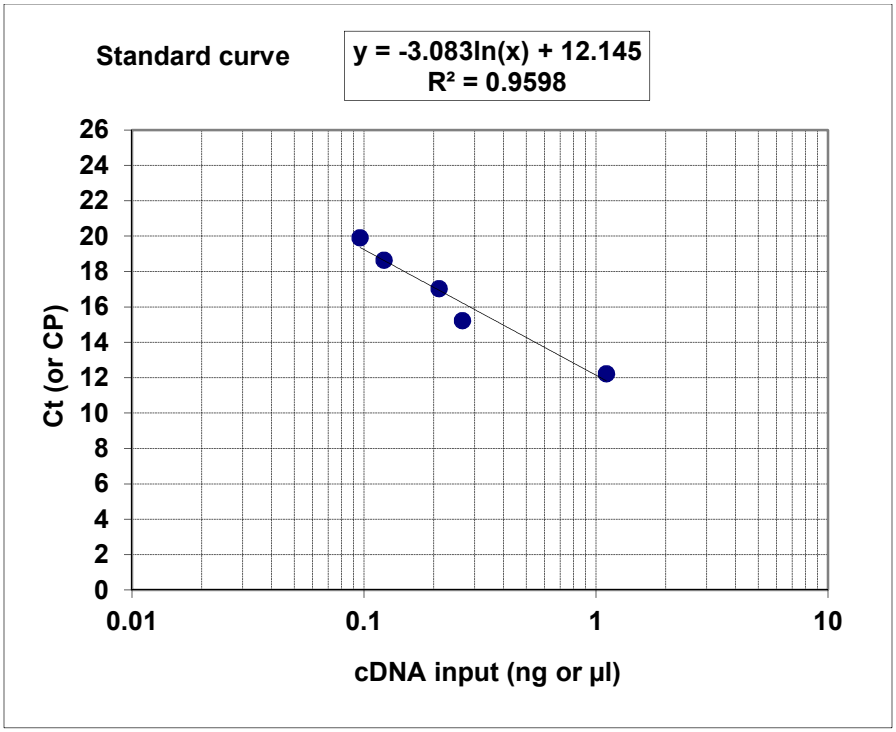
The PCR efficiency calculated for the reference gene standard curve in this sheet is directly used in sheets 3 and 4: fold induction and fold induction (tripl.) to calculate up- or downregulation of a certain gene (normalized to a reference gene) for a given treatment as compared to a control.

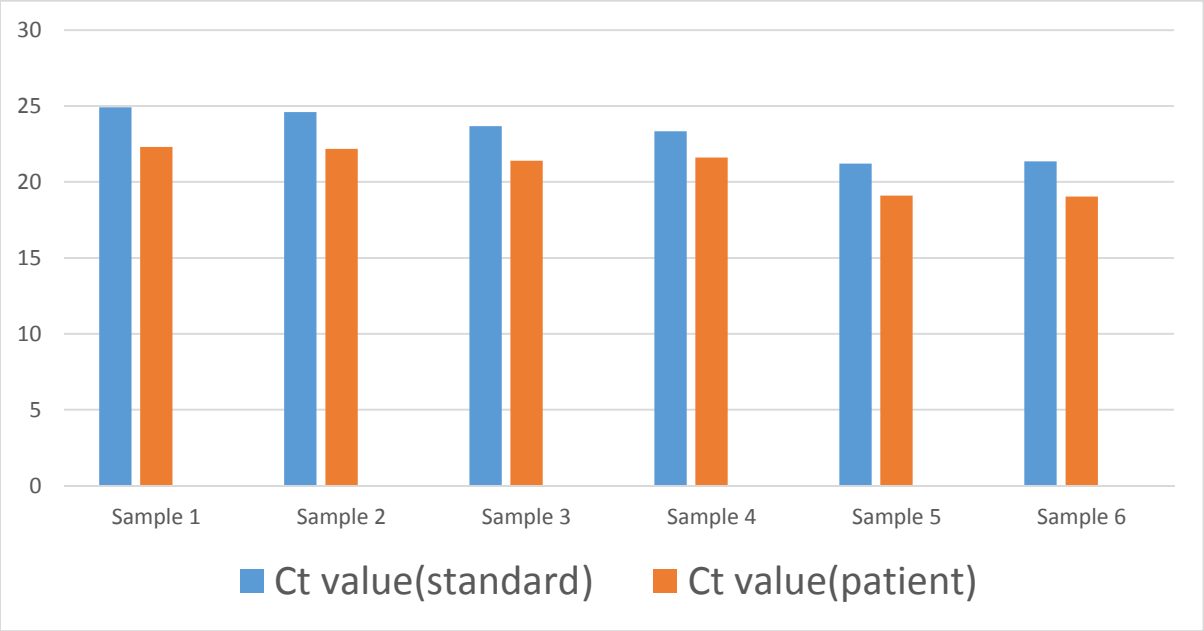

Standard curve Ct versus ln (cDNA)
y = -3.0831x + 12.145
R² = 0.9598
Ct (crossing threshold)
25
20
15
10
5
0
-2.5
-2
-1.5
-1
-0.5
0
0.5
ln(cDNA)



Sample	Ct- value	Mean	Patient	Mean
S-1	24.51 25.83 24.42	24.92	24.51 22.03 23.09	22.30
S-2	25.25 23.32 25.33	24.60	23.11 23.30 20.20	22.17
S-3	23.51 23.01 24.52	23.68	22.02 21.19 21.20	21.40
S-4	24.40 23.40 22.22	23.34	21.21 21.21 22.22	21.61
S-5	22.30 21.10 20.20	21.20	20.86 19.10 18.10	19.09

S-6	22.51 22.43 21.12	21.35	20.01 19.22 18.10	19.01

Comparative study of Ct.value of standard and Ct value of telomere length in Smokers and Non-smokers samples:



Note: Above result are showing down regulated values, therefore they all are positive.

CONCLUSION

Although relying on earlier experiment we performed direct conventional PCR to check comparative length of telomere, we fail to do so. I think it's not possible to verify different length of telomere using conventional PCR

In conventional PCR, which DNA sequence will be targeted is a matter of concern. From various literature we have come to know that 36b4 gene can be targeted to design primer.

Data collected from Real time PCR suggest variations but only in first case i.e. different age group. Although variation is observed in rest two cases also, data interpretation is not quite satisfactory. Therefore, we conclude telomere can be used for biomarker in ageing.

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